Baculovirus Replication: Purification and identification of the *Trichoplusia ni* Nuclear Polyhedrosis Virus-induced DNA Polymerase

By X. WANG AND D. C. KELLY*

NERC Institute of Virology, Mansfield Road, Oxford, OX1 3SR, U.K.

(Accepted 30 June 1983)

**SUMMARY**

DNA polymerase activity present in *Trichoplusia ni* multiple nucleocapsid nuclear polyhedrosis virus-infected *Spodoptera frugiperda* cells has been analysed by chromatography on DNA-cellulose and phosphocellulose columns. In infected cells a new fraction of activity was found to bind to the columns more strongly than did polymerase activity in uninfected cells. The infected-cell-specific DNA polymerase was purified by a combination of DNA-cellulose chromatography of crude extracts and phosphocellulose chromatography of the semi-purified activity. The final product contained a single polypeptide of molecular weight 126 000 which was not found in uninfected cells. The purified enzyme was inhibited by aphidicolin and [E]-5-(2-bromovinyl)-2'-deoxyuridine triphosphate, but not by bromovinyldeoxyuridine. The enzyme was shown to be an early enzyme, probably a delayed early protein, since it was present in cells inhibited by aphidicolin which were locked into the synthesis of early proteins by the drug.

**INTRODUCTION**

A number of enzymes concerned with DNA synthesis are stimulated in baculovirus-infected cells including deoxypyrimidine kinase and DNA polymerase (Kelly, 1981, 1982; Miller *et al.*, 1981; Wang *et al.*, 1983). Although the enzymes have not yet been shown to be virus-coded, the virus-stimulated enzymes differ in properties from the normal cell enzymes, and it is probable that the stimulated enzymes are virus-specific. Predictably, the DNA polymerase is an early function in infected cells, and it is stimulated in activity in cells which are locked into early protein synthesis by an inhibitor of DNA synthesis (cytosine arabinoside) and probably is a delayed early (β) protein (Kelly, 1981; Kelly & Lescott, 1981). The virus-specified enzyme is probably intimately involved in the synthesis of viral progeny DNA.

DNA polymerase may also be an essential enzyme in the transition from early to late functions in baculovirus-infected cells. In this paper we have identified a single infected-cell-specific polypeptide (ICSP) associated with purified DNA polymerase activity obtained by DNA-cellulose and phosphocellulose chromatography. In addition we have identified some early DNA-binding proteins in virus-infected cells.

The DNA polymerases stimulated by baculoviruses have been partly characterized in crude preparations derived from cell culture (Kelly, 1981) or partially purified from larval extracts (Miller *et al.*, 1981). The enzyme activity is inhibited by aphidicolin and bromovinyldeoxyuridine triphosphate (Miller *et al.*, 1981; Wang *et al.*, 1983) and it was of interest to investigate the effect of these drugs on the purified enzyme, since drug-resistant mutants will probably help to elucidate baculovirus DNA replication.

Aphidicolin is a mycotoxin that specifically inhibits nuclear DNA replication in eukaryotes (Spadari *et al.*, 1982). Bromovinyldeoxyuridine (BVdU) is a chemical derivative that is a potent inhibitor of some virus-specific DNA polymerases, notably those of certain herpesviruses, and is a promising antiviral agent (De Clercq *et al.*, 1980).
METHODS

Materials. Aphidicolin was obtained from Dr M. Suffness, Department of Health and Human Services, National Institute of Health, Bethesda, Md., U.S.A. [E]-5-(2-Bromovinyl)-2'-deoxyuridine and [E]-5-(2-bromovinyl)-2'-deoxyuridine triphosphate were gifts from Dr E. de Clercq, Rega Institute, B-3000 Leuven, Belgium. [Me-3H]Thymidine and [Me-3H]thymidine 5'-triphosphate were purchased from Amersham International. DNA-cellulose was purchased from Sigma and phosphocellulose from Uniscience Ltd, Cambridge, U.K.

Virus and cells. Trichoplusia ni multiple nucleocapsid nuclear polyhedrosis virus (T. ni MNPV) was grown and titrated in Spodoptera frugiperda cells as previously described (Kelly & Lescott, 1981; Kelly & Wang, 1981).

Radiolabelling of cells. Uninfected and virus-infected cells were radiolabelled with [35S]methionine, 20 μCi/ml, 3 × 10^5 cells/ml in methionine-deficient medium for 2 h. Infected cells were routinely labelled 12 to 14 h post-infection. The general procedures were as described by Kelly & Lescott (1981).

Preparation of cell extracts for DNA polymerase assay and purification. Routinely, 1 × 10^6 S. frugiperda cells were used. Cells were infected at a m.o.i. of 20 p.f.u./cell. Extraction of cells was performed 14 h later, when viral DNA synthesis and polymerase activity is maximal (D. C. Kelly, X. Wang, M. D. Ayres & T. Lescott, unpublished observations). Cells were pelleted by low-speed centrifugation, washed in ice-cold phosphate-buffered saline and finally resuspended in 8 ml of extraction buffer (50 mM-Tris-HCl pH 7.5, 10 mM-2-mercaptoethanol, 1 mM-EDTA, 10 mM-sodium bisulphite, 10% glycerol). The cell suspensions were then disrupted for 10 min in a sonicating water-bath. The material was then centrifuged for 30 min at 82,500 g and the supernatant containing the DNA polymerase activity was used for further purification.

DNA-cellulose chromatography. The 82,500 g cell extract supernatant was applied to a column (1.5 by 4.5 cm) of DNA-cellulose equilibrated in extraction buffer. After five 10 ml washes of extraction buffer, the DNA polymerase was eluted with a 100 ml linear gradient of 0 to 0.72 M-KCl in the same buffer. Fractions of 2 ml were collected and 50 μl of each fraction was used to determine DNA polymerase activity and where appropriate for acid-insoluble [35S]methionine radioactivity. The absorbance of fractions was determined at 280 nm by examining 300 μl samples in a 1 cm light path in a Pye Unicam SP1800 spectrophotometer.

Phosphocellulose chromatography. The 82,500 g cell extract supernatant or peaks of DNA polymerase activity obtained from DNA-cellulose chromatography were applied to 1:5 by 5 cm phosphocellulose columns in extraction buffer. Semi-purified DNA polymerase preparations were dialysed against extraction buffer before application. The column was prepared as described by Powell & Purifoy (1977). Briefly, it was pre-washed with 50 ml buffer containing 500 μg bovine serum albumin per ml, and then washed with 50 ml of extraction buffer, to prevent non-specific adsorption. After loading, the column was washed five times with 10 ml of extraction buffer and the DNA polymerase was eluted by adding increasing concentrations of KCl: 0 ml each of 0.05 M, 0.15 M, 0.35 M, 0.55 M, 1.0 M and 1.5 M in extraction buffer, or by using a linear 0 to 0.72 M-KCl gradient. Fractions of 2.5 ml were collected and 50 μl samples of these were used to assay DNA polymerase activity or where appropriate acid-insoluble [35S]methionine radioactivity.

Assay of DNA polymerase activity. Purified DNA polymerase was dialysed against extraction buffer and either used immediately or stored at −20°C until assay. The reaction mixture (280 μl) contained 50 mM-Tris–HCl pH 7.5, 2.5 mM-MgCl2, 150 mM-KCl, 10 mM-dithiothreitol, 0.4 mM-EDTA, 180 μM each of dATP, dGTP and dCTP, 0.72 μg nuclease-nicked DNA primer, 0.5 μCi [Me-3H]thymidine 5'-triphosphate (47 Ci/mmol) and 50 μl purified DNA polymerase or column fractions. The reaction mixture was incubated at 28°C for 2 h and the reaction was stopped by adding 10 ml ice-cold 10% trichloroacetic acid containing 50 μg calf thymus DNA. The acid-insoluble radioactivity was determined by liquid scintillation spectrometry.

SDS–polyacrylamide gel electrophoresis. Samples with 2% SDS, 2% 2-mercaptoethanol, 10% glycerol, 0.01% bromophenol blue in 1:0 M-Tris–HCl pH 6.8 were resolved on 16 cm-long 12% polyacrylamide slab gels using the buffer system of Laemmli (1970) as described by Elliott et al. (1977).

RESULTS

Purification of DNA polymerases from T. ni MNPV-infected cells

Chromatography of DNA polymerase activity from infected and uninfected cells on phosphocellulose columns is shown in Fig. 1. A single DNA polymerase fraction eluting with 0.4 M-KCl was obtained in uninfected cells. In infected cells an additional fraction eluting with 0.6 M-KCl was detected. This is an observation similar to that made by Miller et al. (1981) on enzymes found in infected insect larvae. Attempts to purify the enzymes further by DNA–cellulose chromatography were not successful; no activity was recovered from these columns.

Chromatography of DNA polymerase activity from infected and uninfected cells on DNA–
Fig. 1. Chromatography of DNA polymerase activity present in *S. frugiperda* cells and *T. ni* MNPV-infected *S. frugiperda* cells on phosphocellulose and DNA-cellulose columns. (a) Uninfected cell extract on phosphocellulose; (b) infected cell extract on phosphocellulose; (c) uninfected cell extract on DNA-cellulose; (d) infected cell extract on DNA-cellulose. An extra activity binding strongly to the columns is observed in the infected cells. ○, Absorbance at 280 nm; ---, KCl concn. gradient; □, acid-insoluble methionine; ●, DNA polymerase activity.

Cellulose columns is also shown in Fig. 1. In infected cells an additional DNA polymerase peak was detected which eluted at a higher molarity of salt (0.35 M) than the single polymerase activity present in uninfected cells (0.15 M). Further analysis of the two components on phosphocellulose columns (Fig. 2) showed that the semi-purified uninfected cell polymerase contains two components: a major component eluting in 0.35 M-KCl and a minor component eluting in 0.55 M-KCl. The semi-purified infected-cell-specific DNA polymerase also contained two components (one minor constituent) eluting in 0.55 M-KCl and in 1 M-KCl. For subsequent studies, the uninfected cell DNA polymerase eluted in 0.35 M-KCl and the infected-cell-specific DNA polymerase used was that obtained in the 1 M-KCl eluate. The DNA polymerase activity that eluted from an infected-cell sample at 0.55 M-KCl showed properties similar to infected-cell-specific DNA polymerase, not that of the uninfected cells. The minor components present are probably a result of the stepwise elution from the column and incomplete desorption and should not be regarded as representing heterogeneity.
Fig. 2. Chromatography of semi-purified DNA polymerase obtained by DNA-cellulose chromatography and applied to phosphocellulose columns. (a) DNA polymerase activity (●) from uninfected cells; (b) DNA polymerase activity from infected cells, eluting from DNA-cellulose in low salt (---); (c) DNA polymerase activity from infected cells, eluting from DNA-cellulose in high salt (the infected-cell-specific DNA polymerase).

Fig. 3. SDS-polyacrylamide gel electrophoresis of [35S]methionine-labelled polypeptides fractionated on a DNA-cellulose column after extraction from (a) T. ni MNPV-infected S. frugiperda cells and (b) uninfected S. frugiperda cells. It can be seen that four infected-cell-specific polypeptides bind to the columns. The numbers refer to the fraction number (every second fraction was analysed in this experiment). The cell polymerase eluted in fractions 8 to 14 and the infected-cell-specific polymerase eluted in fractions 18 to 24 which contain ICSP 126000. I, Total infected cell profile 12 to 14 h post-infection; U, uninfected cell profile.

Identification of DNA polymerase

Fractionation of [35S]methionine-labelled polypeptides associated with DNA polymerase activities is shown in Fig. 3. The experiment was designed to purify DNA polymerase and not to identify DNA-binding proteins. Four ICSPs bind to the DNA column and are eluted on washing with KCl. A high molecular weight polypeptide (mol. wt. 126000) eluted in fractions containing the infected-cell-specific DNA polymerase activity together with a number of other infected-cell-specific and uninfected cell polypeptides which spanned fractions not containing the activity. This polypeptide is not present in the uninfected cell DNA polymerase. Subsequent analysis of the [35S]methionine-labelled polypeptides associated with the infected-cell-specific DNA polymerase fractions contained a single polypeptide (Fig. 4) recovered from phospho-
Baculovirus DNA polymerase

Fig. 4. SDS-polyacrylamide gel electrophoresis of [35S]methionine-labelled polypeptides present in (a) uninfected cells, (b) T. ni MNPV-infected S. frugiperda cells, (c) ICSP eluted from DNA-cellulose by 0.15 M-KCl, (d) ICSP eluted from DNA-cellulose by 0.35 M-KCl, (e) ICSP eluted in 1 M-KCl from phosphocellulose after DNA-cellulose chromatography, (f) ICSP eluted by 0.55 M-KCl from phosphocellulose after DNA-cellulose chromatography, (g) T. ni MNPV-infected S. frugiperda cells and (h) uninfected cells.

cellulose. The uninfected cell DNA polymerase comprised a number of polypeptides and should be regarded as semi-pure.

Properties of the purified DNA polymerase

The optimum conditions for assay of the infected-cell-specific DNA polymerase were determined to be those described in Methods. Aphidicolin, BVdU and BVdU triphosphate have been shown to inhibit both cell and virus-stimulated enzymes in crude and partially purified preparations (Miller et al., 1981; Wang et al., 1983). BVdU and aphidicolin both inhibit baculovirus replication (Wang et al., 1983; X. Wang & D. C. Kelly, unpublished results). BVdU triphosphate had a marked inhibitory effect on purified infected-cell-specific DNA polymerase and also, although to a lesser extent, on the cell DNA polymerase (Fig. 5a). BVdU had no effect on either enzyme. Aphidicolin also inhibited both infected-cell-specific DNA polymerase and, to a lesser extent, the uninfected cell DNA polymerase (Fig. 5b).

Effect of aphidicolin on T. ni MNPV-infected-cell-specific polypeptide synthesis

Since aphidicolin preferentially inhibited the activity of virus-stimulated DNA polymerase, and it also inhibits virus replication, it was of interest to determine its effect on T. ni MNPV infected-cell-specific polypeptide synthesis and the synthesis of DNA polymerase. Fig. 6 shows polypeptide synthesis in aphidicolin-treated and untreated cells. Aphidicolin is a potent inhibitor of infected-cell-specific polypeptide synthesis and at concentrations of 0.1 μg/ml locked the synthesis into the β (delayed early) phase, including the synthesis of the infected-cell-specific DNA polymerase-associated polypeptide. Higher concentrations of aphidicolin
Fig. 5. Effect of bromovinyldeoxyuridine (BVdU), BVdU triphosphate and aphidicolin on purified *T. ni* MNPV DNA polymerase and partially pure *S. frugiperda* DNA polymerase activity. (a) *T. ni* MNPV polymerase treated with BVdU (■), *S. frugiperda* cell polymerase treated with BVdU (■), *T. ni* MNPV polymerase treated with BVdU triphosphate (○) and *S. frugiperda* cell polymerase treated with BVdU triphosphate (○). (b) Effect of aphidicolin on purified *T. ni* MNPV DNA polymerase (●) and *S. frugiperda* cell DNA polymerase (○).

Fig. 6. Effect of aphidicolin on *T. ni* MNPV ICSP synthesis in the presence of various concentrations of the drug. (a) Uninfected *S. frugiperda* cells; (b) *T. ni* MNPV-infected cells 19 h after infection, showing a typical late profile; (c) infected cells treated with 0·1 µg aphidicolin/ml showing the synthesis of only early proteins (arrowed); (d) infected cells treated with 1 µg aphidicolin/ml; (e) infected cells treated with 10 µg aphidicolin/ml. The cells were radiolabelled with [35S]methionine from 18 to 19 h post-infection.
Baculovirus DNA polymerase

completely suppressed virus-infected-cell-specific polypeptide synthesis although no effect was observed on host cell metabolism.

DISCUSSION

We have identified a high mol. wt. protein associated with an infected-cell-specific DNA polymerase activity found in *T. ni* MNPV-infected cells. The properties of the enzyme in terms of conditions of assay and drug sensitivity (with the exception of BVdU) were similar to that found in crude preparations (Kelly, 1981; Miller *et al*., 1981). The identification of the DNA polymerase will aid the physical mapping of DNA polymerase on the baculovirus genome. Conclusive evidence that the protein is actually the virus-coded DNA polymerase requires the isolation of DNA polymerase mutants of the virus, and correlation of the physical and genetic maps. The fact that the protein is an 'early' high mol. wt. protein which is not found in uninfected cells and which does co-chromatograph exactly with the novel infected-cell-specific DNA polymerase provides strong circumstantial evidence that the polypeptide is the virus-coded DNA polymerase.

The baculovirus-infected-cell-specific DNA polymerase of mol. wt. about 126000 is similar in size to DNA polymerases induced by other large DNA viruses such as herpesviruses (approx. 150000: Allen *et al*., 1977; Powell & Purifoy, 1977) and vaccinia virus (approx. 110000: Challberg & Englund, 1979). The polypeptide is present in cells locked into the β (delayed early) phase of ICSP synthesis by aphidicolin and is therefore an early protein. The protein was not originally identified in an earlier study (Kelly & Lescott, 1981) and so was not classed into α (immediate early) or β ICSP by function-blocking experiments. By the nature of its function as a DNA polymerase it is probably a β polypeptide. An early α polypeptide of mol. wt. 121000 has been described (Kelly & Lescott, 1981) and this polypeptide is not associated with DNA polymerase activity.

In a previous study on the effect of BVdU on baculovirus replication (Wang *et al*., 1983) it was shown that both BVdU and its triphosphorylated derivative inhibited DNA polymerase activity in crude cell extracts, and the direct effect of BVdU on DNA polymerase activity was attributed to its phosphorylation by cell or viral deoxypyrimidine kinases present in the extract. By analogy with certain herpesviruses, only BVdU triphosphate should block DNA polymerase activity (De Clercq *et al*., 1980). In this study we have shown that BVdU has no effect on purified DNA polymerase and so it is indeed probable that the effect of BVdU in crude extracts is because it becomes triphosphorylated. Both purified uninfected cell and infected-cell-specific DNA polymerases were inhibited by BVdU triphosphate although the uninfected cell DNA polymerase was less susceptible.

As originally shown by Miller *et al*., (1981), aphidicolin inhibits the infected-cell-specific DNA polymerase as well as the uninfected cell polymerase. Aphidicolin has been shown to be a potent inhibitor of eukaryotic DNA polymerase α (Huberman, 1981; Spadari *et al*., 1982) and DNA polymerase induced by large DNA viruses such as herpes and vaccinia (Pedrali-Noy & Spadari, 1980). Miller *et al*., (1981) showed that aphidicolin inhibited the virus-induced and cell enzymes equally. Using purified polymerase activity we found that the infected-cell-specific enzyme was more susceptible to aphidicolin, in contrast to the observations of Miller *et al*., (1981). Since there was a differential effect of aphidicolin on the two DNA polymerases, we looked at the effect of the drug on *T. ni* MNPV ICSP synthesis. Aphidicolin had a marked inhibitory effect on ICSP synthesis and only at the low 0·1 μg/ml level was ICSP synthesis (which had not progressed beyond the β phase) detected above the cell background. It is remarkable that at higher concentrations aphidicolin suppressed ICSP synthesis while apparently having no effect on cell protein synthesis and it is possible that transcription of the viral genome is inhibited at concentrations of 1 μg/ml and higher.

The infected-cell-specific DNA polymerase polypeptide bound to a DNA-cellulose column and eluted at high KCl showing it is probably a DNA-binding protein. A number of other polypeptides bound to the DNA columns, including ICSP 16 and 18 (Kelly & Lescott, 1981), and required fairly high concentrations of KCl to elute them. Whether these polypeptides are truly
DNA-binding proteins or proteins that bind to DNA-binding proteins awaits further experimenta-
tion.

We were unable to recover DNA polymerase activity from DNA-cellulose columns loaded
with semi-purified polymerase from phosphocellulose columns. The reason for this is not clear,
although it is possible that the enzyme becomes unstable. The purified polymerases were,
however, remarkably stable on storage.

REFERENCES

induced DNA polymerase. *Virology* 76, 395-408.

CHALLBERG, M. D. & ENGLUND, P. T. (1979). Purification and properties of the deoxyribonucleic acid polymerase
induced by vaccinia virus. *Journal of Biological Chemistry* 254, 7812-7819

DE CLERCQ, E., DESCAMPS, J., MAUDGAL, P. C., MISSOTTEN, L., LEYTEN, R., VERHELIST, G., JONES, A. S., WALKER, R. T.,
deoxyuridines and -2'-deoxycytidines, In *Developments in Antiviral Therapy*, pp. 21-42. Edited by L. H. Collier &

isolated from *Tipula* sp. with two other iridescent viruses (types 2 and 22). *Virology* 25, 336-350.


KELLY, D. C. (1981). Baculovirus replication: stimulation of thymidine kinase and DNA polymerase activities in
*Spodoptera frugiperda* cells infected with *Trichoplusia ni* nuclear polyhedrosis virus. *Journal of General Virology* 52,
313-319.


305-308.


multiple nuclear polyhedrosis virus by \[E\]-5-(2-bromovinyl)-2'-deoxyuridine. *Journal of General Virology* 64,
1221-1227.

(Received 14 March 1983)