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Binding of Deoxyribonucleic Acid-Dependent Deoxyribonucleic Acid Polymerase to Poxvirus

K. B. TAN[1] AND B. R. McAUSLAN

Department of Cell Biology, Roche Institute of Molecular Biology, Nutley, New Jersey 07110

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Poxvirus has a deoxyribonucleic acid polymerase activity that remains associated with the virus despite repeated centrifugation through sucrose gradients. Highly purified poxvirus preparation can adsorb deoxyribonucleic acid polymerase from cytoplasmic extracts of cells containing such an activity. These results indicate that caution must be used in assuming that an enzyme associated with a purified virus is necessarily an integral part of the virion.

Four types of polymerase, deoxyribonucleic acid (DNA)-dependent ribonucleic acid (RNA) polymerase (5), DNA-dependent DNA polymerase (10, 13), RNA-dependent RNA polymerase (12), and RNA-dependent DNA polymerase (2, 16), have been reported to be constituents of purified virus. These polymerases and other virus-associated enzymes, namely, ligase (8), deoxyribonucleases (8, 9), and nucleotide phosphohydrolases (3), probably play significant roles in the early stages of cell transformation or lytic infection. Recently, Salzman reported DNA polymerase in Kilham rat virus (10). We report below our finding on the association of DNA-dependent DNA polymerase with two other deoxyviruses, vaccinia virus and frog virus 3 (FV_3). At least for vaccinia virus, it appears that the polymerase activity is due to adsorption of enzyme to virus.

MATERIALS AND METHODS

FV_3 was grown in baby hamster kidney cells and purified as described recently (15).

Poxvirus was grown in HeLa cells (14).

Semipurified virus. The cytoplasmic extract of infected cells prepared as described elsewhere (14) was homogenized with Freon 113 (Allied Chemicals) for 10 min at 0 C and then centrifuged for 10 min (2,000 rev/min, Sorvall RC-3). The supernatant fluid was then layered over a cushion of 36% (w/v) sucrose in tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (0.01 M, *p*H 8.5) and centrifuged (45,000 × *g*, 45 min). The pellet (semipurified virus) was resuspended in 0.01 M Tris-hydrochloride, *p*H 7.5.

Purified poxvirus. Semipurified virus prepared as described above was further purified by repeated banding in sucrose density gradients as described in the legend to Fig. 1.

[1] Roche Institute postdoctoral fellow. Present address: The Wistar Institute, Philadelphia, Pa. 19104.

Polymerase assay. A standard mixture (160 μliters) consisted of: 10 μmoles Tris-hydrochloride (*p*H 7.0); 2 μmoles $MgCl_2$; 3 μmoles dithiothreitol (DTT); 25 nmoles each of deoxyadenosine triphosphate, deoxycytidine triphosphate, and deoxyguanosine triphosphate; 1.6 nmoles of a mixture of unlabeled thymidine triphosphate (TTP) and 1 μCi ^3H-labeled TTP (specific activity 11 Ci/mmole, New England Nuclear Corp.) having a final activity of 330 counts per min per pmole; 50 μg of bovine serum albumin, 40 μg of salmon sperm DNA, and 13 μg of viral or cellular protein. The reaction mixture was incubated for 1 hr at 37 C. Radioactivity in precipitates obtained after addition of carrier DNA and 13% trichloroacetic acid to the samples was determined by liquid scintillation spectrometry. Radioactivity in samples containing no protein was subtracted from each value. Activated DNA is native salmon sperm DNA (Sigma Chemical Co.) treated with pancreatic deoxyribonuclease as described recently (11).

RESULTS

When highly purified preparations of vaccinia virus were assayed for DNA polymerase activity with exogenous DNA primer, DNA polymerase activity was consistently found (Table 1). Even higher activities were found in virus preparations that had been subjected to fewer purification steps, and, for this reason, preliminary characterization of viral DNA polymerase was carried out with semipurified virus. Uninfected extracts processed the same way as semipurified virus had comparatively low activity (Table 1).

Characteristics of the polymerase activity. One striking feature of the DNA polymerase reaction is a marked stimulation by added primer. This is in contrast to the viral RNA polymerase which transcribes viral DNA enclosed within the core (5). DNA activated by deoxyribonuclease treat-

ment (11) was the most active template, whereas native DNA was two to three times more active than heat-denatured DNA (Table 1). Therefore, activated DNA was used in all subsequent experiments. Significant enzyme activity was measured with as little as 0.75 μg of DNA per assay and a saturating level of primer was reached at 1 to 1.5 mg of DNA per mg of viral protein. The assay was linear with time for at least 2 hr.

TABLE 1. *Activity of poxvirus-associated deoxyribonucleic acid (DNA) polymerase*[a]

Virus prepn	^{3}H-TMP[b] incorporated per mg of protein per hr (pmoles)
Uninfected HeLa cytoplasmic extract + activated DNA	500
Purified vaccinia + activated DNA	131
Semipurified vaccinia − activated DNA	53
Semipurified vaccinia + activated DNA	3,302
Semipurified vaccinia + native DNA	335
Semipurified vaccinia + heat-denatured DNA	121
Semipurified vaccinia + activated DNA, + 0.1% Nonidet P-40	2,454

[a] Activity was assayed as described in Materials and Methods for a standard reaction mixture. The cytoplasmic fraction of uninfected cells (prepared as described in reference 14) was homogenized with Freon 113, centrifuged for 10 min at 2,000 × *g*, and the supernatant fluid was used for assay.

[b] Thymidine monophosphate.

The nonionic detergent Nonidet P-40 (NP-40) is not essential for the DNA polymerase activity. In fact, NP-40 depressed the DNA polymerase activity (Table 1). DTT (1 to 20 μmoles per assay) stimulated the polymerase activity 2.4- to 3-fold.

For maximum activity, the polymerase reaction requires magnesium ions, all four deoxynucleoside triphosphates, and a *p*H of 7.0. The product was completely hydrolyzed by pancreatic deoxyribonuclease (100 μg/ml, at 37 C for 60 min), but not by alkali or ribonuclease, and therefore had properties of DNA. The amount of DNA synthesized was directly proportional to the viral protein concentration from 3 to 40 μg of protein per assay.

Association of polymerase with virus. When semipurified virus was sedimented in a sucrose gradient, a peak of polymerase activity was found in the position of the virus band. About 34% of the total polymerase activity of the sample was associated with virus particles; the rest was located in the upper portions of the gradient.

To determine whether polymerase activity was indeed firmly associated with virus particles,

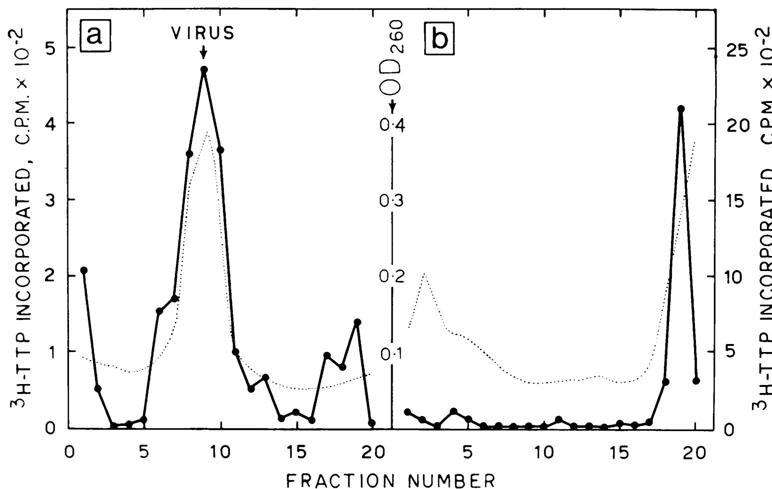

FIG. 1. *Association of DNA polymerase activity with vaccinia virus. Semipurified virus (see Table 1) was purified by repeated banding in sucrose gradients: twice in 20 to 40% (w/v) sucrose in 0.02 Tris-hydrochloride, pH 7.5 (35,300 × g, 35 min, 4 C), and twice in 40 to 80% (w/v) sucrose (126,400 × g, 2 hr, 4 C). One sample of purified virus (a) was then centrifuged (360,000 × g, 2 hr, 4 C) on a 4.0-ml 40 to 80% (w/v) sucrose gradient made on top of a 0.5-ml cushion of 65% (w/w) sucrose. An equal sample of purified virus (b) was incubated for 30 min at 37 C with 0.5% NP-40 and 0.06 M dithiothreitol before sedimentation on a sucrose gradient. Fractions (0.25 ml) were collected from the bottom of the centrifuge tubes. After diluting each fraction with 0.2 ml of 0.01 Tris-hydrochloride, pH 7.0, 100-μliter samples were assayed for polymerase activity with the standard reaction mixture (see Table 1). Optical density of the remainder of each sample was measured. Radioactivity (●), optical density (····). Sedimentation is from right to left.*

semipurified virus was banded five times in sucrose gradients. Most of the polymerase activity was now found associated with the virus band (Fig. 1a). The slight activity near the top of the gradient probably resulted from breakdown of the virus. Another sample of purified virus was treated with NP-40 and DTT, conditions which form subviral particles or "cores," (7) before equilibrium sedimentation on sucrose gradients. Essentially all the polymerase activity was liberated from the virus particles and banded near the top of the gradient (Fig. 1b); virus cores were found in the cushion at the bottom of the gradient.

Adsorption of polymerase to poxvirus. Since the DNA polymerase is liberated by NP-40 treatment, it could be an integral part of the viral envelope. On the other hand, there is a marked induction of DNA polymerase in the cytoplasm of vaccinia-infected cells when DNA synthesis is inhibited by fluorodeoxyuridine. In our hands, this was represented by an increase from 0.2 nmoles ^{3}H-thymidine monophosphate incorporated per mg of protein per hr to a value of 5.09 nmoles between 0 and 6 hr postinfection in the presence of fluorodeoxyuridine. It is likely that this enzyme could adsorb to virus particles. To test this idea, purified virus was mixed with the soluble cytoplasmic fractions from uninfected cells or cells infected for 6 hr with vaccinia virus

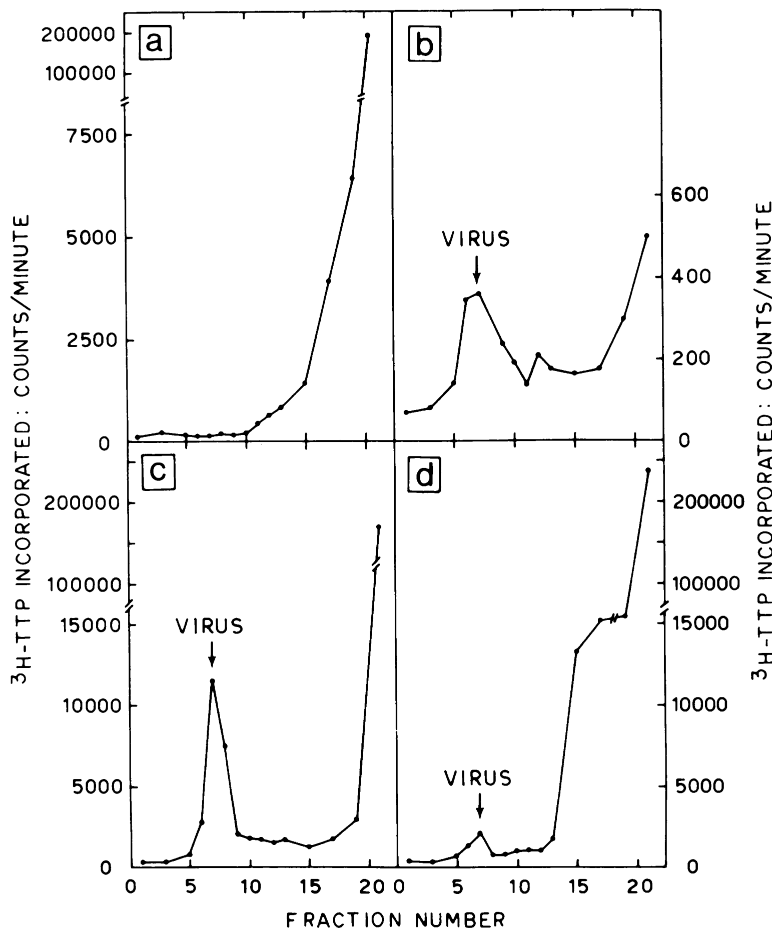

FIG. 2. *Adsorption of induced polymerase to purified vaccinia virus. (a) Uninfected-cell cytoplasmic extract, 1 ml, (3.8 mg of protein; polymerase activity = 4.4 $\times$ 10^{6} counts per min per ml); (b) purified virus, 300 µg; (c) purified virus, 300 µg, incubated at 37 C for 15 min with 1 ml of cytoplasmic extract prepared from cells which were infected with virus for 6 hr (1.7 mg of protein, polymerase activity = 2.8 $\times$ 10^{6} counts per min per ml); (d) a mixture of a + b incubated at 37 C for 15 min. Centrifugation of samples on sucrose gradients and assay of polymerase activity in gradient fractions were performed as described (Fig. 1 and Table 1). Infected- and uninfected-cell cytoplasmic extracts prepared by homogenization of cells in hypotonic buffer (14) were centrifuged at 100,000 $\times$ g for 45 min, and the supernatant fractions were used in the above experiment.*

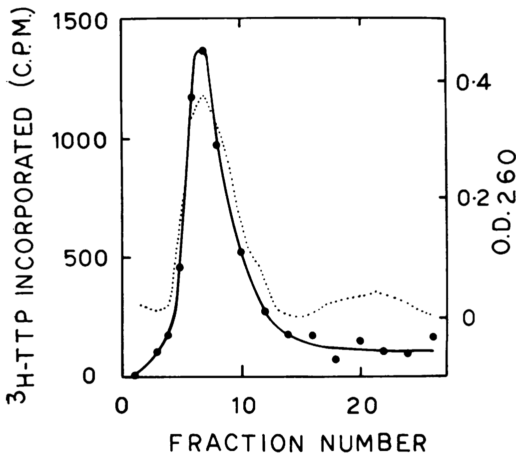

FIG. 3. *Recentrifugation of virus-associated DNA polymerase. The remainder of fractions 6 to 9 (Fig. 2c) were pooled and virus was pelleted. The virus pellet was suspended in 0.3 ml of 0.01 Tris-hydrochloride, pH 7, and layered on a 40 to 80% (w/v) sucrose gradient. The conditions of centrifugation and assay of polymerase activity of gradient fractions were as described (Fig. 1 and Table 1). Enzyme activity (●), optical density (····).*

TABLE 2. *Activity of frog virus (FV_3)-associated deoxyribonucleic acid (DNA) polymerase*[a]

Virus prepn	^{3}H-TMP[b] incorporated per mg of protein per hr (pmoles)
Purified FV_3 + activated DNA	16
Purified FV_3 − activated DNA	7
Purified FV_3 + activated DNA + 0.06% Nonidet P-40	123
Purified FV_3 + activated DNA + 0.5% Nonidet P-40	126

[a] In FV_3 polymerase assays, the standard reaction system (Table 1 and Materials and Methods) was modified to contain 1 μmole $MgCl_2$, 1 μg of DNA, 2 μCi ^{3}H-thymidine triphosphate (specific activity 11 Ci/mmole; New England Nuclear Corp), 8 μg of purified virus, and the pH of the buffer was 8.0. FV_3 was incubated with Tris-hydrochloride, dithiothreitol, and Nonidet P-40 at room temperature for 10 min before the rest of the reaction mixture was added, followed by incubation at 37 C for 1 hr.

[b] Thymidine monophosphate.

in the presence of fluorodeoxyuridine to inhibit DNA synthesis (4).

The virus was then sedimented on sucrose gradients. There was a striking increase (14.4-fold) in virus-associated polymerase activity when virus was first mixed with the soluble cytoplasmic fraction from infected cells (Fig. 2c). When fractions 6 to 9 (Fig. 2c) were pooled and recentrifuged on a sucrose gradient, most of the polymerase remained firmly associated with virus particles, indicating that virus firmly adsorbed polymerase (Fig. 3).

Polymerase activity associated with FV_3. To determine whether DNA polymerase also associates with other deoxyviruses, we investigated FV_3, which also replicates in the host cell cytoplasm (15). Subsequently we found comparatively low DNA polymerase activity associated with purified FV_3. In contrast to vaccinia DNA polymerase, the FV_3 enzyme is strongly stimulated by NP-40 (Table 2). NP-40 treatment disrupts FV_3 outer proteins, but the viral cores appear intact (1).

DISCUSSION

Although we are able to demonstrate DNA polymerases associated with two purified deoxyviruses, vaccinia and FV_3, it is difficult to establish their significance. DNA polymerase, presumably that activity induced by vaccinia infection, bound firmly to purified vaccinia virus. Thus, the polymerase usually found in purified virus could merely represent residual adsorbed polymerase. However, the possibility that a DNA polymerase is present as an integrated component of vaccinia virus is difficult to exclude, since some enzyme activity remains in our most highly purified preparations. The DNA polymerase was not associated with cores produced by treating virus with NP-40 and with DTT. Under similar conditions, the RNA polymerase (5), nucleotide phosphohydrolase (3), and two deoxyribonucleases (9) are all firmly associated with cores. On the other hand, the DNA polymerase in purified vaccinia virus could be located either on or within the viral envelope or even loosely associated with cores. The situation with FV_3 is more complex. Since the enzyme activity is demonstrable by disruption of virus with detergent, it suggests that polymerase, whether a contaminant or an essential component, is enclosed by capsid proteins. Enzymes, notably RNA and DNA polymerases, reported to be constituents of purified viruses, have been demonstrated or speculated to be essential for virus functions. There are no a priori reasons why complex deoxyviruses should carry a structural DNA polymerase. Whether or not DNA polymerase is a contaminant remains to be proved. However, in view of our finding that DNA polymerase binds firmly to purified virus, assertions (10) that enzymes associated with purified virus are in fact constituents should be made with extreme caution.

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