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Induction of Epstein-Barr Virus-associated DNA Polymerase by 12-*O*-Tetradecanoylphorbol-13-acetate

PURIFICATION AND CHARACTERIZATION*

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The diterpene ester promoter of mouse skin tumors, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), efficiently induces Epstein-Barr virus (EBV)-associated DNA polymerase (DNA nucleotidyltransferase) activity in the EBV-producing lymphoblastoid cell line, P3HR-1. With the use of intervent dilution chromatography followed by sequential DEAE-cellulose and phosphocellulose column chromatography, the virus-associated enzyme has been isolated and purified 300-fold. The partially purified EBV DNA polymerase activity could be distinguished from cellular polymerases by its activation with salt and its degree of sensitivity to *N*-ethylmaleimide and phosphonoacetic acid. The enzyme showed maximum activity for copying activated calf thymus DNA in the presence of 100 mM ammonium sulfate. In the absence of salt, the enzyme utilized with high efficiency deoxyoligomer-homopolymer templates, but failed to copy poly(rA)·oligo(dT)$_{10}$ and oligo(dT)$_{10}$, showing that the enzyme had properties distinct from DNA polymerase γ, reverse transcriptase, and terminal deoxynucleotidyltransferase. The partially purified enzyme is strongly inhibited by acyclovir triphosphate and thus has properties similar to herpes simplex virus DNA polymerase.

DNA polymerase activity increases in various mammalian cells after infection with most herpes group viruses (1–10). The virus-induced enzymes differ from the cellular enzymes with respect to activity with different templates, physical properties, immunological specificity, and sensitivity to chemical inhibitors. Epstein-Barr virus DNA synthesis and late viral antigen expression are inhibited by phosphonoacetic acid (11–13). The specific inhibition of viral DNA synthesis led to a suspicion that EBV[1] may also have its own DNA polymerase. Since then a number of attempts have been made to purify this enzyme (14–16), but the lack of a fully permissive cell system for EBV replication has made the purification and characterization of the enzyme difficult. Moreover, interpretation of the results was complicated by the fact that the partially purified enzymes contain appreciable amounts of host polymerase activities (14, 16). Clearly it is essential to purify the virus-associated enzyme in order to study interaction with antiviral drugs. This effort is also important for an understanding of the regulation of replication of EBV genomes found in virus-producing and nonproducing cells.

Recently Zur Hausen *et al.* (17, 18) and Lin *et al.* (19) have shown that the tumor-promoting agent, 12-*O*-tetradecanoylphorbol-13-acetate, induces 80 to 90% of P3HR-1 cells containing latent EBV genomes into the viral productive cycle resulting in a large increase in virus production. We took advantage of this effect and report here that treatment of P3HR-1 cells with TPA results in rapid induction of virus-associated DNA polymerase activity. The large increase in the new DNA polymerase activity enabled us to purify and characterize the enzyme. The properties of the purified enzyme are similar to other known herpes virus enzymes with some differences (1–10).

RESULTS[2]

Induction of Salt-resistant DNA Polymerase Activity after TPA Treatment—Fig. 1*A* shows the kinetics of induction of EBV-associated DNA polymerase activity in the nuclear extract of TPA-treated and untreated P3HR-1 cells. The nuclear extract of cells grown in the presence of TPA produced a large increase in DNA polymerase activity when assayed in the presence of 100 mM ammonium sulfate which inhibits host polymerases drastically (26). Induction of the enzyme was at its maximum after 2 days of TPA treatment; thereafter, the level of activity fell.

With the increase in enzyme activity there was also an increase in the number of EBV EA/VCA-positive cells (results not presented). The maximum number of EA/VCA-positive cells occurs after 5 days of treatment, whereas the maximum level of DNA polymerase activity is attained on the 2nd day. This result may be due to the fact that after 5 days of TPA treatment some cells start to produce virus and die. In contrast, both the EBV-associated polymerase activity and percentage of EA/VCA-positive cells in nontreated cultures re-

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[1] The abbreviations used are: EBV, Epstein-Barr virus; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; EA/VCA, early antigen/viral capsid antigen; PAA, phosphonoacetic acid; ACV, acyclovir[9-(2-hydroxyethoxymethyl)guanine].

[2] Portions of this paper (including "Materials and Methods," Figs. 2S through 6S and Tables IS and IIS) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Maryland 20014. Request Document No. 79M2437, cite author(s), and include a check or money order for $1.65 per set of photocopies.

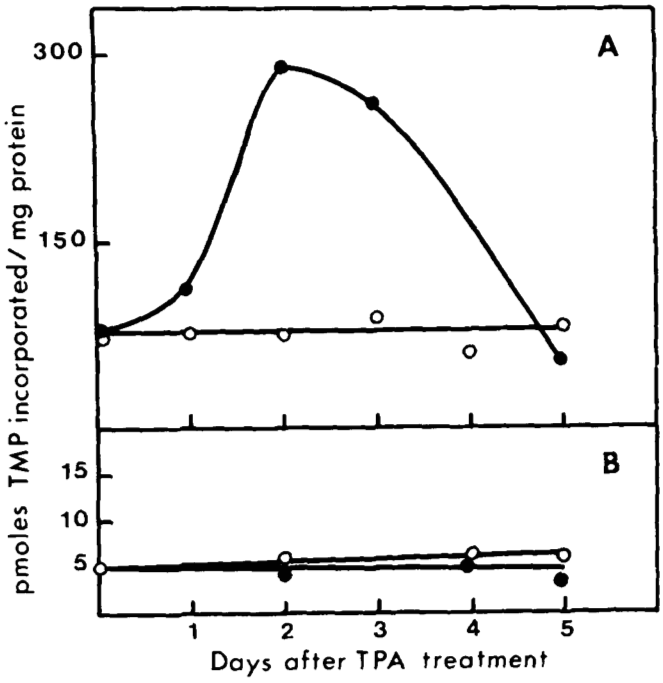

FIG. 1. **Kinetics of induction of EBV-associated DNA polymerase activity upon TPA treatment.** P3HR-1 (*A*) and BJAB (*B*) cells grown in suspension at 10^6 cells/ml were treated with TPA at a concentration of 25 ng/ml. At the indicated days cells were removed, washed, and nuclei prepared as described under "Materials and Methods." For control experiments, cells were grown under the same conditions but without TPA. The nuclear lysates were assayed for DNA polymerase activity in the presence of 100 mM ammonium sulfate. DNA polymerase activity in TPA-treated (●——●) and non-treated (○——○) cells.

mained the same after 5 days of growth. Thus the parallel increase in DNA polymerase activity and EA/VCA-positive cells suggests that this enzyme is virus-induced. As a control we also treated the EBV-negative BJAB cell line with TPA under the same conditions. The data in Fig. 1*B* show that there is no difference in the activity of DNA polymerase even after TPA treatment of BJAB cells. The low level of activity in BJAB cells is due to the fact that most of the host activities are drastically inhibited under the conditions of the assay. Since 80% of the activity was located in the nuclei, we decided to purify EBV-associated DNA polymerase from nuclear extract. The detailed method of purification is presented under "Materials and Methods."[2]

Effect of Salt—Almost all the herpes virus-specific DNA polymerases with the exception of Marek's disease virus can be distinguished from host cell polymerases by their high salt requirement for optimal activity (1–10). Reports on the effect of salt on EBV-induced DNA polymerase are inconsistent (14–16). As shown in Fig. 5S, the activity of the purified EBV-associated enzyme preparation is clearly dependent upon increased salt concentration with the maximum activity attained at 100 mM ammonium sulfate, a 5-fold increase. In contrast, host DNA polymerases, α and β, are inhibited almost 90 to 100% by the same concentration of salt. This characteristic, therefore, distinguishes the EBV-associated enzyme from host α and β polymerases.

Reaction Requirements—Maximum activity requires all four deoxyribonucleoside triphosphates, DNA, Mg^{2+}, and ammonium sulfate. The optimum pH in 50 mM Tris-HCl buffer is 7 to 7.5, the reaction rates at pH 6.0 and 9.0 being 80% and 63%, respectively, of the rate at the optimum. A divalent metal cation is required for optimal activity, the optima for $MgCl_2$ being 10 mM; Ca^{2+} is inhibitory to the reaction. The apparent K_m for TTP is about 1.25 μM.

Effect of PAA and N-Ethylmaleimide—Since all the herpes virus DNA polymerases have been reported to be inhibited specifically by PAA (27–30), we checked the effect of this inhibitor on the EBV DNA polymerase (Fig. 6S*A*). As expected, the EBV-associated enzyme is sensitive to PAA with 90% of the activity abolished by 6 μg/ml of the inhibitor. However, the α polymerase was also sensitive, and 40% of the activity was abolished under the same conditions (Fig. 6S*A*). In contrast, β polymerase is more resistant to the inhibitor; about 70% activity remains at 50 μg/ml.

EBV DNA polymerase is also inactivated by *N*-ethylmaleimide. At a concentration of 0.5 mM almost 70% of the activity is inhibited. This is also true for α polymerase (Fig. 6S*B*), but β polymerase activity is only slightly inhibited by 2 mM *N*-ethylmaleimide. Thus the effect of salt (Fig. 5S) and inhibitors (Fig. 6S) clearly distinguishes the viral enzyme from the host polymerases.

Template Primer Requirement for EBV Polymerases—The use of various template primers is another way of differentiating between various host polymerases and the viral polymerase (1–10). As shown in Table IIS, all the DNA polymerases tested effectively use activated calf thymus DNA as template primers, but poorly utilize denatured and native calf thymus DNA as templates. The EBV-associated polymerase utilizes synthetic template primers such as poly(dA)·oligo(dT)$_{12-18}$ much more efficiently than any of the host polymerases. In contrast, synthetic initiated ribopolymers such as poly(rA)·oligo(dT)$_{10}$ and deoxyoligomers such as oligo(dT)$_{10}$ are not utilized well by any of these polymerases.

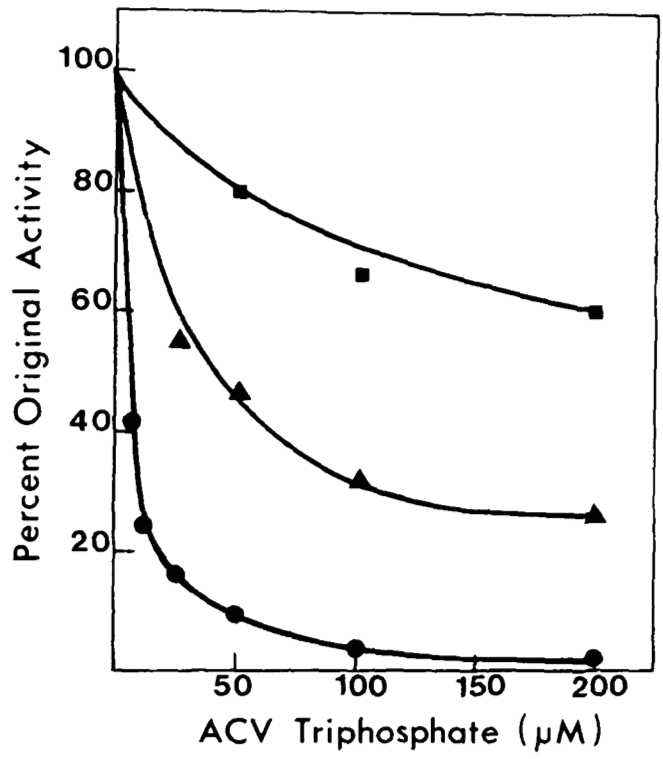

FIG. 7. **Effect of acyclovir (*ACV*) triphosphate on different DNA polymerase activities.** EBV-associated DNA polymerase (●——●), α-DNA polymerase (▲——▲), and β-DNA polymerase (■——■). The method of assay has been described under "Materials and Methods" with the difference that indicated amounts of acyclovir triphosphate were included in the assay. For assay of viral-associated enzyme, 100 mM ammonium sulfate was included in the reaction mixture.

These results thus indicate that EBV-associated DNA polymerase is an enzyme with characteristics distinct from γ polymerase (26, 31), reverse transcriptase (32), and deoxyterminal transferase (33).

Effect of Acyclovir Triphosphate—Studies on the mechanism of action of acyclovir, a potent antiviral compound, have shown that the compound is converted to triphosphate in the herpes virus-infected cell; the first step of phosphorylation is carried out by virus-specified thymidine kinase. The triphosphorylated compound thus formed inhibits viral DNA synthesis by inhibiting viral DNA polymerase (34). In Fig. 7 it is shown that EBV-associated DNA polymerase is also strongly inhibited by the triphosphorylated form of the drug; only 25% of the residual activity is detected at a concentration of 6 μM, whereas α and β polymerases show 55% and 90% of the residual activity, respectively, at the same concentration of the drug.

DISCUSSION

We report here a rapid and simple procedure for purification of EBV-associated DNA polymerase from an EBV-producing cell line. The purification resulted in about a 300-fold increase in specific activity of the enzyme compared to crude nuclear extract with concomitant separation from host α, β, and γ polymerases. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of the purified protein shows four major and several minor bands. Because the native enzyme does not enter polyacrylamide gels in the absence of sodium dodecyl sulfate, it has not yet been possible to determine which stainable band represents active enzyme protein; neither do we know the subunit composition (if any) of the protein. The preparation does have a low amount of deoxyribonuclease (possibly exonuclease) activity, but at this point we cannot say whether it is DNA polymerase-associated activity or any other independent activity. The success of this procedure is dependent upon two major steps. First, the use of TPA to obtain a sufficient quantity of virus-associated DNA polymerase activity (Fig. 1*A*). The inability of the EBV-negative BJAB cell line to produce salt-resistant enzyme after TPA treatment (Fig. 1*B*) provides strong evidence that the induced enzyme is virus-associated and not due to other biochemical side effects of TPA (35). Second, different DNA polymerases have different affinities for DNA and can be extracted from the nuclei at different salt concentrations (36). This differential affinity of the enzymes toward DNA interferes with the chromatographic separation of the enzymes and results in artifacts.

The use of intervent dilution chromatography, a procedure originally devised by Kirkegaard *et al.* (37, 38) and subsequently applied by Valenzuela *et al.* (39) to purify eukaryotic RNA polymerases, has helped to circumvent the problem. The procedure combines ion exchange and gel filtration in the same column (Fig. 2S). By adjusting the pH and ionic strength of the column it was possible to elute the viral enzyme and α polymerase in the excluded peak (*Peak I*) of noninteracting macromolecules and before the liquid column volume so that the enzyme is eluted in the sieving range. Under the conditions of loading (as described under "Materials and Methods"), nucleic acids interact much more strongly with the ion exchanger and are easily resolved from the enzyme. The enzymes (or proteins) which have higher affinity for the DNA are retarded and eluted in the *Peak II* fraction (Fig. 2S). The *Peak II* fraction contained β polymerase activity as detected by *N*-ethylmaleimide resistance with some α polymerase cross-contamination. Although the *Peak II* fraction had some poly(rA)·oligo(dT)$_{10}$ activity, it was negligible compared to the β polymerase activity. This may be due to the fact that the starting material of our enzyme preparation is nuclei and not the whole cell. Thus the intervent dilution chromatography step helped to detect both α and β polymerase activities in the same column.

In the next step of DEAE-cellulose chromatography, most of the virus-associated activity elutes in the flow-through fraction leaving contaminating α polymerase activity bound to the column which can be eluted with 160 mM ammonium sulfate (Fig. 3S). During phosphocellulose column chromatography (Fig. 4S) a small shoulder of activity occasionally appears along with the major activity peak. We could not detect any difference between the two peaks in terms of salt stimulation and inhibitor sensitivities, but to avoid any possibility of cross-contamination we did all of our studies with the major phosphocellulose peak fraction.

Weissbach *et al.* (2) and several other workers (6–9) reported that most of the herpes virus-related polymerases with the exception of Marek's disease virus polymerase (5) are stimulated severalfold in the presence of 100 to 150 mM ammonium sulfate or potassium sulfate. However, several other reports on the salt stimulatory property of EBV-associated DNA polymerase are conflicting (14–16). This is probably due to the fact that the enzyme preparations were not pure and were contaminated with host enzymes which are sensitive to high ionic strength (26). Our unpublished data suggest that the amount of stimulation by salt is also dependent upon the quality of the activated DNA. Allaudeen and Bertino (40) reported detection of an enzyme from a patient's tissue with American Burkitt's lymphoma which is also stimulated by 100 mM ammonium sulfate.

A number of studies (27–31) have been published on the specificity of inhibition of viral DNA synthesis by PAA. This effect was attributed to specific inhibition of virus-induced DNA polymerase activity. Our studies, like others (11, 16, 27), also show that PAA inhibited not only EBV-associated DNA polymerase, but also host α polymerase and that the viral enzyme is more sensitive than α polymerase (Fig. 6S*A*). Similarly both EBV and α polymerases are inhibited by *N*-ethylmaleimide (Fig. 6S*B*), but β polymerase in contrast is relatively resistant to both inhibitors. These results are consistent with the data obtained with intact EBV nuclei (41) as well as for other herpes virus-induced DNA polymerases (1–10) and probably indicate that sensitivity to PAA and *N*-ethylmaleimide is a general property of all the herpes virus-induced DNA polymerases. Our results on salt activation and PAA and *N*-ethylmaleimide inhibition are in agreement with the results of Bolden *et al.* (42) and others (14, 16) but differ significantly from the results of Goodman *et al.* (15).

There are several reasons to believe that the enzyme activity we are measuring is not due to mycoplasma DNA polymerase activity (43). First, the induction of salt-stimulated activity in P3HR-1 cells by TPA with respect to identically subcultured P3HR-1 cells in the absence of TPA makes it unlikely (Fig. 1*A*). Second, the isolation procedure is carried out with partially purified nuclei isolated after hypotonic shock. Lastly, the mycoplasma enzyme is insensitive to *N*-ethylmaleimide and PAA, and is inhibited by 100 mM ammonium sulfate (43).

The EBV-associated DNA polymerase can efficiently use activated calf thymus DNA and synthetic deoxyoligomer-homopolymers, poly(dA)·oligo(dT)$_{12-18}$, as primer templates, but native and denatured calf thymus DNA was relatively inactive. This property resembles somewhat that of an enzyme found in the herpes simplex system (2). No significant reverse transcriptase or deoxyterminaltransferase activities are detected in our EBV DNA polymerase preparation.

The different chromatographic behavior, the high efficiency

of poly(dA)·oligo(dT)$_{12-18}$ as template primer, and the inability to detect such activity in EBV-negative BJAB cells (21) are all consistent with the hypothesis that this virus-associated DNA polymerase is coded by the virus genome. These observations do not, however, rule out the possibility that the appearance of this new DNA polymerase is due to derepression of a host cell enzyme that is not detectable in normal cells or to a modification of a pre-existing host enzyme as a result of the presence of virus genome within the cell. The final proof that this enzyme is not a modified cellular enzyme will come from its total purification and characterization and by detailed genetic studies. Moreover, purification of the enzymes will enable us to study in detail the sensitivity and interaction of the enzyme to antiviral drugs, in particular to [9-(2-hydroxyethoxymethyl)guanine], a new antiviral compound effective against herpes group viruses including EBV (34) (Fig. 7).[3] Work is in progress in this direction.

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INDUCTION OF EPSTEIN-BARR VIRUS-ASSOCIATED DNA POLYMERASE BY 12-O-TETRADECANOYLPHORBOL-13-ACETATE

Purification and Characterization

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MATERIALS AND METHODS

Cells. The Burkitt lymphoma-derived cell line P3HR-1 (gift of Dr. E. Kieff) (20), was maintained between 5 x 10^5 and 10^6 cells/ml in RPMI-1640 medium containing 10% heat-inactivated fetal calf serum supplemented with 100 IU/ml penicillin and 100 µg/ml streptomycin. The EBV-negative lymphoblastoid cell line, BJAB (21) (obtained from Dr. G. Klein) was grown and maintained under the same conditions.

Chemicals. TPA was from Sigma Chemical Corporation, synthetic deoxy and ribohomopolymer-oligomer templates from P-L Biochemicals and [^{3}H]-labeled nucleoside triphosphates were from New England Nuclear Corporation. Acyclovir [9-(2-hydroxyethoxymethyl)guanine] (ACV) triphosphate was the kind gift of Dr. G. B. Elion of Burroughs Wellcome Company, Research Triangle Park.

Induction with TPA: P3HR-1 cells were seeded at 10^6 cells/ml in culture medium. After 24 hr of cultivation at 37°, TPA was added at a final concentration of 25 ng/ml. The culture was incubated at 37° for the indicated period of time. The determination of EBV EA/VCA-positive (early antigen/viral capsid antigen) cells was carried out on cell smears by the indirect immunofluorescence test as described by Henle and Henle (22).

Assay of DNA Polymerase. DNA polymerase was assayed as follows: The reaction mixture (100 µl) contained 25 mM Hepes, pH 8.0, 10 mM $MgCl_2$, 2 mM dithiothreitol (DTT), 0.2 mM EDTA, 20 µg/ml of dialyzed bovine serum albumin (BSA), 10 µg of activated calf-thymus DNA, 4 µM [^{3}H]-TTP (1000-1200 cpm/pmol), 20 µM each of dATP, dGTP and dCTP and the required amount of enzyme. Ammonium sulfate (100 mM) was included in the assay mixture as indicated. The reaction was carried out at 37° for 30 min, after which it was stopped with 10% TCA. The precipitate was collected on GF/C filter paper, washed with 2% TCA and alcohol and radioactivity determined in toluene-based solvent. At 37° the reaction is linear for at least one hour.

Activated calf-thymus DNA was prepared according to the method described previously (23) with some modifications. In brief, DNA at a concentration of 500 µg/ml in 75 mM Tris-HCl pH 7.6, 20 mM NaCl, 7.5 mM $MgCl_2$ and 750 µg/ml BSA was incubated for 15 min at 32° with 10 ng/ml of DNase and was followed by another incubation for 5 min at 77°. The DNA treated in this manner was rendered 25% acid-soluble. Denatured DNA was prepared by heating a DNA solution at 100° for 15 min followed by immediate chilling in ice.

One unit of enzyme is defined as that amount which incorporated one pmol of TMP into DNA under the standard assay condition described. The specific activity is defined as units per mg of protein. Protein was estimated by the method of Lowry et al. (24).

Preparation of Resins. DE-52 cellulose and phosphocellulose (P 11) (Whatman) were washed according to the method described by Buhler et al. (25). DEAE-Sephadex A-25 (Pharmacia Inc. 3.5 ± 0.5 meq/g of dry resin) was used for intervent dilution chromatography. Before use, the gel was washed with 10-20 volumes of 0.1 M NaOH, followed by sufficient 0.2-0.5 M HCl to make the chloride form of the resin. After excess acid was removed with water, the ion-exchange gel was equilibrated with the appropriate buffer until the pH of the wash buffer was same as the input buffer.

Buffers. Buffer A contained 50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 5 mM DTT, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.2% NP-40, 20% glycerol, and 300 mM ammonium sulfate, Buffer B had 50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF, and 20% glycerol. Buffer C was made of 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF and 20% glycerol.

Preparation of DNA Polymerases α and β. To compare EBV-induced DNA polymerase with host α and β-polymerases, partially purified cellular polymerases were prepared. DNA polymerase α is isolated as a by-product of DE-52 cellulose chromatography (Fig. 3S). For preparation of β-polymerase activity, the fractions (Peak II) from the DEAE-Sephadex (A-25) column were pooled and dialyzed against Buffer C containing 20 mM ammonium sulfate. The dialyzed fraction was loaded on a DE-52 cellulose column equilibrated with the dialyzing buffer. Most of the β-polymerase activity comes out in the flow-through fraction as indicated by N-ethylmaleimide (NEM) resistance. The flow-through fraction was pooled and loaded on a phosphocellulose column equilibrated with Buffer B containing 50 mM ammonium sulfate. After loading, the column was washed with the same buffer, and the enzyme was eluted with Buffer B containing 300 mM ammonium sulfate. The activity was checked for NEM resistance and was used for comparative studies.

Preparation of Nuclear Extract. Cells were harvested after 2 days of TPA treatment and washed with ice-cold PBS. For preparation of nuclei, the washed cells were kept suspended in hypotonic buffer (50 mM Tris-HCl, pH 7.5, 10 mM KCl, 5 mM $MgCl_2$, and 0.25 M sucrose) for 10-15 min. A cell homogenate was prepared with a Dounce homogenizer and centrifuged at 800 x g for 5 min. To the supernatant fraction DTT was added to a concentration of 5 mM and centrifuged at 10,000 x g for 30 min, and at 100,000 x g for 60 min. This supernatant material was used as the cytoplasmic fraction. The crude nuclear pellet obtained by low-speed centrifugation was washed two more times with hypotonic buffer and suspended in Buffer A. The viscous suspension was sonicated 2 or 3 times at maximum output for 15 sec on a Branson Sonifier with a 1-min interval between each sonication. The suspension was centrifuged at 10,000 x g for 30 min, and at 100,000 x g for 60 min. The clear supernatant fluid obtained was called "nuclear lysate".

Purification of EBV-Associated DNA Polymerase from Nuclei. All the operations were carried out at 0-4°C unless otherwise stated.

Intervent Dilution Chromatography. Nuclear extract (2.5 ml) from P3HR-1 cells treated with TPA for 2 days was loaded on a column (2.3 x 18 cm) packed with DEAE-Sephadex A-25. The column was previously equilibrated with Buffer B, containing 100 mM ammonium sulfate. When the sample goes completely into the bed, the column was developed with the same buffer containing 300 mM ammonium sulfate. Fig. 2S shows the elution pattern and the enzyme activity. There are two major absorbancy peaks at 280 nm. When the assay for activity is carried out in the presence of 100 mM ammonium sulfate, the first peak (I), which had lower activity than the second peak (II) in the absence of salt, is stimulated almost 3-fold, whereas the Peak II activity almost completely disappears. Since almost all the herpesviruses produce salt-stimulated DNA polymerase activity, we selected Peak I for isolation of EBV-induced DNA polymerase. This step produced almost 6-fold purification (Table IS) in comparison to nuclear extract. Moreover this step led to considerable removal of nucleic acids which interferes with the chromatographic behavior of the enzyme; the A_{260}/A_{280} ratio went from 0.50 to 1.45. Also, there is an apparent increase in polymerase activity after this step (Table IS) perhaps due to loss of inhibitory materials during purification.

DE-52 Cellulose Column Chromatography. Fractions from the DEAE-Sephadex (A-25) column were pooled and dialyzed for 4 hours against Buffer C containing 20 mM ammonium sulfate with one change. The precipitate which sometimes formed during dialysis had no activity and was removed by centrifugation. The dialyzed enzyme fraction was further purified through a DE-52 cellulose column. DE-52 cellulose suspended in the dialysis buffer was deaerated and packed into a chromatographic column (1.5 x 20 cm). After equilibration with the same buffer, the column was charged with the sample slowly. After washing, the elution was carried out with Buffer C containing a linear gradient of ammonium sulfate from 20 mM to 300 mM. Both unabsorbed and gradient fractions (2 ml each) were collected. Fig. 3S shows results of one such chromatographic procedure. Most of the salt-stimulated activity comes out in the flow-through fraction leaving some activity bound to the column which was identified as α-polymerase and is eluted with about 160 mM ammonium sulfate. A further 2.5-fold purification was achieved with this step with almost 100% recovery of the virus-associated enzyme. In addition this step led to removal of contaminating host α-polymerase.

Phosphocellulose Column Chromatography. The flow-through fraction from the DE-52 cellulose column was diluted twice with Buffer B and loaded on a phosphocellulose column (1.1 x 5 cm) prequilibrated with Buffer B containing 10 mM ammonium sulfate. After washing the column, the elution was carried out with Buffer B containing a linear gradient of ammonium sulfate from 10 mM to 200 mM. The bulk of the protein was eluted in the wash fraction (Fig. 4S). The enzyme was eluted in the fractions between approximately 20 mM and 100 mM ammonium sulfate, but the major activity is eluted at about 40 mM salt (Fig. 4S). The first three peak fractions (fractions 18-20) having highest specific activities were pooled and used for most of the experiments described here. The phosphocellulose step produced maximum purification of the enzyme with about 23-fold purification from the DE-52 cellulose step. A 300-fold purification is achieved with about 63% recovery of units as calculated from nuclear extract (Table IS).

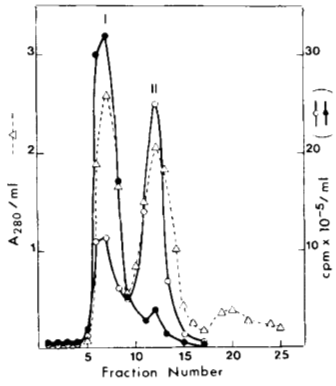

Figure 2S. Intervent dilution column chromatography of nuclear extract from TPA-treated P3HR-1 cells. Details of the procedure are described under Materials and Methods. Fractions (2.5 ml) were collected at 3 minute intervals and assayed for protein by absorbance at 280 nm (---△---) and DNA polymerase activity in presence (-●-) and absence (-○-) of 100 mM ammonium sulfate.

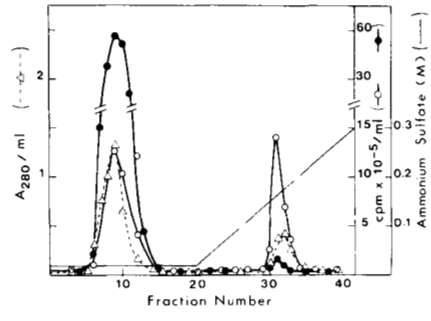

Figure 3S. DE-52 cellulose column chromatography. Elution was carried out as described in Materials and Methods. Each fraction were assayed for protein by absorbance at 280 nm (---△---) and DNA polymerase activity in presence (-●-) and absence (-○-) of 100 mM ammonium sulfate.

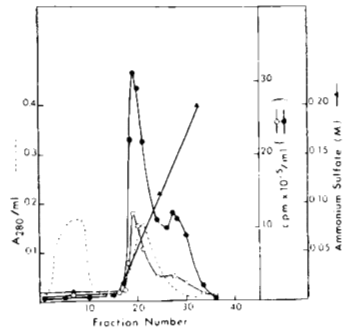

Figure 4S. Phosphocellulose column chromatography. A linear gradient elution was carried out with increasing concentration of ammonium sulfate from 10 mM to 200 mM in Buffer B; 1 ml fractions were collected. Assay of DNA polymerase was carried out in presence (-●-) and absence (-○-) of 100 mM ammonium sulfate.

TABLE IS

PURIFICATION OF EBV-ASSOCIATED DNA POLYMERASE FROM TPA-INDUCED P3HR-1 CELLS

Fraction	Protein (mg/ml)	Total Protein (mg)	Total* Activity (units)	Recovery %	Specific Activity (Units/mg)	Purification (fold)
Crude Nuclear	32.0	80	20,000	100	250	-
DEAE-Sephadex (A-25)	3.2	24	34,275	171	1,428	5.7
DE-52 Cellulose	1.1	9.9	32,400	162	3,273	13
Phospho-cellulose	0.08	0.17	12,600	63	75,000	300

*Assays were done in presence of 100 mM ammonium sulfate. Purification was carried out with 10^9 cells.

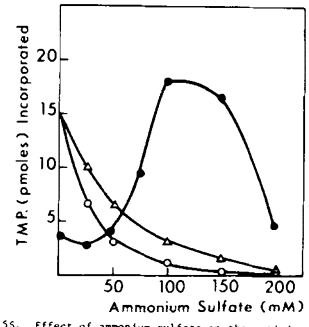

Figure 5S. Effect of ammonium sulfate on the activity of phosphocellulose purified EBV-associated DNA polymerase and its comparison with host polymerase activities. The reaction mixture and other assay conditions are described in "Materials and Methods"; only the concentration of ammonium sulfate was varied. 0.4 µg of purified EBV-associated enzyme was used. (-●-) EBV polymerase, (-○-) α-polymerase and (-△-) β-polymerase.

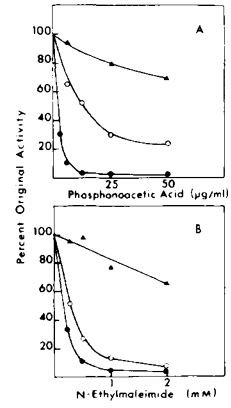

Figure 6S. Effect of phosphonoacetic acid (A) and N-ethylmaleimide (B) on different DNA polymerase activities. EBV-associated DNA polymerase (-●-), α-DNA polymerase (-○-) and β-DNA polymerase (-▲-). The method of assay has been described in the Materials and Methods with the difference that in the experiments described in Fig. 6(B) DTT was omitted from the reaction mixture. For assay of viral-associated enzyme 100 mM ammonium sulfate was included in the reaction mixture.

TABLE IIS

TEMPLATE-PRIMER SPECIFICITY OF EBV-ASSOCIATED DNA POLYMERASE AND ITS COMPARISON WITH HOST POLYMERASES

Template-Primer	Substrate	Concentration (µg/ml)	Percent DNA Polymerase Activity EBV	α	β
No DNA	[^{3}H]-dNTP	0	0	0	0
Activated Calf Thymus DNA	[^{3}H]-dNTP	50	100	100	100
Native Calf Thymus DNA	[^{3}H]-dNTP	50	16	8	12
Denatured Calf Thymus DNA	[^{3}H]-dNTP	50	15	0	8
Poly(rA).oligo(dT)$_{10}$	[^{3}H]-TTP	50	0	0	0
Poly(dA).oligo(dT)$_{12-18}$	[^{3}H]-TTP	50	1220	16	90

Assay was carried out as described in the "Materials and Methods". DNA polymerase activity expressed when assayed with activated calf-thymus DNA was taken as 100%, corresponding to (EBV-DNA polymerase), 4 pmol, (α-polymerase), 32 pmol, and (β-polymerase) 5 pmol of [^{3}H]-TMP incorporated in acid-insoluble product. [^{3}H]-dNTP refers to a mixture of [^{3}H]-TTP and cold dGTP, dCTP, and dATP.