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Isolation of a herpesvirus-specific DNA polymerase from tissues of an American patient with Burkitt lymphoma

(Epstein–Barr virus/DNA nucleotidyltransferase)

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ABSTRACT **A DNA polymerase (DNA nucleotidyltransferase) has been partially purified from a neck mass of an American patient with Burkitt lymphoma and separated from the cellular DNA polymerases. The molecular weight of the enzyme was approximately 90,000. The enzyme differs from the cellular DNA polymerases, but resembles herpes-virus-induced DNA polymerase in its primer template preference, high monovalent cation requirement for activity, and sensitivity to phosphonoacetate. Enzyme activity was inhibited specifically by an antibody directed against herpes-simplex-virus-induced DNA polymerase but not by antibodies directed against DNA polymerase α of HeLa cells and DNA polymerase γ of a normal human lymphoblast cell line, NC37. Although serum of the patient with Burkitt lymphoma contained high Epstein–Barr virus titer, addition of the serum to the assay mixture did not have any effect on the activity of Burkitt lymphoma DNA polymerase. Tissues from spleen and liver of the patient with Burkitt lymphoma did not contain the herpes-virus-induced DNA polymerase. Detection of the herpes virus polymerase in the Burkitt lymphoma tissue provides additional evidence for the association of Epstein–Barr virus with this malignancy.**

A virus etiology of some malignant lymphomas, particularly Burkitt lymphoma, has been suggested by several studies. Epstein–Barr virus (EBV) has been implicated in Burkitt lymphoma as well as in nasopharyngeal carcinoma. However, unlike in some animals, there has been no direct proof of causal relationship. Detection of some viral markers in samples from patients with Burkitt lymphoma has suggested a viral etiology. For example, EBV-specific DNA has been identified in many Burkitt lymphoma biopsies from Africans but in few from Americans (1–4). Antibodies to EBV antigens have been detected in the serum of the patients; Epstein–Barr nuclear antigen has also been detected in the nuclei of some tumor cells (1).

Herpes viruses induce a DNA polymerase (DNA nucleotidyltransferase) in virus-infected cells (5, 6). This enzyme is involved in the replication of the virus DNA (7). The herpes-virus-induced DNA polymerase, require high salt concentration, cellular DNA polymerases α, β, and γ by differences in their properties (6, 8, 9). First, all herpes-virus-induced DNA polymerases, with the single exception of Marek disease herpes-virus-induced DNA polymerase, require high salt concentration, e.g., 100 mM KCl, for maxium activity. At this salt concentration the activities of cellular DNA polymerases α and β are markedly inhibited. Second, they use the synthetic primer template $(dG)_{\sim 15}\cdot(dC)_n$ more efficiently than the cellular DNA polymerases. Third, the virus-induced enzymes are more sensitive to the inhibitor phosphonoacetate. In addition, the virus-induced polymerase can be separated from the cellular enzymes by chromatography on ion-exchange columns.

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Although considerable attention has been directed toward detection of different EBV-specific antibodies in patients and EBV-specific DNA in lymphoma cells, very little is known about the virus-induced DNA polymerase. Twardzik *et al.* (10) compared the total DNA polymerase activities in EBV producer and nonproducer cells in culture. Miller *et al.* (9) reported studies on the EBV-specific DNA polymerase in Burkitt cells in culture induced with IdUrd. However, to our knowledge, no such EBV-induced DNA polymerase has been isolated from tissues of patients with Burkitt lymphoma.

This report describes isolation of a DNA polymerase from tissues of an American patient with Burkitt lymphoma having some properties of the herpes-simplex-virus (HSV)-induced DNA polymerase (6, 8) and an EBV-specific DNA polymerase of Burkitt cells in culture (9).

MATERIALS AND METHODS

Case Report. The patient was a 27-year-old white man with stage IV American Burkitt lymphoma. The patient's serum had a positive EBV titer. The clinical details of this case have been reported (11). Three months before the patient's illness, his in-laws returned from a vacation in Tanzania and Kenya. They purchased a shirt for him from an African village, which he wore frequently. He gave no history of mononucleosis.

The chemicals used and the enzyme assay systems have been described (12). Antibodies directed against HSV DNA polymerase and DNA polymerase α of HeLa cells were kindly provided by A. Weissbach, Roche Institute of Molecular Biology, Nutley, NJ. Antibodies directed against DNA polymerase γ of a normal human lymphoblast cell line, NC37, and DNA polymerase γ of the homologous cell line were gifts from M. Robert-Guroff, National Institutes of Health, Bethesda, MD. A double-antibody immunoprecipitation assay (13) was used to determine binding of the antibody directed against DNA polymerase γ with DNA polymerase from Burkitt lymphoma.

Isolation of DNA Polymerase from Burkitt Lymphoma Tissue. The tissue from a neck mass removed from the patient was used for isolation of the polymerases. The details of the isolation procedure have been described (12). Essentially, the tissues were minced and suspended in 5 vol of 0.5% Triton X-100/1 M KCl and disrupted with a Dounce homogenizer. An equal volume of buffer A (50 mM Tris·HCl, pH 7.0/1 mM dithiothreitol/0.5 mM EDTA/20% glycerol) was added. Nucleic acids were removed by passing the total cell extract through a DEAE-cellulose column that had been equilibrated in buffer A containing 0.3 M KCl. The extract was applied to a phosphocellulose column that had been equilibrated with buffer A (pH 8.0) and developed with a linear gradient of 0–0.6 M KCl.

Abbreviations: EBV, Epstein–Barr virus; HSV, herpes simplex virus.

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The fractions were assayed with activated DNA and $(dT)_{\sim 15}\cdot(A)_n$ both with and without 100 mM KCl in the assay mixture. Samples of the same fractions were also assayed for reverse transcriptase and terminal deoxyribonucleotidyltransferase activities. A similar procedure was used to isolate the DNA polymerases from spleen tissue of the same patient. HSV-induced DNA polymerase was isolated from HeLa cells after they were infected with the virus by the procedure described by Weissbach *et al.* (6).

RESULTS

Presence of a Monovalent Cation-Dependent DNA Polymerase Activity. The fractions of the phosphocellulose column contained all the three cellular DNA polymerase activities (Fig. 1*A*). DNA polymerases α, β, and γ eluted at 0.19, 0.45, and 0.24 M KCl, respectively. Besides the cellular DNA polymerase activities, there was an additional enzyme activity that eluted at 0.3 M KCl. When samples of the phosphocellulose fractions were assayed with activated DNA in the presence of 100 mM KCl, the only major activity peak obtained was at this 0.3 M KCl region. The column fractions contained neither reverse transcriptase nor terminal deoxyribonucleotidyltransferase activities.

General Properties of the DNA Polymerase. The enzyme that eluted at 0.3 M KCl was used for further analyses; it represented 27–30% of the total cellular DNA polymerase activity. Cellular DNA polymerases α, β, and γ represented approximately 31, 19, and 1%, respectively. The estimations were based on their activities with activated DNA template. The salt-dependent polymerase activity was maximum with Tris·HCl, pH 8.0; phosphate buffer inhibited the enzyme reaction. DNA synthesis was linear up to at least 2 hr at 37°C.

Molecular Weight of the Polymerase. The molecular weight of the enzyme was determined by velocity sedimentation in glycerol gradient and gel filtration with a calibrated Sephadex G-150 column as described (12). Ovalbumin, bovine serum albumin, and aldolase were used as standard protein markers. The molecular weight of the enzyme was approximately 90,000 (Fig. 2).

Effect of Monovalent Cation on Activity of the Polymerase. One of the distinguishing properties of the herpes-virus-induced DNA polymerase is its requirement for high salt for activity. Activity with activated DNA template with increasing concentrations of ammonium sulfate is shown in Fig. 3. DNA polymerase α and β activities decrease with increasing concentrations of the salt, while the Burkitt lymphoma DNA polymerase activity was maximum at 100 mM ammonium sulfate. There was a 3- to 4-fold increase in the activity. A similar stimulatory effect was observed when ammonium sulfate was replaced by either K_2SO_4 or NaCl (see Table 2).

Primer Template Specificity. The ability of the Burkitt lymphoma DNA polymerase to use activated DNA and various synthetic primer templates was tested. Table 1 compares the Burkitt lymphoma DNA polymerase with the cellular DNA polymerases α and γ isolated from L1210 cells (14). As with other DNA polymerases, the Burkitt lymphoma enzyme uses activated DNA. The enzyme also efficiently copies $(dG)_{\sim 15}\cdot(dC)_n$; however, it copies $(dT)_{\sim 15}\cdot(dA)_n$ and $(dT)_{\sim 15}\cdot(A)_n$ with only moderate but equal efficiency. Although the Burkitt lymphoma DNA polymerase can copy $(dT)_{\sim 15}\cdot(A)_n$, it does not compare with DNA polymerase γ, which has an excellent ability to copy this primer template.

Effect of Omission of Deoxyribonucleoside Triphosphates. To determine whether there was any difference between the cellular and Burkitt lymphoma DNA polymerases, we tested the activities of Burkitt lymphoma DNA polymerase and DNA polymerases α and β of L1210 cells (14) in the absence of one or more of the deoxyribonucleoside triphosphates. Activity of all the three enzymes was markedly decreased; for example, the Burkitt lymphoma DNA polymerase, DNA polymerase α, and DNA polymerase β incorporated 19, 9, and 18%, respec-

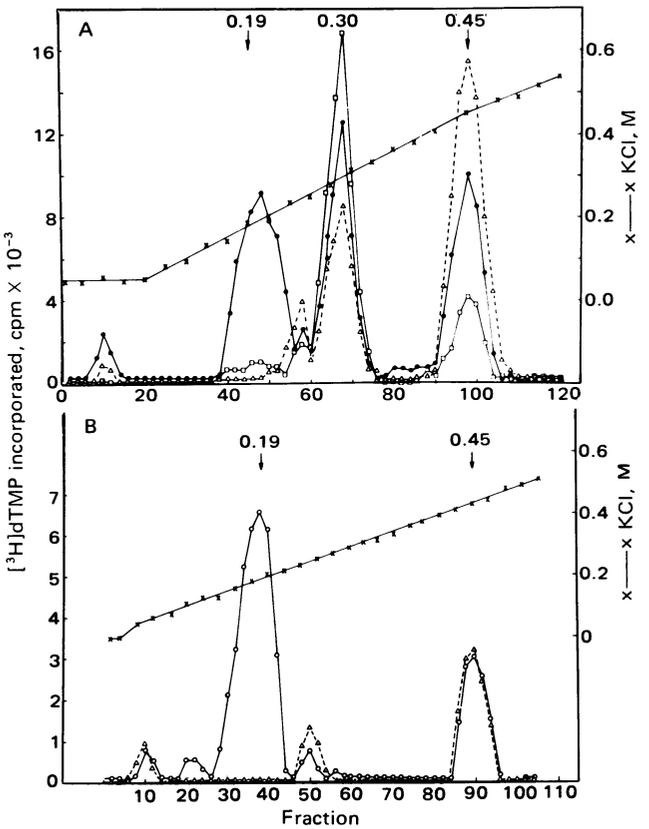

FIG. 1. (*A*) Phosphocellulose chromatography. DEAE-cellulose eluate of total cell extract was dialyzed in buffer A (pH 8.0). Phosphocellulose column (20 ml) was equilibrated in the same buffer. Extract was applied to the column and developed with 200 ml of a linear gradient 0–0.6 M KCl. Fractions of 2 ml were collected, and 10 μl of alternate fractions were used for assay of DNA polymerase activities with activated DNA (●—●) and $(dT)_{\sim 15}\cdot(A)_n$ (△- - -△). Samples of the fractions were also assayed with activated DNA after 100 mM KCl was added to the reaction mixture (□—□). DNA polymerase α activity was assayed in a 50-μl standard reaction mixture that contained 50 mM Tris·HCl (pH 7.5); 2 mM dithiothreitol; 8 mM $MgCl_2$; 100 μM each of dATP, dCTP, and dGTP; 80 μM [^{3}H]dTTP (450 cpm/pmol); 12.5 μg of activated salmon sperm DNA; and enzyme. Incubation was at 37° for 30 min. Acid-insoluble radioactivity was collected on a Gelman nitrocellulose filter, washed several times with 5% trichloroacetic acid containing 2 mM sodium pyrophosphate and once with 70% ethanol, and measured in a liquid scintillation counter. DNA polymerase β activity was assayed under similar conditions except that pH 8.5 buffer and 40 mM KCl were used. DNA polymerase γ activity was assayed in a 50-μl reaction mixture that contained 50 mM Tris·HCl (pH 8.0), 2 mM dithiothreitol, 1 mM $MnCl_2$, 100 mM KCl, 20 μM [^{3}H]dTTP (1800 cpm/pmol), 1 μg of $(dT)_{\sim 15}\cdot(A)_n$, and enzyme. The reaction mixture for assay of reverse transcriptase activity (12) contained the following ingredients in a total volume of 50 μl: 50 mM Tris·HCl (pH 8.0), 1 mM $MnCl_2$, 80 mM KCl, 2 mM dithiothreitol, 40 μM [^{3}H]dGTP (900 cpm/pmol), 0.5 μg of $(dG)_{\sim 15}\cdot(C^m)_n$, and enzyme. Terminal deoxyribonucleotidyltransferase activity was assayed in a 50-μl reaction mixture that contained 50 mM Tris·HCl (pH 8.0), 2 mM dithiothreitol, 0.6 mM $MnCl_2$, 2 μg of $(dA)_{\sim 15}$, 200 μM [^{3}H]dGTP (300 cpm/pmol), and enzyme (14). (*B*) Phosphocellulose chromatography of DNA polymerases of spleen tissue from the same patient with Burkitt lymphoma. Other details are as described for *A*.

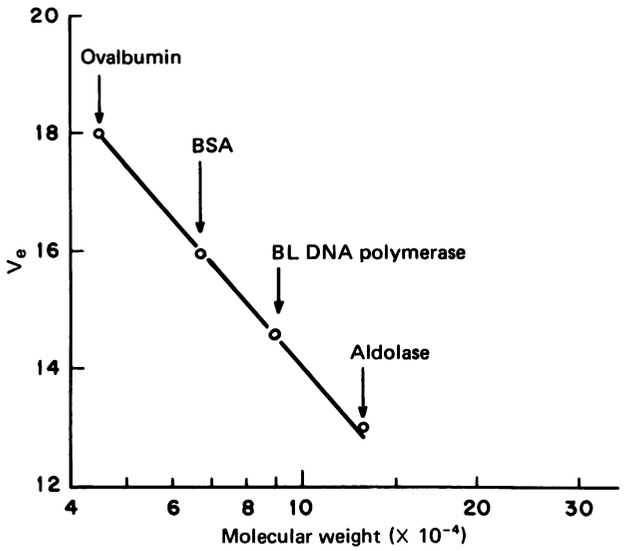

FIG. 2. Column of Sephadex G-150 equilibrated in buffer A containing 0.3 M KCl was used for determination of molecular weight of Burkitt lymphoma (BL) DNA polymerase. Aldolase, bovine serum albumin (BSA), and ovalbumin (5 mg each) were used as protein markers. Elution positions of these proteins were determined by measurement of absorbance at 280 nm.

tively, of radioactive dTMP in the absence of all the other three nucleoside triphosphates. Further nucleotide deletion experiments with one or more of the triphosphates omitted did not reveal any significant difference among the enzymes tested.

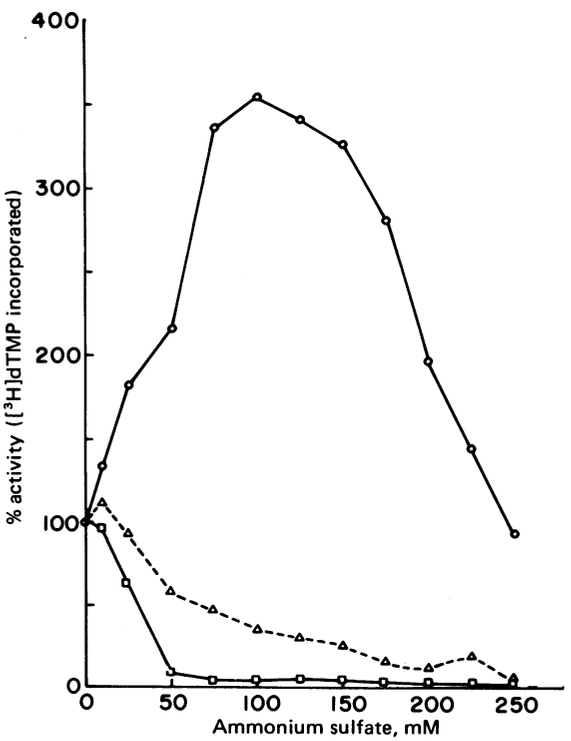

FIG. 3. Effect of monovalent cation on the activities of the DNA polymerases. Enzyme assay conditions were as described in the legend to Fig. 1 except for addition of ammonium sulfate. One hundred percent of Burkitt lymphoma DNA polymerase (●—●), DNA polymerase α (□—□), and DNA polymerase β (△- - -△) activities represents 14, 53, and 27 pmol of [^{3}H]dTMP incorporated, respectively, in assays with activated DNA template.

Table 1. Template preference of the Burkitt lymphoma DNA polymerase

Primer-templates	[^{3}H]dNMP incorporated	% activity: BL DNA polymerase	% activity: DNA polymerase α	% activity: DNA polymerase γ
Activated DNA	dTMP	100	100	100
$(dT)_{\sim 15}\cdot(dA)_n$	dTMP	49.8	33.0	285
$(dT)_{\sim 15}\cdot(A)_n$	dTMP	47.0	0.5	725
$(dT)_m\cdot(dA)_n$	dTMP	21.8	17.0	115
$(dG)_{\sim 15}\cdot(dC)_n$	dGMP	203.1	20.8	413
$(dG)_{\sim 15}\cdot(C)_n$	dGMP	<0.01	<0.01	29
$(dA)_{\sim 15}$	dGMP	<0.01	<0.01	<0.01

The assay conditions were as described in the legend to Fig. 1; 12.5 μg of activated DNA, 1 μg each of the synthetic primer templates, and 2 μg of $(dA)_{\sim 15}$ were used. DNA polymerases α and γ of L1210 cells were used as controls. One hundred percent of Burkitt lymphoma (BL) DNA polymerase, DNA polymerase α, and DNA polymerase γ activities represents 32, 76, and 3 pmol of [^{3}H]dTMP incorporated, respectively.

Effect of Phosphonoacetate and *N*-Ethylmaleimide. We compared the relative inhibition by phosphonoacetate of the DNA polymerase isolated from Burkitt lymphoma tissue, HSV-induced DNA polymerase, and cellular DNA polymerases α and β (Fig. 4). Of the enzymes tested, the HSV-induced DNA polymerase was the most sensitive. The Burkitt lymphoma DNA polymerase was very similar to the HSV DNA polymerase in its sensitivity. DNA polymerase α was also inhibited at similar low concentrations. Cellular DNA polymerase β was the least sensitive enzyme. This pattern of phosphonoacetate inhibition is similar to that reported earlier for the herpes-virus-induced DNA polymerase and cellular DNA polymerases (14, 15).

A comparison of the effect of *N*-ethylmaleimide, phosphonoacetate, and monovalent cations on the polymerase activity is illustrated on Table 2. *N*-Ethylmaleimide, a sulfhydryl inhibitor, at 10 mM, significantly reduced DNA polymerase α activity but not DNA polymerase β activity; DNA polymerase γ activity was only partially inhibited. Burkitt lymphoma DNA polymerase activity was not affected.

Antibody Inhibition of Burkitt Lymphoma DNA Polymerase. To determine the immunological relationship of the

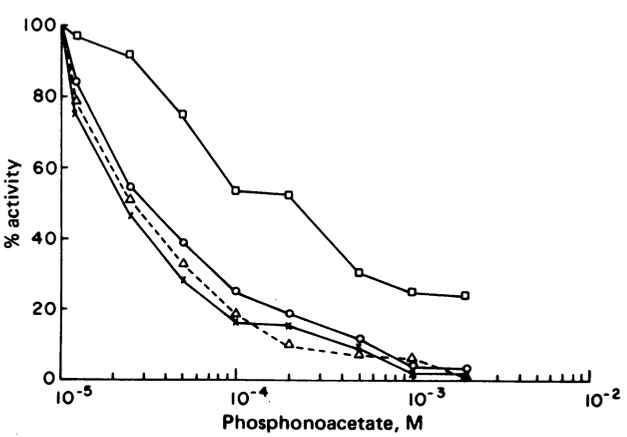

FIG. 4. Effect of addition of phosphonoacetate on activities of the polymerases. The assay conditions were as described in the legend to Fig. 1 except that known concentrations of phosphonoacetate were added as indicated. One hundred percent of HSV DNA polymerase (×—×), Burkitt lymphoma DNA polymerase (△- - -△), DNA polymerase α (○—○), and DNA polymerase β (□—□) activities represents 6, 9, 53, and 27 pmol of [^{3}H]dTMP incorporated, respectively.

Table 2. Effect of inhibitors and monovalent cations on DNA polymerase activity

Addition	% activity			
	BL DNA polymerase	DNA polymerase α	DNA polymerase β	DNA polymerase γ
None	100	100	100	100
N-Ethylmaleimide: 10 mM	104.4	2.1	98.4	38.9
Phosphonoacetate: 2.5×10^{-5} M	50.2	54.5	91.8	98.1
1.0×10^{-4} M	18.0	25.0	53.9	69.2
NaCl: 50 mM	191	61.6	167.6	119.0
100 mM	292	6.6	61.6	93.0
$(NH_4)_2SO_4$: 50 mM	216	9.8	58.2	98.1
100 mM	354	4.1	36.1	49.1

All conditions were as described in Table 1. One hundred percent activity of DNA polymerase β of L1210 cells represents 28 pmol of [^{3}H]dTMP incorporated. BL, Burkitt lymphoma.

DNA polymerase isolated from Burkitt lymphoma tissue, we studied its interaction with antisera directed against the herpes-virus-induced DNA polymerase and the cellular DNA polymerases α and γ. Fig. 5 shows that the HSV DNA polymerase antiserum inhibited Burkitt lymphoma DNA polymerase activity while the HeLa cell DNA polymerase α antiserum did not. This DNA polymerase α antiserum, however, inhibited the DNA polymerase α isolated from Burkitt lymphoma tissue. In addition, the binding of the antibody directed against the DNA polymerase γ of human lymphoblast cells with Burkitt lymphoma DNA polymerase was examined by a double immunoprecipitation assay; the antibody did not crossreact with the Burkitt lymphoma polymerase; however, 8 μg of the antibody bound to 63% of the DNA polymerase γ of human lymphoblast cells.

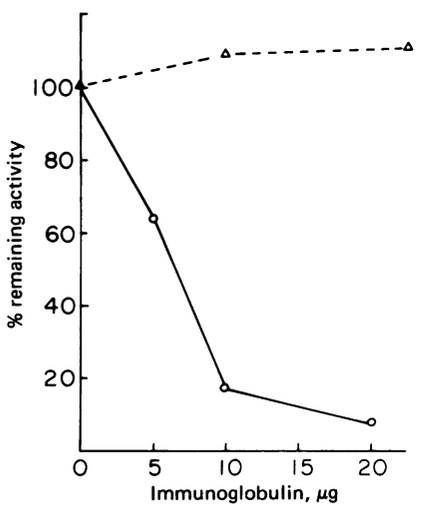

FIG. 5. Titration of fixed amounts of Burkitt lymphoma DNA polymerase against increasing concentrations of antibodies directed against HSV DNA polymerase (O—O) and DNA polymerase α of HeLa cells (Δ- - -Δ). One hundred percent of Burkitt lymphoma DNA polymerase represents 12 pmol of [^{3}H]dTMP incorporated in assays with activated DNA template. Other conditions are described in the legend for Fig. 1. HSV DNA polymerase and DNA polymerase α of HeLa cells were used as controls to test the effect of antibodies from the homologous systems under assay conditions optimum for the respective enzymes. Ten micrograms of the antibody directed against HSV DNA polymerase inhibited 78% of the HSV DNA polymerase activity. Twelve micrograms of the antibody directed against DNA polymerase α inhibited 57% of the DNA polymerase α activity of HeLa cells. Furthermore, 12 μg of the DNA polymerase α antibody inhibited 48% of DNA polymerase α of Burkitt lymphoma tissue. One hundred percent of the HSV DNA polymerase, DNA polymerase α of HeLa cells, and DNA polymerase α of Burkitt lymphoma tissue activities represents 9, 48, and 27 pmol of [^{3}H]dTMP incorporated, respectively.

Effect of Serum of the Burkitt Lymphoma Patient on Activity of the Polymerase. Since the serum of the patient was positive for EBV antibodies, we tested the effect of addition of serum on the activity of the viral DNA polymerase. Preincubation of the enzyme for 10 min with the serum did not bring about any significant change in the activity of either the Burkitt lymphoma DNA polymerase or the cellular DNA polymerases α and β.

Absence of Salt-Dependent DNA Polymerase in Spleen and Liver of the Burkitt Lymphoma Patient. Tissues from spleen and liver obtained from the same patient were also examined for the presence of salt-dependent DNA polymerase activity by a similar isolation procedure. No evidence for a virus-induced DNA polymerase activity was present in the crude extracts or in the fractions from phosphocellulose column chromatography. However, the tissues contained cellular DNA polymerase activities. The elution positions in phosphocellulose column of the polymerases of spleen from the patient are shown on Fig. 1*B*.

DISCUSSION

Burkitt lymphoma from endemic regions of Africa and from America appears to be similar in histopathology, surface marker characteristics, kinetic properties, and response to chemotherapy. However, differences between endemic and nonendemic Burkitt lymphoma have been observed with respect to the association with EBV. High titers of antibodies to EBV-related antigens have been detected in the sera of African patients with Burkitt lymphoma, and the presence of EBV genomes has been demonstrated in nearly all tumor biopsies tested. The majority of American patients with Burkitt lymphoma, on the other hand, lack the serological or viral genome association with EBV. Only recently, evidence for the association of EBV genome with American Burkitt lymphoma has been reported (1–4).

The isolated enzyme from the Burkitt lymphoma tissue resembles the HSV DNA polymerase and the polymerase isolated from Burkitt cells in culture induced with IdUrd in its high salt requirement for activity, primer template preference, and inhibition by phosphonoacetate. Furthermore, serological analyses indicate that the enzyme is immunologically related to the HSV-induced DNA polymerase but different from DNA polymerase α and DNA polymerase γ of human cells. Although the serum of the patient had a high titer for the virus capsid antigen, addition of samples of the serum to the reaction mixture did not inhibit the activity of the polymerase. Thus, it is unlikely that antibodies to the herpes virus DNA polymerase were present in the serum. The autopsy samples of spleen and liver of the patient did not contain any measurable herpes virus polymerase.

DNA polymerase γ and mitochondrial DNA polymerase resemble the herpes-virus-induced DNA polymerase in its high salt requirement for activity; however, DNA polymerase γ and mitochondrial DNA polymerase generally account for approximately 1% of the total cellular DNA polymerase activity, whereas the salt-dependent DNA polymerase activity in the Burkitt lymphoma tissue accounts for 30% of the total DNA polymerase activity. Furthermore, the primer template preference of DNA polymerase γ is distinctly different from that of the Burkitt lymphoma polymerase. The Burkitt lymphoma DNA polymerase differs from DNA polymerase γ also in its sensitivity to phosphonoacetate and *N*-ethylmaleimide; the Burkitt lymphoma DNA polymerase is very sensitive to phosphonoacetate, while DNA polymerase γ is only moderately sensitive. The Burkitt lymphoma DNA polymerase is insensitive to *N*-ethylmaleimide; in this respect it resembles the Marek disease herpes-virus-induced DNA polymerase (16). It has been claimed that the majority of African patients with Burkitt lymphoma show evidence for the presence of oncornavirus components in tumor cells (17). The Burkitt lymphoma tissue that we examined did not contain any detectable reverse transcriptase activity.

An examination of lymphoma tissue from other patients may determine if the presence of the herpes virus DNA polymerase is a general phenomenon or unique to Burkitt lymphoma. The unique property of the herpes-virus-induced polymerases to function optimally at high salt concentration enables its identification even in crude extracts without interference from the cellular DNA polymerases since the activities of the cellular enzymes are inhibited at high salt concentation. Identification of this biochemical marker in Burkitt lymphoma tissue provides further evidence for the viral etiology of this malignancy and a new lead for studying the molecular biology of the induction of malignancy by Epstein–Barr virus.

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