Hall, B. G., & Hartl, D. L. (1974) *Genetics 76*, 391–400.
Hall, B. G., & Hartl, D. L. (1975) *Genetics 81*, 427–435.
Hall, B. G., & Clarke, N. (1977) *Genetics 85*, 193–201.
Hall, B. G., & Zuzel, T. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 3529–3533.
Marshall, P., Reed, C. G., Sinnott, M. L., & Souchard, I. J. L. (1977) *J. Chem. Soc., Perkin Trans. 2*, 1198–1202.
O'Farrell, P. H. (1975) *J. Biol. Chem. 250*, 4007–4021.
Rolseth, S. J., Fried, V. A., & Hall, B. G. (1980) *J. Bacteriol. 142*, 1036–1039.
Sinnott, M. L., & Souchard, I. J. L. (1973) *Biochem. J. 133*, 89–98.

Characterization of Purified Epstein–Barr Virus Induced Deoxyribonucleic Acid Polymerase: Nucleotide Turnover, Processiveness, and Phosphonoacetic Acid Sensitivity†

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ABSTRACT: The Epstein–Barr (EB) virus induced DNA polymerase has been further purified and characterized with respect to nucleotide turnover activity, processiveness of synthesis, and interaction with phosphonoacetic acid (PAA). The polymerase as purified through denatured DNA–cellulose chromatography was inseparable from a labile nuclease activity associated with an equally labile DNA-dependent nucleotide turnover function. The EB virus induced DNA polymerase even in the absence of detectable nuclease or nucleotide turnover activity was less processive in its synthesis than were lymphocyte α polymerase or procaryotic polymerases, and this processiveness decreased with increasing purity of the enzyme. PAA was shown to inhibit nucleotide incorporation by the EB virus induced DNA polymerase in the presence of nuclease-activated native DNA template in the manner of a pyrophosphate analogue. Under conditions in which the concentration of 3′-hydroxyl termini in the template was more limited, PAA was not inhibitory. PAA likewise failed to significantly decrease the processiveness and the nucleotide turnover function of the polymerase.

The Epstein–Barr (EB) virus induced DNA polymerase found in certain EB virus transformed lymphocytes has been associated with productive viral DNA replication (Goodman et al., 1978; Ooka et al., 1979; Datta et al., 1980) and resembles DNA polymerase activities induced by other herpes viruses (Weissbach et al., 1973; Boezi et al., 1974; Huang, 1975; Allen et al., 1977; Knopf, 1979). The EB virus DNA polymerase has also been shown to copurify through initial chromatography steps with an EB virus induced DNase activity containing inseparable exo- and endonuclease functions (Clough, 1979, 1980). Similar nuclease activities have been reported to copurify partially with the herpes simplex virus induced DNA polymerase (Weissbach et al., 1973; Hoffman & Cheng, 1978, 1979; Knopf, 1979; Ostrander & Cheng, 1980). We report here the further purification of the EB virus DNA polymerase by using chromatography with denatured DNA–cellulose. The resultant enzyme preparation represents a high level of recovery and degree of purity, enabling us to undertake studies not previously feasible on various aspects of enzyme function. Also, polymerase characteristics at varying levels of enzyme purity have been compared.

This study characterizes the purified EB virus induced DNA polymerase with regard to nucleotide turnover, processiveness of synthesis, and inhibition by phosphonoacetic acid (PAA).[1] The purified polymerase was observed to mediate nucleotide turnover from triphosphate to monophosphate in the presence of activated DNA template and more extensively in the presence of poly[d(A-T)] template. Such DNA-dependent turnover of nucleotide is characteristic of procaryotic DNA polymerases, which are thought to mediate this process with an associated nuclease (Huang & Lehman, 1972). The nucleotide turnover exhibited by the EB virus induced DNA polymerase, however, showed certain differences from the procaryotic polymerase associated turnover. The processiveness of a DNA polymerase refers to the average number of nucleotides incorporated with each polymerase–template binding event. When this parameter was determined for the EB virus induced DNA polymerase in the presence or absence of associated turnover and nuclease activities, the number of nucleotides added per polymerase–template binding event appeared to decrease with increasing purity of the enzyme preparation and in all cases was a lower number than the processiveness of lymphocyte α polymerase or procaryotic polymerases. Studies of PAA inhibition showed that PAA was most inhibitory to the EB virus induced polymerase under assay conditions where a template with a high concentration of 3′-hydroxyl termini was present. PAA had little effect on polymerase processiveness or nucleotide turnover. From these data, we concluded that PAA did not always function as a pyrophosphate analogue as previously suggested (Leinbach et al., 1976).

Materials and Methods

Materials. [*methyl*-^{3}H]Thymidine 5′-triphosphate (55 Ci/mmol) was from Schwarz/Mann, and [α-^{32}P]deoxy-

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[1] Abbreviations used: PAA, phosphonoacetic acid; EBV, Epstein–Barr virus; PEI, poly(ethylenimine); Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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adenosine 5′-triphosphate (2000 Ci/mmol) was from Amersham. RPMI media and fetal calf sera were from Gibco. Calf thymus DNA, DNase I, micrococcal nuclease, and spleen phosphodiesterase were purchased from Worthington. Unlabeled deoxynucleotide triphosphates and insulin were from Sigma. Cellulose was a gift of the Brown Paper Co., Berlin, NH. Phosphonoacetic acid was a gift of Abbott Laboratories.

Cell Growth and Preparation of Cell Extracts. Cell growth and cell extracts were as previously described (Goodman et al., 1978; Clough, 1979). The preparation and assay of intracellular EB virus induced DNA polymerase by DEAE-cellulose and phosphocellulose chromatography were as previously reported by this laboratory (Goodman et al., 1978) with the modifications noted (Clough, 1979).

Preparation of DNA Template for Polymerase Assays. High molecular weight DNA was prepared by overnight solubilization using gentle stirring of 1 mg of calf thymus DNA per mL in distilled water. Activation of this DNA with DNase I as described (Aposhian & Kornberg, 1962) produced a very active substrate for DNA polymerase assays. Activated DNA was not viscous as was the untreated DNA and was rendered about 7% acid soluble by activation, compared to 1% for unactivated DNA.

Denatured DNA–Cellulose Chromatography. Denatured DNA–cellulose was prepared as described (Litman, 1968), except that the calf thymus DNA was sheared by five rapid passages through an 18-gauge needle and then heated for 40 min in a boiling water bath before binding to cellulose. Denatured DNA–cellulose (1.5 g) was packed in a 1.4 × 10 cm column. This was equilibrated with several column volumes of buffer C (Clough, 1979) to which 10 μg of insulin per mL was added to stabilize the enzyme. The EB virus induced DNA polymerase containing phosphocellulose fractions which had been dialyzed against buffer C were slowly (0.2 mL/min) passed into the column, and the flow was stopped for 30 min to allow the enzyme to adhere to the DNA–cellulose. The column was washed with 20 mL of buffer C and then eluted with 60 mL of a linear gradient of 0–0.9 M KCl in buffer C. The column was then washed with 10 mL of 0.9 M KCl in buffer C. One-milliliter fractions were collected throughout and assayed for DNA polymerase and exonuclease activity.

Determination of Polymerase Processiveness. The lysis-defective amber mutant φX174am3 was obtained from Dr. H. Shizuya and was used to prepare a high titer stock of virions (Sinsheimer, 1966). Single-stranded DNA was prepared by virion purification in CsCl density gradients and phenol extraction of virion DNA (Thomas & Abelson, 1966). The double-stranded replicative form (RF) was prepared by infection of *Escherichia coli* C followed by amplification through addition of 20 μg of chloramphenicol per mL to the culture for 15 h at 37 °C. The cells were lysed, chromosomal DNA was removed (Clewell & Helinski, 1969), and the RF was purified as described by Zasloff et al. (1978) followed by chromatography on Sepharose 2B (Pharmacia) to remove small oligonucleotides. The RF was digested to completion with *Hae*III (Bethesda Research Laboratories), and the resulting fragments were separated on a 0.75% agarose slab gel. The fragments were eluted by diffusion during overnight incubation of the macerated gel slices (Smith, 1980). For each separate processiveness determination, 5 μg of the single-stranded φX174am3 DNA was mixed with an equimolar (polymer, not nucleotide) amount of the 1353-base-pair *Hae*III fragment of the RF. These were placed in a 100 °C bath for 3 min in TE (1 mM Tris-HCl, pH 8.0, and 1 mM EDTA, pH 8.0) followed by hybridization at 60 °C for 10 min. After chilling of the DNA, 25 μL of a DNA polymerase sample and 45 μL of a mixture were added to the φX174 DNA, producing a 100-μL assay volume with 80 mM Tris-HCl, pH 8.0, 5 mM magnesium acetate, 10 μM each of dGTP, dATP, and dCTP, 5 μM [^{3}H]dTTP (3.6×10^4 cpm/pmol, determined by counting from PEI plates), 1 mM dithiothreitol, and 0.05% Triton X-100. Incubations were for 15 min at 37 °C and were stopped with 0.2 mL of 200 mM sodium pyrophosphate; 25 μL of a 1 mg/mL stock solution of activated calf thymus DNA was added as carrier, and the samples were precipitated by addition of 1 mL of 15% trichloroacetic acid. After 15 min on ice, the precipitates were collected by centrifugation for 15 min at 1500*g* and were dissolved in 0.5 mL of 0.1 N NaOH. This was neutralized with 1 mL of 1 M Tris-HCl, pH 8.0, followed by 36 h of dialysis against four changes of 1 L each of TE and by ethanol precipitation. The precipitated samples were dissolved in 70 μL of TE buffer to which were added 1 μL of micrococcal nuclease (1 mg/mL, 18 530 U/mg), 4 μL of 1 M glycine buffer, pH 9.2, 4 μL of 50 mM $CaCl_2$, and 27 μL of H_2O. After a 3.5-h incubation at 37 °C, the following additions were made: 10 μL of 200 mM ammonium acetate, 2 μL of 150 mM potassium phosphate buffer (pH 6.5), 4 μL of 200 mM acetic acid, and 11 μL of spleen phosphodiesterase (1.1 mg/mL). These mixtures were incubated at 37 °C for 3.5 h, after which they were centrifuged at 1500*g* for 20 min; 60 μL of each supernatant was spotted, 5 μL at a time, onto freshly washed poly(ethylenimine) thin-layer chromatography (PEI) plates (Brinkmann) in the presence of 0.1 μmol each of 3′-dTMP and thymidine. The plates were developed in 0.5 M LiCl and dried, and the spots corresponding to 3′-dTMP and thymidine were cut out. Each spot was eluted by shaking in 1.0 mL of 0.5 N HCl at room temperature for 45 min; 5 mL of Triton scintillation fluid (Clough, 1979) was mixed in, and each sample was counted in a liquid scintillation counter.

DNA Polymerase and Nuclease Assays. The DNA polymerase assays for column fractions and PAA inhibition were 100 μL containing 80 mM Tris-HCl, pH 8.0, 10 μM each of dATP, dCTP, and dGTP, 5 μM dTTP, 10 μCi/100 μL assay [^{3}H]dTTP, 1 mM dithiothreitol, 0.05% Triton X-100, and 15 μg/100 μL assay of activated or unactivated calf thymus DNA. An enzyme sample (25 μL) (sometimes diluted as indicated with buffer C) was included in each reaction. When appropriate, the concentration of activated DNA substrate or unlabeled deoxynucleoside triphosphate was varied as indicated. The incubations were for 30 min at 37 °C. Determination of incorporation of labeled nucleotide into the template DNA by using trichloroacetic acid precipitation was as described by Goodman et al. (1978).

When nucleotide turnover was to be determined (Table I, Figure 2), the assays were the same, except dTTP was 10 μM, dATP was 5 μM, and 1 μCi of [α-^{32}P]dATP was used per assay as the labeled nucleotide. The template was either activated DNA or poly[d(A-T)] (at 15 μg/assay or as indicated). Control experiments were always performed in which TE buffer was substituted for template. After incubation for the times indicated, the tubes were removed to ice. Twenty microliters was removed to tubes containing 10 μL of a solution containing 10 mM each of dATP and dAMP as markers and 5 μL of 250 mM EDTA; 25 μL of this mixture was spotted, 5 μL at a time, onto freshly washed PEI plates. The plates were developed in 1 M LiCl, and the nucleotide spots were cut out and counted for radioactivity as described above. The remaining 80 μL of the original 100-μL reaction volume was used to determine nucleotide incorporation as follows: 0.2 mL

Table I: Purification of the EB Virus Induced DNA Polymerase: Relative Recoveries of Copurifying Nuclease and Nucleotide Turnover Activities

recovery of	purification stage of EB virus induced polymerase		
	DEAE-cellulose	phosphocellulose	denatured DNA-cellulose
polymerase activity[a]	69400 U (100%)	49800 U (72%)	31600 U (46%)
total protein[b]	243 mg	4.7 mg	*e*
nuclease activity[c]	4063000 U (100%)	488000 U (12%)	5145 U (0.1%)
poly[d(A-T)]-dependent turnover of [α-^{32}P]dATP[d]	89%	93%	95%

[a] DNA polymerase assays were performed as described under Materials and Methods in the presence of activated DNA template. One unit of polymerase activity was defined as that amount of enzyme incorporating 1 pmol of [^{3}H]dTMP (1500 cpm/pmol) per 30 min at 37 °C. [b] Total protein was determined by the method of Lowry et al. (1951). [c] Nuclease assays were performed as described under Materials and Methods in the presence of [^{3}H]poly[d(A-T)] substrate. One unit of nuclease activity was defined as that amount of enzyme releasing 1 pmol of dTMP per 30 min at 37 °C. [d] Poly[d(A-T)]-dependent turnover of [α-^{32}P]dATP was determined as described under Materials and Methods. The figures shown above represent that percentage of the [α-^{32}P]dATP which had been inserted into the template by the polymerase and which was turned over. [e] Due to the instability of the associated nucleotide turnover function, polymerase preparations were purified in the presence of added insulin or bovine serum albumin at this stage. Therefore, accurate protein determinations of nuclease-active polymerase preparations of this degree of purity are not available.

of 200 mM sodium pyrophosphate was added followed by 5 μL of bovine serum albumin (50 mg/mL) and 1 mL of 15% trichloroacetic acid. The precipitates were collected by centrifugation at 1500*g* for 20 min, dissolved in 0.5 mL of 0.1 N NaOH, then reprecipitated, and redissolved. They were precipitated again and collected onto GF/A filters (Whatman); the precipitates were washed with 0.5 N HCl and 70% ethanol, and the filters were dried and counted.

The nuclease assays were 200 μL and contained 5 mM $MgCl_2$, 10 mM mercaptoethanol, 50 mM Tris-HCl, pH 8.3, 1–2 μg of [^{3}H]poly[d(A-T)] [(1.5–2.5) × 10^4 cpm/μg], and 25 μL of enzyme sample. After incubation at 37 °C for 15 min, the tubes were removed to ice, and 200 μL of bovine serum albumin (250 μg/mL) was added, followed by addition of 150 μL of 6% perchloric acid. The precipitates were removed by centrifugation at 1500*g* for 20 min, and the supernatants were poured into scintillation vials. A scintillation fluor (5 mL) containing Triton X-100 (Clough, 1979) was mixed in, and the vials were counted.

Results

Further Purification of Phosphocellulose-Purified EB Virus Induced DNA Polymerase Using Denatured DNA–Cellulose Chromatography. Purification of the EB virus induced DNA polymerase through columns of DEAE-cellulose and phosphocellulose has been described (Goodman et al., 1978; Clough, 1979). The phosphocellulose column fractions containing EB virus induced DNA polymerase activity were pooled and dialyzed against low phosphate buffer C (see Materials and Methods) for 2–3 h. As measured by conductivity, this removed most of the phosphate used for elution of proteins from the phosphocellulose. More extended dialysis led to significant loss of polymerase activity. The dialyzed fractions were loaded slowly onto a column of denatured calf thymus DNA–cellulose. (The enzyme was found not to bind to native DNA–cellulose; data not shown.) After the sample had entered the column bed it was allowed to bind for 30 min and then eluted with a linear gradient of 0–0.9 M KCl in buffer C (see Materials and Methods). The elution profile in Figure 1 shows that the DNA polymerase elutes from denatured DNA–cellulose in the lower third of the salt gradient.

Phosphocellulose column chromatography partially separated the EB virus induced polymerase from the EB virus induced nuclease (Clough, 1979). While this EB viral nuclease activity has been shown to function as an exonuclease, a copurifying endonucleolytic function has been detected (Clough, 1979, 1980). Further separation of the DNA polymerase and the exonuclease activities was seen upon chromatography of

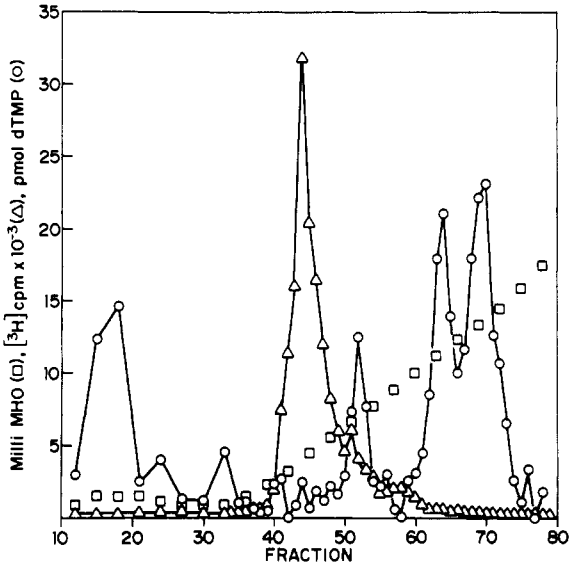

FIGURE 1: Denatured DNA–cellulose chromatography of EB virus induced DNA polymerase. Phosphocellulose-purified enzyme was chromatographed on denatured DNA–cellulose in the presence of low phosphate buffer C and a 0–0.9 M KCl gradient as described under Materials and Methods and Results. Fractions were assayed for conductivity (□), for DNA polymerase activity in the presence of activated DNA template (△), and for nuclease activity measuring pmol of dTMP released from [^{3}H]poly[d(A-T)] substrate (○).

the polymerase on denatured DNA–cellulose, where the bulk of the nuclease activity eluted at different KCl concentrations (Figure 1) than did the polymerase activity. This nuclease showed a substrate preference for [^{3}H]poly[d(A-T)] rather than [^{3}H]DNA isolated from Raji lymphocytes labeled in vivo with [^{3}H]thymidine (Clough, 1980). A similar preference for poly[d(A-T)] was observed for nuclease isolated from herpes simplex virus infected cells (Weissbach et al., 1973; Hoffman & Cheng, 1978; Knopf, 1979).

The first stage at which the EB virus DNA polymerase is separated from the cellular polymerases is during the gradient elution from DEAE-cellulose; this is therefore the first stage at which EB virus DNA polymerase activity can be quantified. The recovery of activity through phosphocellulose and denatured DNA–cellulose chromatography is 72% and 46%, respectively, with the DEAE-cellulose-purified sample normalized to 100% (Table I).

Table II shows that the DNA–cellulose-purified EB virus DNA polymerase can be readily distinguished from the cellular α and γ DNA polymerases by the utilization of different templates. The viral polymerase uses poly[d(A-T)] as template

Table II: Comparative Template Specificities of the EB Virus Induced, Lymphocyte α, and Lymphocyte γ DNA Polymerase Activities[a]

template	EB virus induced	lymphocyte α	lymphocyte γ
activated DNA	100	100	100
unactivated DNA	6	16	50
poly[d(A-T)]	17	126	252
poly(dC)·$(dG)_{12-18}$	266	77	1551
poly(rA)·$(dT)_{12-18}$	6	2	1030

[a] The EB virus induced DNA polymerase was DNA-cellulose purified, and the lymphocyte α and γ polymerases were phosphocellulose purified [see text and Goodman et al. (1978); Clough (1979)]. DNA polymerase assay conditions were as described under Materials and Methods using activated or unactivated calf thymus DNA template (150 μg/mL) or poly[d(A-T)] (Miles, 2.3 OD_{260} units/mL) or poly(rA)·$(dT)_{12-18}$ (P-L Biochemicals, 1.6 OD_{260} units/mL). The assays containing poly(dC)·$(dG)_{12-18}$ (Miles, 2.5 OD_{260} units/mL) measured incorporation of [^{3}H]dGTP over 30 min at 37 °C and were as described by Goodman et al. (1978). The values obtained with activated DNA as template were set at 100% and were DNA-cellulose-purified EB virus induced DNA polymerase, 25 320 cpm, α polymerase, 294 860 cpm, and γ polymerase, 34 250 cpm.

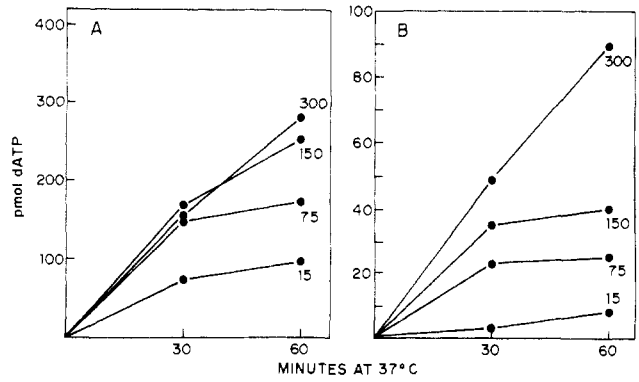

FIGURE 2: Nucleotide turnover as compared to nucleotide incorporation by the DNA-cellulose-purified EB virus induced DNA polymerase in the presence of various concentrations of poly[d(A-T)] template. Nucleotide turnover (A) and incorporation (B) were determined as described under Materials and Methods using [α-^{32}P]dATP in the presence of the indicated concentrations of poly[d(A-T)] (15, 75, 150, and 300 μg/mL). The nucleotide turnover function had a saturating template concentration of 150 μg of poly[d(A-T)]/mL, apparently far lower than the saturating template concentration for nucleotide incorporation.

far less efficiently than do the other enzymes. Conversely, the EB virus polymerase uses poly(dC)·$(dG)_{12-18}$ more efficiently than α polymerase uses this template. This observation has been reported by others for this polymerase (Miller et al., 1977; Ooka et al., 1979) and for the polymerases of other herpes viruses (Miller & Rapp, 1976; Huang, 1975). The EB virus polymerase uses poly(rA)·$(dT)_{12-18}$ very poorly compared with γ polymerase.

Template-Dependent Nucleotide Turnover Associated with EB Virus Induced DNA Polymerase. Nucleotide turnover refers to the DNA-dependent conversion of deoxynucleoside triphosphates to the corresponding monophosphate. In the case of procaryotic polymerases, such turnover activity is thought to be due to nuclease-mediated excision of a nucleotide previously inserted into the template. A nucleotide turnover reaction was observed for the EB virus induced DNA polymerase when poly[d(A-T)] was used as template (Figure 2A) and to a lesser extent with an activated DNA template. The turnover values shown represent deoxynucleoside monophosphate generated by the action of the DNA–cellulose-purified enzyme in the presence of poly[d(A-T)] template. All turnover assays were done in the presence of controls, which were identical assays performed in the absence of template. In the case of less purified polymerase preparations, some deoxynucleoside monophosphate was produced in such control experiments, but this template-independent nucleotide turnover diminished greatly with enzyme purification and was virtually absent from assays performed with DNA–cellulose-purified polymerase such as depicted in Figure 2A. The poly[d(A-T)]-independent turnover present in less pure enzyme preparations most probably resulted from contaminating nucleoside triphosphatase that was removed through purification.

As shown in Table I, the amount of nuclease activity that copurified with polymerase progressively decreased during three purification stages. The assay of the denatured DNA–cellulose column fractions for nuclease activity with [^{3}H]-poly[d(A-T)] (Figure 1) or with [^{3}H]DNA (data not shown) showed low but significant activity over background in the region of the polymerase activity. The data in Table I also show that the fraction of nucleotide which was inserted into the poly[d(A-T)] template by the DNA polymerase and which was turned over was not decreased by progressive purification. Therefore the turnover observed was not due to the nuclease activity that was separated from the polymerase by phosphocellulose and denatured DNA–cellulose chromatography but was due to the residual nuclease activity copurifying with polymerase through denatured DNA–cellulose chromatography. The fact that the fraction of inserted nucleotide which was turned over was unaltered by progressive purification implies the existence of a specific association between the turnover and polymerase functions.

Figure 2B shows the nucleotide incorporation achieved by the polymerase in the same set of experiments for which nucleotide turnover was measured (Figure 2A). It can be seen that the saturating template concentration is significantly lower for the nucleotide turnover function than for the nucleotide incorporation function of the DNA–cellulose-purified EB virus induced DNA polymerase.

Both nuclease and turnover activities were found to decay in 7–10 days upon storage of DNA–cellulose-purified polymerase at −80 °C, although the polymerase activity remained undiminished. Storage of DNA–cellulose-purified polymerase under these conditions was the only way that we have been able to obtain polymerase completely free of nucleotide turnover or residual nuclease activities since rechromatography of the polymerase-active fractions from columns such as that depicted in Figure 1 did not separate any further nuclease activity from the polymerase.

Processiveness of the EB Virus Induced DNA Polymerase. The average number of nucleotides incorporated by a DNA polymerase each time a polymerase–template binding event occurs is termed the processiveness of the polymerase. This parameter was measured for the EB DNA polymerase with a modification of the method of Das & Fujimura (1979). The template in this assay was circular single-stranded DNA of the bacteriophage φX174 that had been primed with a restriction fragment from the double-stranded replicative form of φX174. This method involved a limited synthesis by the polymerase so that only a fraction of the 3′-hydroxyl ends available received any nucleotide addition. The synthetic product was isolated and cleaved to 3′-monophosphonucleosides with micrococcal nuclease followed by spleen phosphodiesterase (see Materials and Methods). The resultant limit digestion products contained nucleosides that were only from the termini of the product of the limited synthesis by the DNA polymerase. 3′-Monophosphonucleosides were released from nucleotides that were not at termini, in other words, that were internal

Table III: Comparative Processiveness of the EB Virus Induced, α, and Procaryotic DNA Polymerases[a]

polymerase	(a) processiveness (to one standard deviation)	(b) processiveness in the presence of 50 μg of PAA/mL
EB virus induced		
DEAE-cellulose purified	9.7 ± 1.5	9.2 ± 3.2
phosphocellulose purified	10.3 ± 1.7	ND[b]
DNA–cellulose purified	6.1 ± 2.5	6.3 ± 5.4
α (lymphocyte)	19.8 ± 4.5	17.1 ± 6.4
Escherichia coli poll	19.8 ± 2.3	ND
T4 bacteriophage	23.2	ND

[a] Determination of polymerase processiveness was performed by using primed phage φX174 DNA template as described under Materials and Methods. Incubations were for 15 min at 37 °C using 2.5–6 units of polymerase activity per determination. One unit polymerase activity was defined as that amount of enzyme incorporating 1 pmol of [^{3}H]dTMP per 30 min at 37 °C into highly activated DNA template in the presence of the other assay conditions detailed under Materials and Methods. Lymphocyte α polymerase had been purified through DEAE-cellulose and phosphocellulose chromatography (Goodman et al., 1978; Clough, 1979) and was free of nuclease activity. Purified bacteriophage T4 DNA polymerase was the gift of M. Goodman and S. Watanabe. *E. coli* polI DNA polymerase was purchased from Boehringer Mannheim. [b] ND, not done.

within the DNA segment synthesized by the DNA polymerase. The total synthesis was restricted so that a fraction (approximately one-third) of the available 3′-hydroxyl termini had received any nucleotide addition. This increased the likelihood that the synthesis observed was the result of a single binding event of the DNA polymerase to the template.

The data in Table III showed that the EB virus induced DNA polymerase was one-half to one-third as processive on the primed φX174 DNA template as the lymphocyte α, T4 bacteriophage, or *E. coli polI* DNA polymerases. The processiveness of the EB virus polymerase apparently decreased with purification through denatured DNA–cellulose, although overlap of the standard deviations was apparent. The processiveness of six nucleotides for DNA–cellulose-purified EB virus polymerase was unchanged by the presence or absence of detectable nucleotide turnover or nuclease activity. The lymphocyte α used in these experiments was free of nuclease. The value shown here of 19.8 ± 4.5 nucleotides for lymphocyte α polymerase was higher than the 11 ± 5 nucleotides reported by Fisher et al. (1979) for KB cell α, although there was overlap in the standard deviations. The lymphocyte α polymerase preparation used here was not as fully purified, and processiveness of α polymerase has been shown to depend on associated proteins (Alberts et al., 1975; Bambara & Hockensmith, 1980).

PAA (Phosphonoacetic Acid) Inhibition of EB Virus DNA Polymerase. In HR-1 cells, the EB virus DNA polymerase was the only polymerase found to be markedly sensitive to inhibition by PAA; in the presence of activated DNA template, it was equally sensitive at all stages of purification (Figure 3). Leinbach et al. (1976) had previously shown that a DNA polymerase isolated from cells infected with herpes virus of turkey, when assayed with activated DNA template in the presence of PAA, showed noncompetitive inhibition with varying deoxynucleoside triphosphate concentration and mixed but principally uncompetitive inhibition with varying template concentration. To study the interaction of the EBV-induced

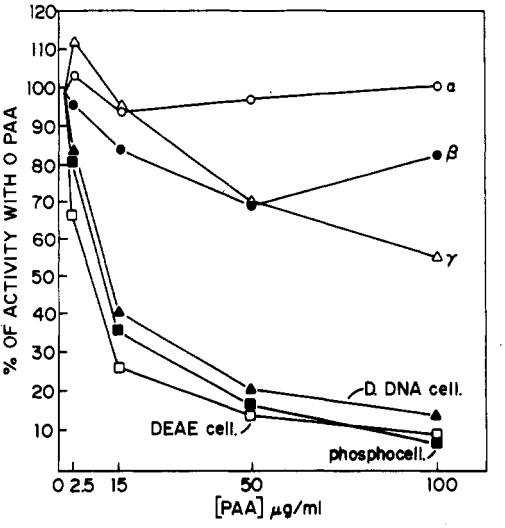

FIGURE 3: Inhibition of various lymphocyte DNA polymerase activities by PAA in the presence of activated DNA template. Lymphocyte α (○), β (●), and γ (△) polymerase activities were purified through DEAE-cellulose and phosphocellulose chromatography as described by Goodman et al. (1978). EB virus induced DNA polymerase was purified through DEAE-cellulose (□), phosphocellulose (■), and subsequently denatured DNA–cellulose (▲) chromatography. All assays were performed as described under Materials and Methods at 37 °C for 30 min in the presence of 15 μg of activated DNA/100 μL of assay mixture as template. PAA concentrations were as indicated. Activities of polymerases in the presence of 0 PAA were EB virus induced, DEAE-cellulose purified, 74 150 cpm, phosphocellulose purified, 25 580 cpm, DNA–cellulose purified, 23 560 cpm, α, 335 080 cpm, β, 120 080 cpm, and γ, 28 090 cpm.

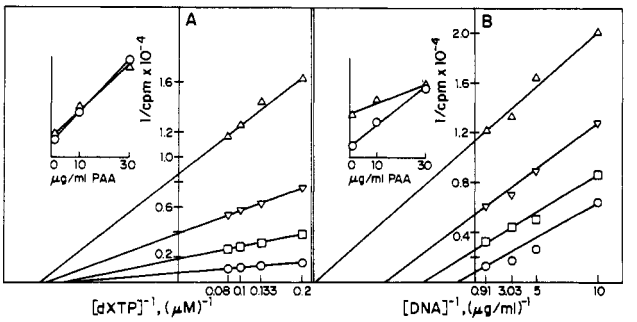

FIGURE 4: Plots of (initial enzyme velocity)$^{-1}$ vs. (A) ([deoxynucleoside triphosphates])$^{-1}$ or (B) ([activated DNA])$^{-1}$. The concentrations of the unlabeled deoxynucleoside triphosphates (μM) or of the activated DNA (μg/mL) were varied in the standard DNA polymerase assay with different PAA concentrations. (○) 0 μg/mL PAA; (□) 10 μg/mL PAA; (▽) 30 μg/mL PAA; (△) 100 μg/mL PAA. The inserts are replots against PAA concentration of the slopes (△) and intercepts (○) of the corresponding double-reciprocal plots, showing a K_{ii} of 8.0 μg/mL and a K_{is} of 5.0 μg/mL for PAA with varying deoxynucleoside triphosphate concentration and a K_{ii} of 5.0 μg/mL and a K_{is} of 46 μg/mL with varying activated DNA concentration.

DNA polymerase with PAA, we initially performed studies similar to those of Leinbach et al. (1976) using phosphocellulose-purified enzyme, which was most similar to the partially purified enzyme preparations used by Leinbach et al. In the presence of activated DNA template, we too were able to show that PAA inhibition of the EBV polymerase was noncompetitive with varying deoxynucleoside triphosphate concentration (Figure 4A) and mixed but principally uncompetitive with varying template concentration (Figure 4B). Leinbach et al. had interpreted these and related studies to mean that PAA was acting as a pyrophosphate analogue. Our data as presented in Figure 4 show that in the presence of activated DNA the EBV DNA polymerase interacts with PAA in a similar manner.

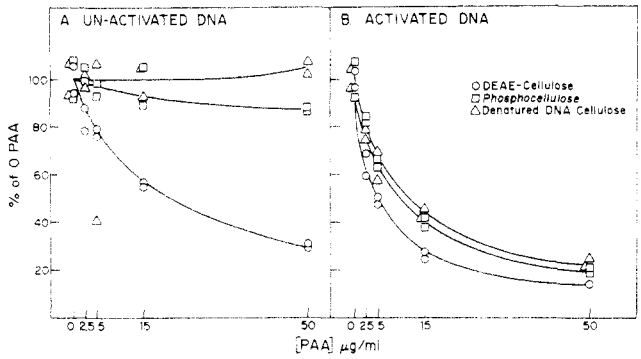

FIGURE 5: EB virus induced DNA polymerase purified through DEAE-cellulose, phosphocellulose, or denatured DNA–cellulose assayed for 30 min with various concentrations of PAA. (A) With unactivated DNA substrate. 100% values are DEAE-cellulose purified, 86 630 cpm, phosphocellulose purified, 14 400 cpm, and denatured DNA–cellulose purified, 2600 cpm. (B) With activated DNA substrate. 100% values are DEAE-cellulose purified, 265 080 cpm, phosphocellulose purified, 278 970 cpm, and denatured DNA–cellulose purified, 9860 cpm.

When the activated DNA template was replaced with calf thymus DNA that had not been activated with DNase I, the pattern of inhibition was different. The data in Figure 5A show that in the presence of intact, unactivated template, the inhibition was found to be the greatest with the DEAE-cellulose-purified EB virus polymerase, while the denatured DNA–cellulose-purified enzyme was insensitive to 50 μg/mL of PAA, a concentration that inhibited the same polymerase as much as 90% when activated DNA was used as template.

The phosphocellulose-purified polymerase always showed a variable but intermediate degree of inhibition by PAA. The only difference between the templates used in the studies shown in parts A and B of Figure 5 was that the activated DNA (Figure 5B) possessed more 3′-hydroxyl ends, which act as primer for the polymerase. The DEAE-cellulose-purified enzyme samples were known to contain significant amounts of EB virus induced nuclease activity, which would activate template DNA not previously activated with DNase I (Clough, 1979, 1980; Table I). We have found phosphocellulose-purified polymerase preparations to be somewhat variable in nuclease content although always containing significantly less nuclease activity than the DEAE-cellulose-purified polymerase. Likewise, the PAA inhibition pattern of phosphocellulose-purified polymerase, while always intermediate between that of DEAE-cellulose- and DNA–cellulose-purified enzyme, has been seen to be somewhat variable in the presence of unactivated DNA template.

Significant amounts of copurifying EB virus induced nuclease in the polymerase preparations would have the effect of partially digesting or activating unactivated DNA template. Only the DNA–cellulose-purified polymerase preparations would have sufficiently small amounts of poly[d(A-T)]-preferring nuclease activity to be unable to significantly activate a DNA template during the course of the assay. Therefore, the DNA–cellulose-purified polymerase may be the only EBV enzyme preparation that can actually be assayed in the presence of unactivated DNA. Since many other viral and even cellular proteins are removed from the DNA polymerase preparation during the course of purification, we cannot rule out the possibility that other accessory proteins have been removed that contribute to the interaction of the polymerase with PAA in the presence of unactivated template.

In summary, under conditions with unactivated DNA template and little or no contaminating nuclease activity, PAA no longer inhibited DNA polymerase activity and therefore no longer behaved as a pyrophosphate analogue. There was limited inhibition by PAA displayed in the presence of restriction fragment primed ϕX174 DNA template, which would be expected both to have a relatively limited concentration of 3′-hydroxyl ends and to be less susceptible to nuclease activation than native calf thymus DNA. Under these template conditions, DEAE-cellulose-purified polymerase activity was inhibited approximately 25% by 50 μg of PAA per mL, and DNA–cellulose-purified enzyme was inhibited in the 5%–10% range. PAA also showed little effect upon the processiveness of the EB virus induced DNA polymerase (Table III, column b). We have not been able to detect significant or reproducible inhibition by PAA of the polymerase-associated nuclease or turnover function. For example, in experiments such as those depicted in Figure 2A, 50 μg of PAA per mL would typically decrease the percent of nucleotides turned over by 10%–15%.

Discussion

The DNA polymerases of procaryotes (Kornberg, 1980) and of lower eucaryotes (Banks & Yarranton, 1976; Wintersberger, 1978) have been found to possess associated 3′–5′-exonucleases. These exonucleolytic activities are thought to maintain the fidelity of synthesis by "proofreading" the synthesis of the polymerase. The DNA polymerases of higher eucaryotes have not been shown to be associated with such an associated exonuclease. The data presented here show that the EB virus induced DNA polymerase possesses associated nuclease and DNA-dependent nucleotide turnover functions even after multiple purification steps. Although unassociated nuclease contaminating the polymerase sample could in principle account for this turnover, this appeared unlikely since the fraction of nucleotide turned over was independent of total amount of nuclease removed during purification (Table I). The small amount of nuclease present in the denatured DNA–cellulose-purified polymerase samples apparently accounted for all turnover observed. This conclusion was based on the data in Table I and the fact that nuclease and turnover functions disappeared simultaneously upon freezer storage of purified enzyme. The DNA polymerase induced by herpes simplex virus has also been shown to possess an associated exonuclease that has a preference for poly[d(A-T] (Weissbach et al., 1973; Ostrander & Cheng, 1980) and is capable of nucleotide turnover (Knopf, 1979).

Polymerase that was free of nuclease and demonstrated no turnover was inevitably obtained upon storage of the DNA–cellulose-purified EB virus induced polymerase for 7–10 days at −80 °C. This differential rate of inactivation of nuclease and polymerase activities in association with the different levels of template required for saturation (Figure 2A,B) suggested that the polymerase and nuclease resided on different polypeptides or in separate and independent regions of the same polypeptide. In the case of procaryotic enzymes, the usual way to prove that a polymerase possesses an associated nuclease is to purify the polymerase to homogeneity. Any associated nuclease activity can then be attributed to the same polypeptide. However, in the case of herpes virus induced polymerases, purification to homogeneity has not yet been achieved. We can demonstrate no further separation of nuclease activity with rechromatography of peak fractions of purified polymerase activity, and the instability of the associated nuclease activity makes it impractical to attempt further purification of the EB virus induced polymerase at this time.

The DNA of procaryotes is replicated by multienzyme systems of which DNA polymerase is but one component (Modrich & Richardson, 1975; Morris et al., 1975; Schekman et al., 1975), and it is expected that eucaryotic cells will show

at least this degree of complexity (DePamphilis & Wassarman, 1980). The EB polymerase associated nuclease may represent such an accessory protein.

DNA polymerases are expected to behave in a processive manner in vivo. Nonprocessive synthesis requires rebinding of the template by the DNA polymerase for each nucleotide addition, making it inherently less rapid and efficient than processive synthesis. Replication by the reconstituted multienzyme complex of bacteriophage T4 is much more processive than that of the purified T4 DNA polymerase alone (Alberts et al., 1975). α DNA polymerase species have been described that differ in their subunit components and also in the processiveness displayed (Bambara & Hockensmith, 1980). Therefore, some proteins that are removed during purification are responsible for more processive synthesis. A rationale has also been presented which could allow a high level of fidelity of synthesis by the polymerase with or without associated nuclease but would require processive synthesis (Hopfield, 1980). Our data suggest that the processiveness of the EB virus DNA polymerase, like that of α, decreases with increasing purification.

The ability of PAA to inhibit the EB virus DNA polymerase is highly dependent upon the template present and upon the degree of purity of the enzyme. Such variability is not consistent with PAA always acting as a pyrophosphate analogue (Leinbach et al., 1976). First, it is possible that successive purification steps remove accessory proteins that facilitate the interaction of PAA, polymerase, and template. Second, the importance of the degree of activation of the template, whether by pretreatment with DNase I or by endogenous activation by EBV nuclease during the course of the assay procedure, indicates that the concentration of 3′-hydroxyl termini available to the polymerase is critical to the PAA–polymerase–template interaction. Third, PAA has little effect on the processiveness of the polymerase although processiveness is measured in a template system (primed ϕX174 DNA) showing reduced PAA inhibition of polymerase activity. It is possible that PAA interacts directly with the template rather than the enzyme and that interaction is increased in the presence of a high concentration of primer ends. Another possibility is that the interaction of PAA with the enzyme or template occurs preferentially during the first rather than subsequent nucleotide additions to the growing chain. In the latter case, synthesis upon an activated DNA template with many primary ends might be expected to show more sensitivity to PAA inhibition. These two explanations for the data are not mutually exclusive.

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References

Alberts, B., Morris, C. F., Mace, D., Sinha, N., Bittner, M., & Moran, L. (1975) in *DNA Synthesis and Its Regulation* (Goulian, M., & Hanawalt, P., Eds.) pp 241–269, W. A. Benjamin, New York.

Allen, G. P., O'Callaghan, D. J., & Randall, C. C. (1977) *Virology 76*, 395–408.

Aposhian, H. V., & Kornberg, A. J. (1962) *J. Biol. Chem. 237*, 519–525.

Bambara, R. A., & Hockensmith, J. W. (1980) *J. Supramol. Struct., Suppl. 4*, 331.

Banks, G. R., & Yarranton, G. T. (1976) *Eur. J. Biochem. 62*, 143–150.

Boezi, J. A., Lee, L. F., Blakesley, R. W., Koenig, M., & Towle, H. C. (1974) *J. Virol. 14*, 1209–1219.

Clewell, D. B., & Helinski, D. R. (1969) *Proc. Natl. Acad. Sci. U.S.A. 62*, 1159–1166.

Clough, W. (1979) *Biochemistry 18*, 4517–4521.

Clough, W. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 6194–6198.

Das, S. K., & Fujimura, R. K. (1979) *J. Biol. Chem. 254*, 1227–1232.

Datta, A. K., Feighny, R. J., & Pagano, J. S. (1980) *J. Biol. Chem. 255*, 5120–5125.

DePamphilis, M. L., & Wassarman, P. M. (1980) *Annu. Rev. Biochem. 49*, 627–666.

Fisher, P. A., Wang, T. S.-F., & Korn, D. (1979) *J. Biol. Chem. 254*, 6128–6137.

Goodman, S. R., Prezyna, C., & Benz, W. C. (1978) *J. Biol. Chem. 253*, 8617–8628.

Hoffman, P. J., & Cheng, Y.-C. (1978) *J. Biol. Chem. 253*, 3557–3562.

Hoffman, P. J., & Cheng, Y.-C. (1979) *J. Virol. 32*, 449–457.

Hopfield, J. J. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 5248–5252.

Huang, E. S. (1975) *J. Virol. 16*, 298–310.

Huang, W. M., & Lehman, I. R. (1972) *J. Biol. Chem. 247*, 3139–3146.

Knopf, K.-W. (1979) *Eur. J. Biochem. 98*, 231–244.

Kornberg, A. (1980) *DNA Replication*, W. H. Freeman, San Francisco, CA.

Leinbach, S. S., Reno, J. M., Lee, L. F., Isbell, A. F., & Boezi, J. A. (1976) *Biochemistry 15*, 426–430.

Litman, R. M. (1968) *J. Biol. Chem. 243*, 6222–6233.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Miller, R. L., & Rapp, F. (1976) *J. Virol. 20*, 564–569.

Miller, R. L., Glaser, R., & Rapp, F. (1977) *Virology 76*, 494–502.

Modrich, P., & Richardson, C. C. (1975) *J. Biol. Chem. 250*, 5508–5514.

Morris, C. F., Sinha, N. K., & Alberts, B. M. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 4800–4804.

Ooka, T., Lenoir, G., & Daille, J. (1979) *J. Virol. 29*, 1–10.

Ostrander, M., & Cheng, Y.-C. (1980) *Biochim. Biophys. Acta 609*, 232–245.

Schekman, R., Weiner, J. H., Weiner, A., & Kornberg, A. (1975) *J. Biol. Chem. 250*, 5859–5865.

Sinsheimer, R. L. (1966) *Proced. Nucleic Acid Res. 1*, 569–576.

Smith, H. O. (1980) *Methods Enzymol. 65*, 371–380.

Thomas, C. A., & Abelson, J. (1966) *Proced. Nucleic Acid Res. 1*, 553–568.

Weissbach, A., Hong, S. L., Aucker, J., & Muller, R. (1973) *J. Biol. Chem. 248*, 6270–6277.

Wintersberger, E. (1978) *Eur. J. Biochem. 84*, 167–172.

Zasloff, M., Ginder, G. D., & Felsenfeld, G. (1978) *Nucleic Acids Res. 5*, 1139–1152.