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Distinctive properties of DNA polymerases induced by herpes simplex virus type-1 and Epstein–Barr virus*

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Summary

The properties of DNA polymerases induced by two human herpesviruses, herpes simplex virus type-1 (HSV-1) and Epstein–Barr virus (EBV), have been compared. The HSV-1 and EBV polymerases can be distinguished from one another by differences in the elution profiles in phosphocellulose and single-stranded DNA cellulose columns. Although both enzymes require monovalent cations for optimum activity, the HSV-1 enzyme requires ammonium sulfate whereas the EBV enzyme activity is inhibited by it; on the other hand, the EBV polymerase requires KCl. Other reaction requirements are also different for the two viral enzymes. Thus, when the EBV DNA polymerase was assayed under conditions optimum for the HSV-1 DNA polymerase, only 15% of its activity was expressed. Differences were also noted in sensitivities of the two viral enzymes to the 5′-triphosphates of nucleoside analogs with antiherpesvirus activity such as BVdU, IVdU, ACV, FIAC and IdUrd. The HSV-1 polymerase was more sensitive than the EBV DNA polymerase to inhibition by phosphonoacetate, phosphonoformate, aphidicolin and *N*-ethylmaleimide. However, the EBV DNA polymerase was more sensitive than HSV-1 DNA polymerase to heat treatment at 42°C. Thus, the marked differences between the two viral enzymes can be

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Abbreviations used: ACV, 9-(2-hydroxyethoxymethyl)guanine; BVdU, (*E*)-5-(2-bromovinyl)-2′-deoxyuridine; CMV, cytomegalovirus; EBV, Epstein–Barr virus; FIAC, 1-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)5-iodocytosine; HSV-1, herpes simplex virus type-1; HSV-2, herpes simplex virus type-2; IVdU, (*E*)-5-(2-iodovinyl)-2′-deoxyuridine; IdUrd, 5-iodo-2′-deoxyuridine; NEM, *N*-ethylmaleimide; PA, phosphonoacetate; PF, phosphonoformate; ss-DNA, single stranded DNA; VCA, virus capsid antigen; VZV, varicella-zoster virus.

useful in identifying enzyme activities in cells producing the virus and also in studying the biochemical mechanism of action of some of the antiviral agents.

DNA polymerases; HSV-1; EBV

Introduction

One of the common features of the five human herpesviruses (herpes simplex virus type-1, HSV-1; herpes simplex virus type-2, HSV-2; Epstein–Barr virus, EBV; varicella-zoster virus, VZV; and cytomegalovirus, CMV) is that they all induce virus specific DNApolymerases in virus-infected cells [3,10,19,22,24,26]. Of these, the polymerases induced by HSV-1 and HSV-2 have been well characterized [19,22,24,26]. The properties of the viral enzymes are clearly distinguishable from the cellular DNA polymerases, α, β and γ [13]. However, the HSV-1 polymerase is similar to HSV-2 polymerases in many of their properties such as elution position in ion exchange columns, reaction conditions for optimum activity, affinity for the natural triphosphate substrates and inhibition by phosphonoformate; these enzymes are also antigenically similar [22,26].

In recent years several nucleoside analogs have been synthesized and shown to have selective and potent antiherpesvirus activity. The selectivity is due to at least two factors: first, the ability of the virus induced pyrimidine deoxynucleoside kinase to preferentially phosphorylate nucleoside analogs and second, the ability of the 5′-triphosphates to inhibit the virus induced DNA polymerase more than the cellular DNA polymerases [4,7,12,13]. Thus, the virus induced DNA polymerases have become exploitable targets for selective antiviral chemotherapy. These findings have enhanced our interest in examining the properties of the virus induced enzymes. We are particularly interested in distinguishing the viral DNA polymerases from their cellular counterparts as well as in determining differences among the viral enzymes.

One of the nucleoside analogs with selective antiherpesvirus activity studied in detail in this laboratory is (*E*)-5-(2-bromovinyl)-2′-deoxyuridine (BVdU). BVdU is more inhibitory to HSV-1 replication than EBV replication in vitro; BVdU inhibited HSV-1 replication in vitro at concentrations as low as 0.007–0.01 μg/ml whereas inhibition of EBV replication was observed only at higher concentrations [9,29]. Although it is still not certain whether the EBV can induce its own pyrimidine deoxynucleoside kinase, it was of interest to compare the sensitivity of BVdU 5′-triphosphate (BVdUTP) to DNA polymerases of HSV-1 and EBV. We observed that HSV-1 DNA polymerase was more sensitive than EBV DNA polymerase to BVdUTP inhibition. Similar differences were also noticed between the two enzymes in sensitivity to the 5′-triphosphates of other nucleoside analogs with antiherpesvirus activity. The present study deals with these as well as other distinguishing features of the DNA polymerases specific to HSV-1 and EBV.

Materials and Methods

Cells and viruses

Monolayer cultures of Vero cells were grown in minimum Eagle's medium supplemented with 10% calf serum. The HSV-1 strain, Cl101, was used to infect Vero cells. P3HR-1 cells, originally derived from a patient with Burkitt's lymphoma, were grown in RPMI 1640 medium supplemented with 10% calf serum, 100 IU penicillin, 100 μg/ml streptomycin, and 5 μg/ml fungizone [25]. The three sublines used in this study were 4, 8 and 13% virus capsid antigen (VCA) positive, respectively.

Isolation of cellular and viral polymerases

Cellular DNA polymerases α and β were purified from leukocytes of a patient with acute myelogenous leukemia. HSV-1 DNA polymerase was isolated from Vero cells infected with HSV-1 strain Cl101 [2]. EBV DNA polymerase was purified from the P3HR-1 cell line. The isolation procedures of the polymerases were published previously [3,2,19,24].

Chemicals

All nucleoside triphosphates were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Tritiated deoxynucleoside triphosphates were obtained from the New England Nuclear Corp. (Boston, MA, U.S.A.). Calf thymus DNA was converted to the activated form by treatment with DNase I and used as the template in this study.

BVdU, FIAC [1-(2'-deoxy-2'-fluoro-β-D-arabinofuranosyl)-5-iodocytosine] and ACV [9-(2-hydroxyethoxymethyl)guanine] were gifts from Dr. E. De Clercq (Rega Institute, Leuven, Belgium), Dr. J. Fox (Sloan-Kettering Institute for Cancer Research, New York, U.S.A.), and Dr. G.B. Elion (Burroughs Wellcome, Chapel Hill), respectively. Procedures used for the chemical conversion of the nucleoside analogs to the corresponding 5'-triphosphates were described earlier [4]. Their purity was examined by high-performance liquid chromatography on an Altex Model 332 gradient liquid chromatograph and they were >90% pure [4–6]. All other commercially available chemicals used were of the highest purity.

DNA polymerase assay

DNA polymerase α activity was assayed in 50 μl reaction mixtures containing 50 mM Tris–HCl, pH 8.0, 2 mM dithiothreitol (DTT), 8 mM $MgCl_2$, 100 μM each of dATP, dCTP and dTTP, and 3–5 μM [^{3}H]dGTP (1060 cpm/pmol), 10 μg activated calf thymus DNA, 10–20 μg bovine serum albumin (BSA), 5–10% glycerol and enzyme. Incubation was at 37°C for 30 min. Acid-insoluble radioactivity was collected on nitrocellulose filters (Gelman or Millipore, 0.45 μm) washed six times using a Millipore filtration manifold with 5% trichloroacetic acid containing 2 mM sodium pyrophosphate. The filters were finally washed once with 70% ethanol, dried, and the radioactivity measured in a liquid scintillation counter. DNA polymerase β activity was assayed under similar conditions except that Tris–HCl buffer, pH 9.0, 50 μM of non-radioactive triphosphates, 20 μM [^{3}H]dGTP, and 40 mM KCl were used.

The reaction mixture for assaying HSV-1 DNA polymerase activity contained 50

mM Tris–HCl, pH 8.3, 2 mM DTT, 4 mM $MgCl_2$, 10 μM each of dATP, dCTP and dTTP, and 0.5–1.0 μM [^{3}H]dGTP (4300 cpm/pmol), 5 μg activated calf thymus DNA, 50 mM ammonium sulfate, 10 μg BSA, 5–10% glycerol and the enzyme. Other conditions were similar to those described for measuring DNA polymerase α activity.

EBV DNA polymerase activity was assayed under similar conditions for DNA polymerase α except that 4 mM $MgCl_2$, 10 μM [^{3}H]dGTP and 100 mM KCl were used.

In enzyme assays to determine the K_i values of the 5′-triphosphates of nucleoside analogs with antiviral activity, the corresponding radioactive triphosphates of the normal substrates were used. The K_i values for the 5′-triphosphates of nucleoside analogs were determined by procedures described previously [4–6].

Results

Elution positions in ion exchange columns

The enzymes were purified by successive chromatography using DEAE-cellulose, phosphocellulose, and single stranded DNA (ss-DNA)-cellulose columns. We noted differences in the elution profiles of the two enzymes in ion exchange columns. The HSV-1 DNA polymerase eluted from the phosphocellulose column at 0.1 M KCl whereas the EBV DNA polymerase eluted at 0.05 M KCl at pH 8.0. The elution profiles of phosphocellulose columns of DNA polymerases induced by two strains of HSV-1 (Cl101 and HF) were similar; furthermore, the elution profiles of EBV DNA polymerase in three sublines of P3HR-1 with varying amounts of VCA (4, 8 and 13%) were also similar. Thus, there is a consistent difference in the elution profiles between DNA polymerases of HSV-1 and EBV. The elution profiles of these two enzymes are different also in ss-DNA-cellulose column. The EBV DNA polymerase and HSV-1 DNA polymerase eluted from the DNA-cellulose columns at 0.2 and 0.28 M KCl, respectively, at pH 7.5. From these results we conclude that the two polymerases have distinct chemical properties.

Assay conditions for optimum activity

Optimum reaction conditions for DNA polymerases of EBV and HSV-1 have been determined using activated DNA template. Table 1 summarizes the requirements for optimum activities of these two viral enzymes. Most significant differences between the two viral polymerases were observed in their requirement for monovalent cations. The enzymes purified by successive DEAE-cellulose, phosphocellulose and ss-DNA-cellulose columns were used for the determination of monovalent cation requirements for optimum activity. For example, the HSV-1 DNA polymerase required 50 mM ammonium sulfate for maximum activity; its activity was enhanced 5-fold by the addition of ammonium sulfate. However, ammonium sulfate was inhibitory to the EBV DNA polymerase activity especially at higher concentrations; its activity was enhanced 7–9-fold by the addition of 100 mM KCl (Fig. 1A, B). Addition of KCl, however, enhanced the HSV-1 DNA polymerase activity only 2-fold.

Other differences in the optimum reaction conditions between these two enzymes are also illustrated in Table 1. The optimum conditions for the two viral enzymes are

TABLE 1

Optimum assay conditions for the cellular and viral DNA polymerases

DNA polymerase		Template conc.[a] (μg/ml)	Unlabelled dNTP concentration (μM)	Tris–HCl buffer		Mg^{2+} (mM)	KCl (mM)	$(NH_4)_2SO_4$ (mM)
				(pH)	(mM)			
α	Activated calf thymus DNA	200	100	7.5	50	8.0	–	–
β	Activated calf thymus DNA	200	50	9.0	50	8.0	50	–
HSV-1	Activated calf thymus DNA	100	10	8.7	50	4.0	–	50
EBV	Activated calf thymus DNA	200	100	8.5	50	4.0	100	–

[a] Activated calf thymus DNA.

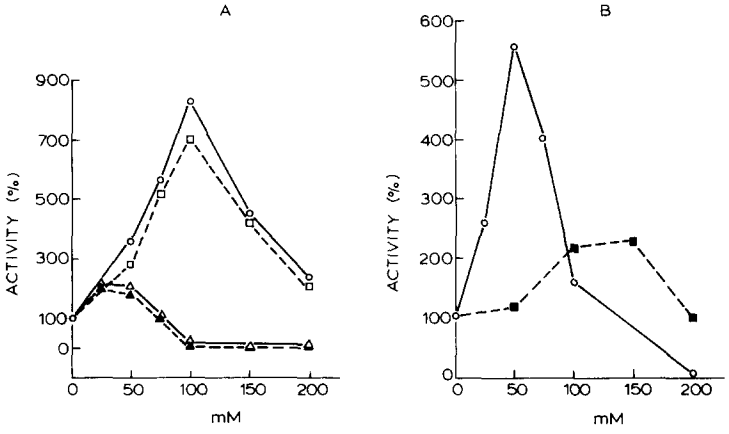

Fig. 1. Effect of monovalent cations on the activities of viral DNA polymerases. (A) EBV DNA polymerase: enzymes from two different EBV producer cell lines are shown. (○—○), Effect of KCl on DNA polymerase activity from P3HR-1 cell line that is 4% VCA positive; (□---□), effect of KCl on DNA polymerase activity from P3HR-1 cell line that is 8% VCA positive; (△—△), effect of ammonium sulfate on DNA polymerase activity from P3HR-1 cell line that is 4% VCA positive; and (△---△), effect of ammonium sulfate on DNA polymerase activity from P3HR-1 cell line that is 8% VCA positive. 100% of activity represents 18–20 pmol of [^{3}H]dTMP incorporation in enzyme assays with activated DNA template. (B) Effect of ammonium sulfate (○—○) and KCl (■---■) on the activity of HSV-1 DNA polymerase. 100% activity represents 14 pmol of [^{3}H]dTMP incorporation in enzyme assays with activated DNA template.

substantially different and therefore it is essential to use the appropriate conditions for each enzyme. To further illustrate this point, each polymerase was assayed under conditions optimum for the other enzyme. When the EBV DNA polymerase was assayed under conditions optimum for the HSV-1 DNA polymerase, only 15% of its activity was expressed; conversely, the HSV-1 DNA polymerase expressed only 62% of its optimum activity when assayed under conditions optimum for the EBV DNA polymerase (Table 2).

The affinities of the two viral enzymes for the natural triphosphate substrates were also compared. The K_m values for all four triphosphates of HSV-1 DNA polymerase were less than 1 μM; this is consistent with observations made in other laboratories [19,24]. However, the K_m values of EBV DNA polymerase were 10–20-fold higher than those of the HSV-1 DNA polymerase (Table 3).

Sensitivity to the 5'-triphosphates of nucleoside analogs

Some of the recently synthesized nucleoside analogs have been demonstrated to selectively inhibit HSV replication both in vitro and in vivo [4,8,9,17,20]. The selectivity is due to their preferential phosphorylation by the HSV induced pyrimidine deoxynucleoside kinase [18] and ability of the 5'-triphosphates to inhibit the HSV induced DNA polymerase more than the cellular DNA polymerases [4–6,8,17]. Therefore, it was of interest to us to compare the effect of the 5'-triphosphates of some of the nucleoside analogs on the activities of HSV-1 DNA polymerase and EBV DNA polymerase. The K_m values for the corresponding natural triphosphates, K_i values of

TABLE 2

Comparison of activities under different assay conditions

DNA polymerase	% Activity under optimal assay conditions for DNA polymerases			
	α	β	HSV-1	EBV
α	100	84.1	10.1	20.9
β	46.4	100	5.8	30.7
HSV-1	54.1	45.8	100	61.9
EBV	47.4	87.0	15.7	100

TABLE 3

K_m values of cellular and viral DNA polymerases

DNA polymerase	K_m values (μM)			
	dATP	dCTP	dGTP	dTTP
α	8.0	5.0	3.3	4.4
β	11.0	7.5	2.9	14.3
HSV-1	0.52	0.41	0.20	0.66
EBV	10.5	9.1	6.2	13.0

the inhibitors and the K_m/K_i ratios of both enzymes have been compared (Table 4). The EBV DNA polymerase was relatively insensitive to all the triphosphates tested; the K_i values are 10–20-fold higher than those for the HSV-1 DNA polymerase.

Inhibition by phosphonoacetate (PA) and phosphonoformate (PF)

PA and PF have been shown to suppress HSV-1 replication both in vitro and in vivo [15,28]. These compounds suppress HSV replication by inhibiting the virus induced DNA polymerase. It has been shown earlier that the HSV-1 DNA polymerase is more sensitive than cellular DNA polymerases to inhibition by PA and PF [15,28]. The sensitivities of DNA polymerases of HSV-1 and EBV to these inhibitors were compared. Fig. 2 shows that the HSV-1 DNA polymerase is sensitive to PA and PF whereas the EBV enzyme was relatively insensitive to the inhibitors.

Inhibition by aphidicolin and NEM

Aphidicolin is a potent inhibitor of HSV-1 DNA polymerase. Spadari and his colleagues as well as others [11,23] have extensively studied the selective inhibition by aphidicolin of DNA replication in HSV-infected cells. Its effect on EBV replication is not known. Therefore, the effect of aphidicolin on the activities of the DNA polymerases of HSV-1 and EBV was examined. As has been shown earlier, the HSV-1 DNA polymerase was completely inhibited by less than 1 μg/ml aphidicolin whereas the EBV DNA polymerase was insensitive; even at 5 μg/ml aphidicolin inhibited only 20% of the EBV DNA polymerase activity. *N*-Ethylmaleimide (NEM) inhibited 50%

TABLE 4

A comparison of inhibition of HSV-1 and EBV DNA polymerase activities by the triphosphates of nucleotides

Analog	HSV-1 DNA polymerase			EBV DNA polymerase		
	K_m (μM)	K_i (μM)	K_m/K_i	K_m (μM)	K_i (μM)	K_m/K_i
BVdUTP	0.66	0.25	2.6	13	16.1	0.81
IVdUTP	0.45	0.12	3.7	11.5	6.0	1.9
ACVTP	0.15	0.03	5.0	3.1	9.8	0.32
FIACTP	0.40	0.26	1.5	8.2	32.2	0.25
IdUrdTP	0.45	0.86	0.6	11.5	15.1	0.76

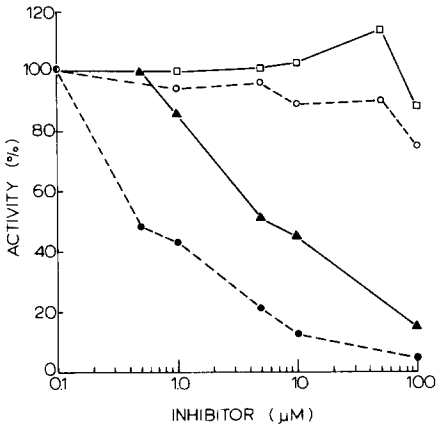

Fig. 2. Effect of PA and PF on the activities of HSV-1 DNA polymerase and EBV DNA polymerase. (□—□), Effect of PA on EBV DNA polymerase; (○---○), effect of PF on EBV DNA polymerase; (▲—▲), effect of PA on HSV-1 DNA polymerase; and (●---●), effect of PF on HSV-1 DNA polymerase. Other conditions are described in Fig. 1.

of HSV-1 DNA polymerase activity. However, it had no significant effect on the EBV DNA polymerase activity; 5 mM NEM inhibited only 34% of its activity (Fig. 3B).

Thermolability of HSV-1 DNA polymerase and EBV DNA polymerase

DNA polymerases in general are sensitive to heat. Dube et al. [12] have shown that the DNA polymerase β is more rapidly inactivated in vitro by elevated temperature than DNA polymerase α. HSV-1 DNA polymerase and EBV DNA polymerase were incubated at 42°C for a given period and the remaining enzyme activities were determined using the appropriate assay conditions. EBV DNA polymerase is more sensitive to heat treatment than HSV-1 DNA polymerase. Incubation at 42°C for 10 min decreased the EBV DNA polymerase activity by 50% whereas the same treatment did not decrease the HSV-1 DNA polymerase at all. Increasing the incubation to 20

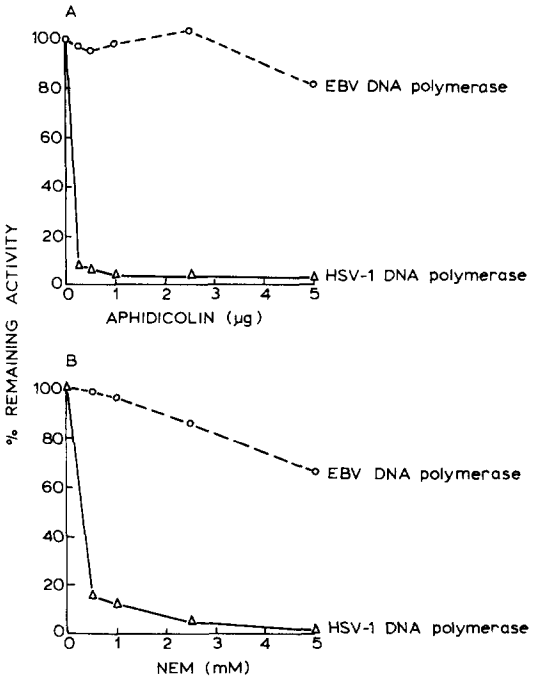

Fig. 3. (A) Effect of aphidicolin on the activities of viral DNA polymerases. (○---○), EBV DNA polymerase; (△—△), HSV-1 DNA polymerase. (B) Effect of NEM on the activities of EBV DNA polymerase (○---○) and HSV-1 DNA polymerase (△—△). Other conditions are described in Fig. 1.

min caused loss of EBV DNA polymerase and HSV-1 DNA polymerase activities by 82 and 15%, respectively. Addition of 1 mg/ml BSA did not significantly alter the sensitivity of HSV-1 DNA polymerase to heat although it offered a limited protection to the EBV DNA polymerase from heat inactivation.

Discussion

Most of the recently developed 'second generation' nucleoside analogs exert their selective antiviral effect by preferentially inhibiting the viral DNA replication. In particular, the virus induced DNA polymerases are more sensitive than the cellular DNA polymerases α and β to the 5'-triphosphates of the nucleoside analogs [4–6,8,17]. Therefore, the study of DNA polymerases induced by the human herpesviruses has gained additional importance particularly in providing the biochemical basis for selective antiviral effect of the nucleoside analogs. Such comprehensive studies have led to the appreciation of differences between the cellular and viral enzymes as well as differences among the viral enzymes. Although the enzymes induced by HSV-1 and EBV share some common properties, the results presented here point out that they are clearly distinct from one another in many respects. More importantly, there was a marked difference in sensitivities between the two viral

enzymes to inhibition by the known antiviral agents. In general, the EBV DNA polymerase is less sensitive than HSV-1 DNA polymerase to inhibition by the 5'-triphosphates of some of the nucleoside analogs tested. The EBV enzyme is also less sensitive to agents such as PA, PF, aphidicolin and NEM. Although agents such as ACV, PA and PF have been shown to inhibit the expression of EBV genome in the virus producing cell lines [7,28,29] the results presented here clearly indicate that the viral enzyme may not be the primary target for their antiviral effect. We have shown earlier that the EBV DNA polymerase is not as sensitive as HSV-1 DNA polymerase to inhibition by the 5'-triphosphate of ACV (ACVTP), although ACV inhibited the VCA expression in the virus producing cell lines [5]. This indicates that inhibition of EBV DNA polymerase activity by ACVTP may not be the primary mechanism responsible for ACV inhibition of EBV replication.

Thus, the EBV DNA polymerase is less sensitive than HSV-1 DNA polymerase to inhibition by many agents including the 5'-triphosphates of certain nucleoside analogs. One of the cellular DNA polymerases, DNA polymerase β is also less sensitive to these agents. However, the EBV DNA polymerase used in this study was distinctly different from DNA polymerase β. First, the elution positions of EBV DNA polymerase and DNA polymerase β on ion exchange columns are clearly different. For instance, on a phosphocellulose column, the EBV DNA polymerase eluted at 0.05 M KCl whereas the DNA polymerase β eluted at 0.35 M KCl. Second, as we have shown earlier [3], the level of EBV DNA polymerase increased in three cell lines producing varying amounts of the virus proportional to the percent of virus producing cells; however, the level of DNA polymerase β in all three cell lines remained constant. Third, the optimum reaction conditions for the viral and cellular polymerases are distinctly different; the EBV DNA polymerase activity was optimum at 100 mM KCl whereas addition of 100 mM KCl inhibited the DNA polymerase β activity considerably. These results are in agreement with those of Ooka et al. [21] who have clearly shown the differences between the viral and cellular DNA polymerases. Thus, at conditions optimum for the EBV DNA polymerase, only 30% of DNA polymerase β activity was expressed (Table 3). Fourth, NEM inhibited EBV DNA polymerase activity, although not as much as HSV-1 DNA polymerase activity; however, the DNA polymerase β was totally resistant to inhibition by NEM at the concentrations tested. Furthermore, the K_m value for the natural substrate dGTP of DNA polymerase β is 2.9 μM, whereas that of EBV DNA polymerase is at least two times higher (Table 3). Therefore, the enzyme that eluted at 0.05 M KCl resembled the EBV DNA polymerase in many of its characteristics as published by other investigators (Ooka et al. [21], Feighney et al. [16] and by us [3]), but was clearly distinct from the cellular polymerase β. Moreover, EBV DNA polymerase is present only in virus producing cell lines but not in non-producer cell line such as Raji. These results are in agreement with those published by Feighney et al. [16].

The results presented here on the K_m values for dGTP and K_i values for ACVTP of EBV DNA polymerase are different from those obtained by Datta et al [7]. Our efforts to verify the values with the viral enzyme isolated from three different cell lines and at a wide range of ACVTP concentrations gave similar results, thus confirming our original observation. It is also important to note that our K_m value of DNA polyme-

rase for dGTP is similar to the values reported by other investigators (1.5–2.5 μM) [17]. However, the K_m value of DNA polymerase α for dGTP reported by Datta et al. [7] is unusually low (0.18 μM).

An appreciation of differences in properties of the herpesvirus induced polymerases is important for other reasons as well. Results presented here indicate that the reaction conditions for optimum activity of HSV-1 DNA polymerase and EBV DNA polymerase are quite different. Under conditions optimum for HSV-1 DNA polymerase, the EBV enzyme activity is suppressed by 85%. Therefore, it is essential to use the appropriate assay conditions in detecting the virus induced DNA polymerase activities in cell extracts. EBV has been etiologically associated with human malignancies such as Burkitt lymphoma and nasopharyngeal carcinoma. Identification of the viral enzymes in human cancer cells provides further evidence for the involvement of viruses in certain malignancies. We have earlier identified an EBV DNA polymerase-like activity in tissues of an American patient with Burkitt lymphoma [2]. More recently, we have identified HSV-2 DNA polymerase-like activity in samples from several patients with cervical carcinoma (Allaudeen, H.S. and Schwartz, P., unpublished observations). Detection of the herpesviral enzymes in tissues from these patients provides further support for the association of the two human herpesviruses with these malignancies.

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