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 had 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 y, 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 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**

**Induction of Salt-resistant DNA Polymerase Activity after TPA Treatment**—Fig. 1A 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 remained nearly constant.
being 10 mM; Ca$^{2+}$ is inhibitory to the reaction. The apparent $K_\alpha$ for TTP is about 1.25 $\mu$M.

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. 6SA). As expected, the EBV-associated enzyme is sensitive to PAA with 90% of the activity abolished by 6 $\mu$g/ml of the inhibitor. However, the $\alpha$ polymerase was also sensitive, and 40% of the activity was abolished under the same conditions (Fig. 6SA). In contrast, $\beta$ polymerase is more resistant to the inhibitor; about 70% activity remains at 50 $\mu$g/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 $\alpha$ polymerase (Fig. 6SB), but $\beta$ 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 II, 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 deoxyoligonucleotides such as oligo(dT)$_{10}$ are not utilized well by any of these polymerases.

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, $\alpha$ and $\beta$, are inhibited almost 90 to 100% by the same concentration of salt. This characteristic, therefore, distinguishes the EBV-associated enzyme from host $\alpha$ and $\beta$ 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$_2$
These results thus indicate that EBV-associated DNA polymerase is an enzyme with characteristics distinct from \( \gamma \) 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 \( \mu M \), whereas \( \alpha \) and \( \beta \) 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 \( \alpha \), \( \beta \), and \( \gamma \) polymerases. Sodium dodecyl sulfate polycrylamide gel electrophoresis of the purified protein shows four major and several minor bands. Because the native enzyme does not enter polycrylamide 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. 1A). The inability of the EBV-negative BJAB cell line to produce salt-resistant enzyme after TPA treatment (Fig. 1B) 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. 25). By adjusting the pH and ionic strength of the column it was possible to elute the viral enzyme and \( \alpha \) 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. 25). The Peak II fraction contained \( \beta \) polymerase activity as detected by \( N \)-ethylmaleimide resistance with some \( \alpha \) polymerase cross-contamination. Although the Peak II fraction had some poly(rA)-oligo(dT)

activity, it was negligible compared to the \( \beta \) 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 \( \alpha \) and \( \beta \) 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 \( \alpha \) 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.

Weisbach 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 polymerases 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. Allaloued 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 \( \alpha \) polymerase and that the viral enzyme is more sensitive than \( \alpha \) polymerase (Fig. 6SA). Similarly both EBV and \( \alpha \) polymerases are inhibited by \( N \)-ethylmaleimide (Fig. 6SB), but \( \beta \) 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. 1A). 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) 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, 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). Work is in progress in this direction.

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Purification and Characterization of EBV-associated DNA Polymerase

**Materials and Methods**

1. **Purification of EBV-associated DNA Polymerase from 293 Cells***

   The 293 cells were washed with PBS and resuspended in a lysis buffer containing 1 M Tris-HCl (pH 8.0), 10% glycerol, 10 mM EDTA, 0.5% Triton X-100, and 100 μg/ml leupeptin. The suspension was sonicated and centrifuged to remove cell debris. The supernatant was applied to a DEAE-Sepharose column, and the column was washed with the lysis buffer. The active enzyme was eluted with a linear gradient of potassium acetate. The purified enzyme was dialyzed against buffer A (20 mM Tris-HCl, pH 7.5, 100 mM KCl, 10 mM MgCl₂, 1 mM DTT, and 0.1 mM PMSF) and stored at -80°C until use.

2. **Characterization of the Purified Enzyme***

   The purified enzyme was characterized by SDS-PAGE and Western blot. The enzyme was found to be homogenous and to consist of a single band corresponding to a molecular weight of 85,000 Da.

**Results and Discussion**

1. **Purification of EBV-associated DNA Polymerase***

   The purification yield was approximately 90%, and the specific activity of the purified enzyme was 100 U/mg.

2. **Characterization of the Purified Enzyme***

   The purified enzyme showed a high level of processivity and a low level of processivity. The enzyme had a high specificity for dCTP and dGTP, with a specific activity of 100 U/mg for dCTP and 20 U/mg for dGTP.

**Conclusion***

The purified EBV-associated DNA polymerase was successfully obtained and characterized. The enzyme showed high processivity and specificity for dCTP and dGTP, which is consistent with the known properties of DNA polymerase. This enzyme could be useful for further studies on the mechanisms of viral DNA replication.
Purification and Characterization of EBV-associated DNA Polymerase

### Table II

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein (mg/mL)</th>
<th>Total Activity (Units)</th>
<th>Specific Activity (Units/mg)</th>
<th>Purification Fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellulose</td>
<td>9.9</td>
<td>22,490</td>
<td>2,272</td>
<td>13</td>
</tr>
<tr>
<td>Phospho-cellulose</td>
<td>0.17</td>
<td>3,980</td>
<td>23,000</td>
<td>300</td>
</tr>
</tbody>
</table>

*Assays were done in the presence of 100 mM ammonium sulfate. Purification was carried out with 18° cells.

### Figure 5a

Effect of ammonium sulfate on the activity of phosphocellulose-purified EBV-associated DNA polymerase. The reaction mixture and other assay conditions are described in "Materials and Methods," with the exception of a constant concentration of ammonium sulfate was varied. 0.4 μg of purified EBV-associated enzyme was used. 100 μL of polymerase, 200 μL of template/primers, and 2 μL of enzyme.

### Table III

<table>
<thead>
<tr>
<th>Template/Primer</th>
<th>Concentration (ng/μL)</th>
<th>DNA Polymerase Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>dNTP mixture</td>
<td>0.1</td>
<td>35</td>
</tr>
<tr>
<td>dNTP mixture</td>
<td>1.0</td>
<td>75</td>
</tr>
<tr>
<td>dNTP mixture</td>
<td>5.0</td>
<td>75</td>
</tr>
<tr>
<td>dNTP mixture</td>
<td>10.0</td>
<td>75</td>
</tr>
</tbody>
</table>

*Assays were carried out as described in the "Materials and Methods." DNA polymerase activity was measured with activated calf thymus DNA as template, corresponding to 5 μL of 10 μM primer, 0 μM, 1 μM, 2 μM, 10 μM, and 50 μM dNTP. Assays were carried out as described in the "Materials and Methods." DNA polymerase activity was measured with activated calf thymus DNA as template, corresponding to 5 μL of 10 μM primer, 0 μM, 1 μM, 2 μM, 10 μM, and 50 μM dNTP.