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Cellular and Epstein-Barr virus specific DNA polymerases in virus-producing Burkitt's lymphoma cell lines

H.S.Allaudeen* and G.Rani

Pharmacology Department, Yale University School of Medicine, New Haven, CT 06510, USA

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ABSTRACT

We have determined the levels of cellular DNA polymerases and Epstein-Barr virus specific DNA polymerase in three Burkitt's lymphoma cell lines producing varying amounts of EBV, one of which was induced by 12-O-tetradecanoylphorbol-13-acetate (TPA). There was a proportional increase in the level of EBV-DNA polymerase with an increase in the percent of virus-producing cells. However, there was a reciprocal relationship between the levels of EBV-DNA polymerase and DNA polymerase α i.e., in cell line containing the highest level of EBV-DNA polymerase, activity of DNA polymerase α, but not of DNA polymerase β, was reduced to an insignificantly low level. TPA does not have any direct effect on activities of either EBV-DNA polymerase or DNA polymerase α. EBV-DNA polymerases isolated from cells grown with or without TPA are indistinguishable in their properties such as elution position on phosphocellulose column, molecular weight, mono and divalent cation requirements, pH optimum, and other requirements for optimum activity. Addition of crude extracts of cells grown in presence of TPA to the purified DNA polymerase α did not inhibit its activity indicating that the observed loss was not due to any specific inhibitor present in TPA treated cells. Raji, a nonproducer cell line, did not contain EBV-DNA polymerase. There was no induction of EBV-DNA polymerase when Raji cells were grown in presence of TPA. The phenomenon of reduction in the levels of DNA polymerase α in cells induced to produce EBV may represent a mechanism by which the host DNA replication is shut off following virus infection.

INTRODUCTION

Epstein-Barr virus (EBV) has been etiologically associated with infectious mononucleosis, Burkitt's lymphoma, and nasopharyngeal carcinoma (1-7). Most human lymphoblastoid cell lines carry EBV in a latent form. In nonproducer lines, cells do not spontaneously enter the viral cycle. In virus producer lines, a small proportion of cells continuously enter viral cycle. Generally, only a low per cent of the producer cell lines contain viral capsid antigen (VCA) at any given time (8-10). In recent years, several factors have been recognized to enhance EBV production by producer cell lines. For example, low temperature and corticosteroids increase viral replication (11). n-Butyrate increases the number of virus producer cells in EBV carrying

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P3HR-1 and B95-8 cell lines (12). 5-Bromo-deoxyuridine (13) and antibodies to human IgM (14) can also induce in some lines the virus production with high efficiency. More recently, zur Hausen et al. (15) have described induction of persisting genomes of oncogenic herpesviruses by tumor promoters of the diterpene ester type. For instance, addition of the tumor promoter, 12-O-tetradecanoyl phorbol-13-acetate (TPA) to virus producing cell lines in culture resulted in a 3 to 5-fold increase in VCA production and a 10 to 20-fold increase in the amount of viral DNA. However, in nonproducer cell lines such as Raji, TPA induces only early antigen (EA) expression (16-18).

Infection of permissive mammalian cells with herpes group viruses results in the induction of a virus specific DNA-polymerase in infected cells (19-21). The essential role of the virus specific DNA polymerase in replication of viral DNA has been established in the case of herpes simplex virus type-1 (HSV_1) and type-2 (HSV_2) (22,23). The virus-specific DNA polymerase can be distinguished from the cellular DNA polymerases by differences in their properties such as elution position in ion exchange column chromatography, primer-template preference, molecular weight, requirement for mono and divalent cations and sensitivity to some inhibitors (19-21). Properties of a partially purified EBV-specific DNA polymerase from virus producing cell lines have been described (24-28). Recently, Datta, et al (29) reported an increase in the level of EBV-DNA polymerase in P3HR-1 cell lines induced with TPA.

As a continuing effort in identification and characterization of EBV-specific DNA polymerase in Burkitt's lymphoma samples (26) and cell lines established from Burkitt's lymphoma patients, we have been interested in the effect of tumor promoter, TPA, on the levels of viral as well as cellular DNA polymerases in virus-producing cell lines and also to examine whether there is any correlation between changes in the level of virus production and virus-specific DNA polymerase.

Results described in this report show that there was an increase in the level of EBV-DNA polymerase when a virus-producing line was grown in presence of TPA; the level of cellular DNA polymerase α, however, decreased concomitantly. The implications of these observations are of considerable importance in understanding the regulation of cellular and viral DNA replication in virus-producing cell lines.

MATERIALS AND METHODS

Tritiated deoxyribonucleoside triphosphates were obtained from the New

England Nuclear Corp., Boston, Mass., and from the ICN Chemical and Radioisotope Division, Irvine, Calif. Nucleoside triphosphates and synthetic primer templates were purchased from P. L. Biochemicals, Milwaukee, Wi. and Sigma Chemical Co., St. Louis, Miss. The oligonucleotides contained 12-18 nucleotides. Calf thymus DNA was converted to the activated form by treatment with DNase I. 12-O-Tetradecanoylphorbol-13-acetate (TPA) was from Sigma Chemical Co.

Cells: Two sublines of P3HR-1, Raji, and BJAB were kindly provided by Dr. G. Klein, Karolinska Institute, Stockholm (30). P3HR-1 is an EBV producer cell line originally cloned from a Burkitt's lymphoma line, Jijoye. Raji is a nonproducer cell line; BJAB was derived from an African Burkitt's lymphoma and is EBV-genome negative. The three cell lines of P3HR-1 used in this study are referred to as cell lines A, B and C. Cell line A produced a low level of EBV (less than 1% of cells were VCA positive); cell line B was 4% VCA positive and cell line C represents line B treated with 20 ng of TPA for 72 hrs and was approximately 14% VCA positive. They are propagated as exponential suspension cultures in RPMI 1640 medium supplemented with 10% calf serum, 100 I.U. pencillin, 100 µg/ml streptomycin, and 5 µg/ml fungizone.

Induction with TPA: P3HR-1 and Raji cells were seeded at 10^5 cells/ml. After 72 hrs of cultivation at 37°C, the cells reached a density of $8x10^5$ cells/ml. TPA was added at a final concentration of 20 ng/ml. Incubation was continued for another 72 hrs, and the cells were harvested by centrifuging them at 1000xg for 10 min. VCA determination of P3HR-1 cells was kindly performed by Dr. J. Robinson, Dept. of Pediatrics and Epidemiology using established procedure (31).

Isolation of the DNA polymerases: The enzymes were isolated by procedures described before (26). A gram of cells (approximately 10^9 cells) were mixed with 5 ml of pellet homogenization buffer (0.5% Triton X-100 in 0.8M KCl) and ground in a chilled mortar and pestle. Equal volume of buffer A consisting of 20 mM potassium phosphate, pH 7.0, 0.5mM EDTA, 1 mM dithiothreitol (DTT) and 50% glycerol v/v was added and mixed well. Nucleic acids from the total cell extract were removed by passing it through a DEAE-cellulose column equilibrated with the same buffer; proteins were eluted with buffer A containing 0.3M KCl. Salt was removed by dialysis and the extract was applied on to a phosphocellulose column equilibrated with buffer B consisting of 50 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, 1 mM DTT and 20% glycerol. The DNA polymerases were fractionated on this column with a linear KCl gradient between 0-0.6M in buffer B.

Gel filtration: Sephadex G-200 superfine (Pharmacia) beads were soaked in a buffer consisting of 50 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, 1 mM DTT, 0.01% Triton X-100, 300 mM KCl and 20% glycerol v/v and poured into a column (1.2x38 cm). Other conditions were described before (26).

Enzyme assays: DNA polymerase α activity was assayed in a 50 µl reaction mixture which contained 50 mM Tris-HCl (pH 7.5), 2 mM DTT, 8 mM $MgCl_2$, 100 µM each of dATP, dCTP and dGTP, and 20 µM [^{3}H]dTTP (530 cpm/pmole), 10 µg of activated calf thymus DNA, 10-20 µg of bovine serum albumin (BSA), 5-10% glycerol, and enzyme. Incubation was at 37^o for 30 min. Acid-insoluble radioactivity was collected on a nitrocellulose filter (Gelman or Millipore, 0.45 µm) washed several times with 5% trichloroacetic acid containing 2 mM sodium pyrophosphate, once with 70% ethanol, dried, and measured in a liquid scintillation counter (32).

DNA polymerase β activity was assayed under similar conditions except that a pH 8.5 Tris-HCl buffer, 50 µM of nonradioactive triphosphates, 40 µM [^{3}H]dTTP and 40 mM KCl were used.

The reaction mixture for assaying EBV-DNA polymerase activity contained 50 mM Tris-HCl, pH 8.5, 2 mM DTT, 4 mM $MgCl_2$, 100 µM each of dATP, dCTP, and dGTP and 40 µM of [^{3}H]dTTP, 100 mM KCl, 10 µg BSA, 5-10% glycerol and enzyme.

RESULTS

Separation of cellular and EBV-specific DNA polymerases from a virus producing cell line: The elution profile of DNA polymerases of P3HR-1 cell line A on a phosphocellulose column chromatography is shown on Fig. 1A. The column fractions contained three DNA polymerase activities eluting at 0.05 M, 0.18 M and 0.35 M KCl, respectively. The enzymes which eluted at 0.18 M KCl and 0.35 M KCl were further analyzed and were identified as the DNA polymerase α and DNA polymerase β, respectively, from their characteristic elution positions on a phosphocellulose column, molecular weights, sensitivity to N-ethylmaleimide (NEM), effect of salt and optimum assay conditions (20,26). Thus, the cell line contained an additional peak of polymerase activity eluting at 0.05M KCl.

Further characterization of the additional DNA polymerase: Further characterization of the DNA polymerase that eluted at 0.05M KCl was undertaken to confirm that it is the viral DNA polymerase but does not represent an alternate form of DNA polymerase α (21,34). EBV DNA polymerase is distinctly different in many respects from the host DNA polymerases α and β. Firstly, conditions optimum for the activities of the viral and cellular polymerases

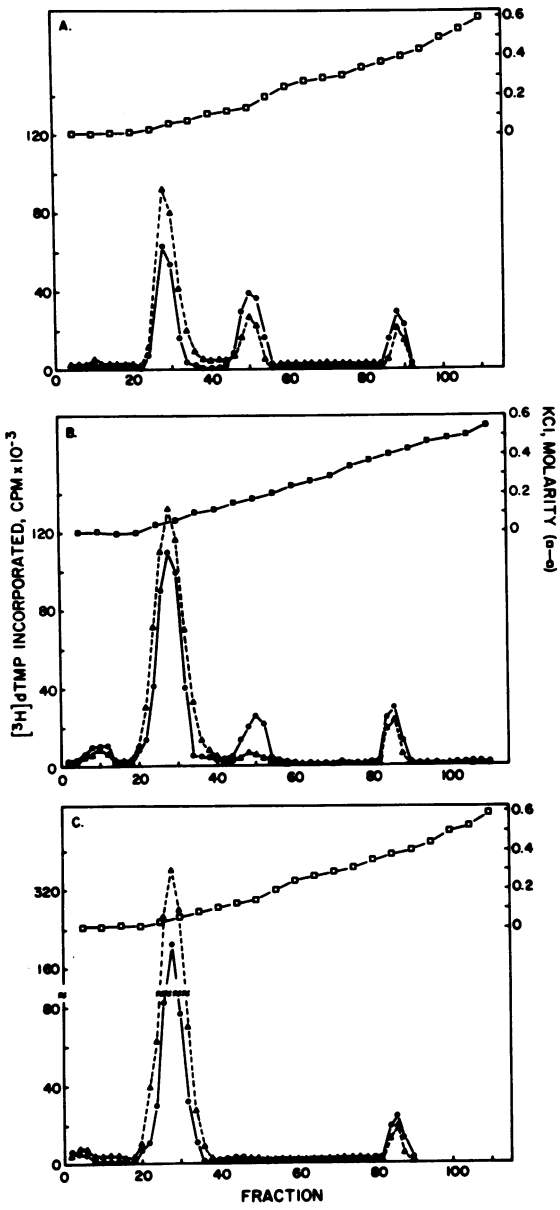

Figure 1

Phosphocellulose chromatography of DNA polymerases from the three cell lines A, B and C described in the legend for Table 1. Samples (10 µl) of every other fraction were assayed under conditions optimum for DNA polymerase α (o——o) and EBV-DNA polymerase (▲----▲).

are different. For example, they differ in their monovalent and divalent cation requirements for optimum activity. EBV DNA polymerase requires KCl for its optimum activity; in contrast, it is inhibitory to DNA polymerase α activity; addition of 100 mM KCl increased the EBV DNA polymerase by 700% whereas it decreased DNA polymerase α activity by 75%. Furthermore, EBV DNA polymerase requires 4 mM Mg^{++} but DNA polymerase α requires 8 mM Mg^{++}. Thus, at conditions optimum for EBV DNA polymerase activity, only 20% DNA polymerase α activity is expressed. Secondly, the viral and cellular DNA polymerases differ markedly in their sensitivities to inhibitors such as aphidicolin and N-ethylmaleimide (NEM). DNA polymerase α is quite sensitive to inhibition by aphidicolin (33) while EBV DNA polymerase is not; Fig. 3 illustrates the effect of aphidicolin on the activities of DNA polymerase α, DNA polymerase β and EBV DNA polymerase; 20 μM aphidicolin inhibited 49% of DNA polymerase α activity while it inhibited the activity of EBV DNA polymerase by only 3%; DNA polymerase β activity was inhibited by 18%. Moreover, addition of 2 mM NEM almost completely inhibited the DNA polymerase α activity whereas it inhibited the EBV DNA polymerase activity by only 19%.

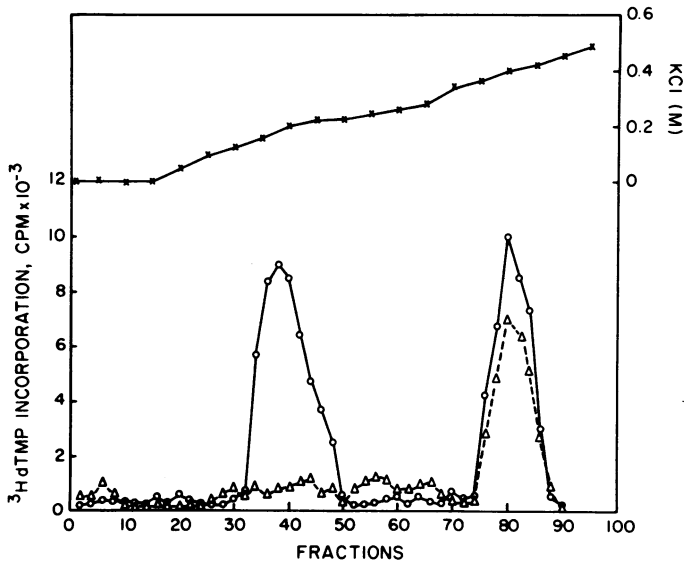

Figure 2

Phosphocellulose chromatography of DNA polymerases from Raji cells. Samples(10 μl) of every other fractions were assayed under conditions optimum for DNA polymerase α (o——o) and EBV-DNA polymerase (△- - -△).

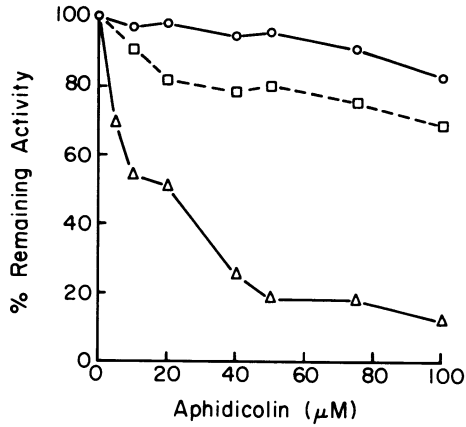

Figure 3

Effect of aphidicolin on the activities of EBV-DNA polymerase, DNA polymerase α and DNA polymerase β. Aphidocolin was kindly provided by Dr. B. Hesp of the Imperial Chemical Industries, England. The polymerases isolated from cell line A (Fig. 1A) were used. DNA polymerase α (Δ——Δ); DNA polymerase β (□- - -□); EBV-DNA polymerase (○——○). One hundred percent activities of DNA polymerase α, DNA polymerase β and EBV-DNA polymerase represent 19, 12, and 17 pmoles of [^{3}H]dTMP incorporated into DNA respectively, under assay conditions optimum for the individual polymerases. Other conditions are described in the text.

Levels of viral and cellular DNA polymerases in three virus producing cell lines: The EBV DNA polymerase and cellular DNA polymerases α and β were isolated from three cell lines referred to as A, B and C (Fig. 1A, 1B and 1C). The levels of the viral and cellular DNA polymerases were quantitated in all the three cell lines using similar procedures. The active fractions from each peak were pooled and total amount of the polymerase activity was determined by assaying the activities under conditions optimum for the individual polymerases. There was a gradual increase in the level of EBV DNA polymerase from cell line A to C. Table 1 illustrates the amount of viral and cellular DNA polymerases in the three cell lines. There was a 5.3-fold increase in the level of EBV DNA polymerase between cell line A and C. Thus, the amount of EBV DNA polymerase correlated with an increase in percent of virus producing cells in a given population. The levels of the cellular DNA polymerases were also compared simultaneously in all the three cell lines. Unlike the level of viral polymerase, the level of DNA polymerase α decreased with an increase in VCA titer. The DNA polymerase α activity in cell line B

TABLE 1. Relative levels of the DNA polymerase activities

Cell line		Enzyme activity/10^6 cells, n moles		
		EBV-DP	DP α	DP β
A. P3HR-1	<1% VCA	237 (100%)	98 (100%)	68 (100%)
B. P3HR-1	4% VCA	363 (153%)	55 (56%)	65 (95.6%)
C. P3HR-1	TPA treated 14% VCA	1250 (527%)	0 (0%)	54 (79.4%)

Active fractions from each peak eluting at 0.05, 0.18 and 0.35M KCl, respectively, were combined and the total activity in each pool was determined by assaying them under conditions optimum for EBV-DNA polymerase, DNA polymerase α and DNA polymerase β,respectively. The enzyme activity is expressed as n moles of [^{3}H]dTMP incorporated into acid precipitable counts in 30 min per 10^6 cells. Cell line A was >1% VCA positive; cell line B was 4% VCA positive and cell line C represents line B treated with TPA.

decreased to nearly half the level of cell line A; it completely disappeared in cell line C. However, there was only a slight decrease in the level of DNA polymerase β activity between cell lines A and C.

Comparison of EBV DNA polymerase from control and TPA treated cells. To determine whether there was any difference in properties between EBV-specific DNA polymerase isolated from the control P3HR-1 cells and TPA treated cells, the properties of enzymes from cell lines B and C were compared. There was no difference in the elution position on phosphocellulose column. The enzymes from these two cell lines were further compared for molecular weight, pH optimum, optimum concentrations of monovalent and divalent cations, and other requirements for optimum activity. The enzyme from these two sources are identical in all the properties examined. For example, EBV DNA polymerase isolated from the control and TPA treated cells have identical molecular weights (approximately 67,000) as determined by gel filtration. The polymerases from both sources have similar requirement for KCl for optimum activity; at 100 mM KCl the activities of both polymerases increased seven fold over the control with no salt; higher concentration of KCl, however, decreased the

activities of both enzymes (Data not shown).

Mixing experiments. To examine whether a decrease in the level of DNA polymerase α in cell line B and a total disappearance in cell line C was due to any inhibitor present in cell line C, the following experiment was performed. The DNA polymerase α purified from the cell line A was mixed with the crude extracts from cell line C. It did not have any effect on the activities of the DNA polymerase α. Furthermore, samples from the phosphocellulose fractions of cell line C eluting at 0.18 M KCl, the region where the DNA polymerase α normally elutes, was mixed with the DNA polymerase α and its effect on the enzyme activity was measured. There was no effect on the enzyme activity. From these results, it is concluded that the apparent loss of the DNA polymerase α in cell line C was not due to any inhibitor specific to DNA polymerase α present in the TPA treated cells.

Effect of TPA on the enzyme activities. Varying concentrations of TPA were added to the enzymes and its effect on their activities were examined. Table 2 shows that TPA did not have any effect on the activities of any of the enzymes tested. This indicated two things: an increase in EBV DNA polymerase activity observed in crude extracts of cells grown in presence of TPA was not due to a stimulatory effect of TPA on the activity of the viral polymerase;

TABLE 2. Effect of TPA on the activities of DNA polymerases

		% Remaining activity in presence of TPA (ng)			
Polymerase	Cell Source	0	1	10	100
EBV-DP	P3HR-1	100(18.8)	99.5	90.6	93.7
EBV-DP	TPA treated P3HR-1	100(43.8)	93.9	101.2	94.9
DP - α	P3HR-1	100(20.0)	97.5	94.4	97.5
DP - β	P3HR-1	100(18.3)	94.9	93.9	103.0

The DNA polymerase activities were determined under conditions optimum for the individual enzymes. TPA was originally dissolved in 50% dimethyl sulfoxide,DMSO, (1 mg/ml). The final concentrations of DMSO in the reaction mixture ranged from 0.005 to 0.5%. Addition of DMSO at this range of concentrations to the reaction mixture did not have any effect on activities of the enzymes used in this experiment. The effect of adding 1, 10 or 100 ng of TPA in 50 µl was determined. The numbers in parenthesis represent pmoles of 100% activity of the polymerases.

moreover, a decrease in the DNA polymerase α activity in TPA treated cells was not due to a direct inhibitory effect of TPA on DNA polymerase α activity.

DNA polymerases from Raji and BJAB cell lines: DNA polymerases were isolated from Raji, an EBV nonproducer cell line, by similar procedures used for P3HR-1 cell line. EBV DNA polymerase consistently elutes at 0.05M KCl on a phosphocellulose column with a linear salt gradient. It is evident from Fig. 2 that there was no peak at 0.05M KCl on this column showing that Raji cells do not contain EBV DNA polymerase activity. This is in agreement with earlier reports of Goodman et al (25) and Feighney et al (28) who clearly demonstrated the absence of EBV DNA polymerase activity in Raji cells. The DNA polymerase α and DNA polymerase β, as expected, eluted at 0.18M and 0.35M KCl, respectively (Fig. 2). Under assay conditions optimum for EBV-DNA polymerase, some activity of DNA polymerase β, but not of DNA polymerase α, is usually expressed. Thus, the enzyme that eluted at 0.35M KCl was DNA polymerase β but not EBV DNA polymerase. Further confirmation that the activity that eluted at 0.35M KCl was DNA polymerase β, was obtained by examining the effect of KCl, dideoxythymidine triphosphate and N-ethylmaleimide on its activity (32). When Raji cells were grown in presence of 20 ng of TPA and DNA polymerases were analyzed using similar procedures, there was no qualitative change in the phosphocellulose profile of DNA polymerases. These results indicate that TPA did not induce EBV DNA polymerase in Raji cells. BJAB, an EBV-genome negative cell line, when analyzed similarly, did not contain EBV DNA polymerase activity; the phosphocellulose profile of DNA polymerases from Raji and BJAB cell lines were similar (data not shown).

DISCUSSION

Tumor promoters of the diterpene ester type induce persisting genomes of oncogenic herpesviruses. It was therefore of interest to investigate the level of viral DNA polymerase in TPA induced cells. The data presented here show a quantitative relationship between an increase in EBV production and the viral DNA polymerase level. In this respect, our results are consistent with those of Datta et al (29), which appeared during the course of this investigation, showing a parallel increase in EBV DNA polymerase activity and VCA positive cells. However, effect of the tumor promoter on the levels of cellular DNA polymerases in EBV producing cell lines has not been examined previously. Evidence that the polymerase that eluted at 0.05M KCl on a phosphocellulose column is the EBV-specific DNA polymerase but not an alternate form of DNA polymerase α is as follows: a) It is an additional poly-

merase present only in EBV-producing cell lines; A nonproducer cell line, Raji, and a viral genome negative cell line, BJAB, did not contain the polymerase eluting at 0.05M KCl on a phosphocellulose column. b) The level of DNA polymerase that eluted at 0.05M KCl increased proportionally to an increase in the percent of virus-producing cells. c) Properties of the additional polymerase such as monovalent and divalent cation requirements for optimum activity, sensitivity to inhibitors such as aphidicolin and NEM resemble those of EBV DNA polymerase reported from several laboratories including ours (24-29) but differ from the properties of the cellular DNA polymerases α and β (20,21,33). The additional polymerase is distinctly different also from DNA polymerase γ both in its quantitative and qualitative characteristics; DNA polymerase γ usually represents only 0.5 to 1% of the total cellular DNA polymerase activity whereas the enzyme that eluted at 0.05M KCl was 60% of the total DNA polymerase activity in the cell line A. In addition, the elution position on phosphocellulose column and certain enzymic properties of the polymerase that eluted at 0.05M KCl are different from those characteristic of DNA polymerase γ (21).

In addition to quantitating the levels of EBV specific DNA polymerase in these cells, we have determined the levels of cellular DNA polymerases α and β as well. Of particular interest is a reciprocal relationship between the levels of EBV DNA polymerase and DNA polymerase α i.e., in cells that have the highest level of EBV DNA polymerase, DNA polymerase α level decreased to an insignificant level. A decrease in DNA polymerase α activity with an increase in viral DNA polymerase activity has been observed during replication of another herpes group virus, herpes simplex virus type-1 in a mouse cell line (35).

We ruled out the possibility that the observed decrease in the level of DNA polymerase α was due to any inhibitor present in TPA treated cells by demonstrating that addition of crude extract from TPA treated cells to DNA polymerase α did not inhibit its activity. Addition of TPA to the reaction mixture did not affect the activities of either viral or cellular DNA polymerases. It is possible that the synthesis of DNA polymerase α is inhibited in cells induced to replicate more EBV. Experiments to determine the half life of the cellular as well as viral DNA polymerases may provide a lead in understanding the mechanism of such a change. It is known that induction of replication of herpesviruses, particularly herpes simplex viruses, leads to shut off of host protein synthesis (36). It is likely that the DNA polymerase α is one such protein the synthesis of which is turned off in virus-

producing cells.

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*To whom correspondence should be addressed.

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