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Purification of Epstein-Barr Virus DNA Polymerase from P3HR-1 Cells

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The Epstein-Barr virus DNA polymerase was purified from extracts of P3HR-1 cells treated with *n*-butyrate for induction of the viral cycle. Sequential chromatography on DNA cellulose, phosphocellulose, and blue Sepharose yielded an enzyme preparation purified more than 1,300-fold. The purified enzyme was distinct from cellular enzymes but resembled the viral DNA polymerase in cells infected with herpes simplex virus type 1 or 2. The active enzyme had an apparent molecular weight of 185,000 as estimated by gel filtration on Sephacryl S-300. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed a major polypeptide corresponding to a molecular weight of ca. 110,000. This polypeptide correlated with the catalytic function of the purified enzyme, whereas the other, less abundant polypeptides did not. By immunoblotting, the 110,000-molecular-weight polypeptide could be identified as a viral polypeptide. It could not be determined whether the native enzyme was composed of more than one polypeptide.

Large DNA viruses of both procaryotic and eucaryotic cells express several enzymes associated with nucleic acid and DNA synthesis (18). Cells infected by herpes simplex virus (HSV) type 1 or 2 contain two well-studied enzymes that bind to DNA in vitro, the HSV DNA polymerase (17, 23, 26, 28) and the alkaline nuclease (12, 24). These are distinct from cellular counterparts and appear to be essential for the replication of the virus.

Epstein-Barr virus (EBV) is a transforming herpesvirus associated with Burkitt lymphoma and nasopharyngeal carcinoma. No in vitro system for the replication of EBV has been described, but an abortive lytic cycle can be induced in certain EBV-carrying cell lines (21).

Recent reports indicate that EBV-producing cells contain a viral nuclease (5, 6) and a DNA polymerase (1, 2, 7, 10, 11, 25). Both enzymes have been partially purified. Data on the viral DNA polymerase are conflicting with respect to both functional and chromatographic properties. Most investigators have described one HSV-like DNA polymerase in EBV-producing cells (1, 2, 7, 10, 25), but other data suggest that two viral enzymes can be demonstrated (11). Neither of the latter two enzymes conforms to properties typical for the HSV-type DNA polymerase, e.g., stimulation by salts and inhibition by PP_i analogs (phosphonoacetic acid [PAA] and phosphonoformic acid [PFA]). The activity of the EBV DNA polymerase has not been correlated with a specific viral polypeptide.

We have previously studied viral protein synthesis in P3HR-1 cells in which the viral cycle was induced by treatment with *n*-butyrate. Over 20 viral polypeptides could be demonstrated by immunoprecipitation (15, 16). Four of the early polypeptides have been shown to bind to DNA in vitro (29). They were designated 152K, 134K, and 55 to 51K, but are here designated 135K, 110K, and 56 to 48K, respectively, to conform to molecular weight designations in other recent studies (14, 27).

The objective of this investigation was to determine the properties of the purified EBV DNA polymerase and to correlate the activity of the purified enzyme with a specific viral polypeptide. The EBV DNA polymerase was isolated by a three-step purification procedure and was not detected in uninduced cells. After induction, it was induced in parallel with a nuclease and the majority of the early viral polypeptides (15, 29). The purified viral enzyme was clearly distinguishable from cellular alpha and beta DNA polymerases but shared properties with the DNA polymerases of HSV types 1 and 2, such as sensitivity to inhibition by PP_i analogs and stimulation by salt. In contrast to the HSV enzyme, the EBV DNA polymerase did not bind to DEAE-Sephacel and was relatively unstable.

MATERIALS AND METHODS

Chemicals. All chemicals were of analytical grade. Nucleoside triphosphates, Pepstatin A, phenylmethylsulfonyl fluoride, bovine serum albumin (BSA) fraction V, and calf thymus DNA were obtained from Sigma Chemical Co. Cellulose CF 11 and phosphocellulose P11 were from Whatman, Inc.; Sephacryl S-300, Blue Sepharose, and DEAE-Sephacel were from Pharmacia Fine Chemicals, Inc. Synthetic polynucleotides were purchased from P-L Biochemicals, Inc., and radiochemicals were from the Radiochemical Centre (Amersham, England).

Tissue culture. P3HR-1 cells were maintained as stationary cultures in 2-liter Roux bottles at 37°C in a humidified atmosphere containing 5% CO_2. The cultures were routinely passaged twice weekly and fed with RPMI 1640 medium fortified with 2% fetal calf serum, penicillin, and streptomycin. Induction of the viral cycle was performed as described previously (21). All induced cultures were screened for mycoplasma contamination by the method of Schneider et al. (30). At the time of harvest, the proportions of cells positive for early antigen and for virus capsid antigen were determined. In general, the number of early antigen-positive

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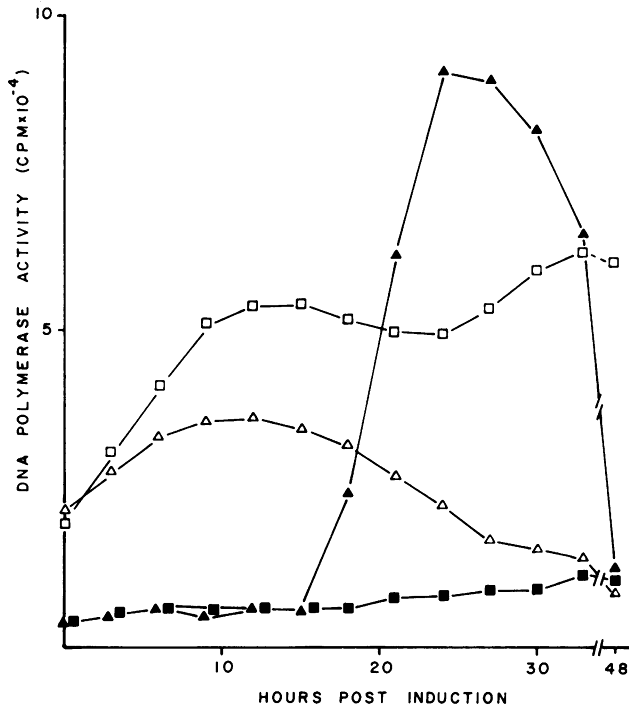

FIG. 1. Induction of salt-stimulated DNA polymerase in *n*-butyrate-treated and untreated P3HR-1 cells. At each time point, 5×10^6 cells were harvested and extracted with 200 μl of buffer as described in the text. Enzyme activity was assayed on 10-μl aliquots of the extracts. Assays were performed in the presence (▲, ■) and absence (△, □) of 100 mM ammonium sulfate, with extracts of butyrate-treated (▲, △) and untreated (■, □) cells.

cells was ca. 25%. Cultures containing less than 15% positive cells were discarded as they were less suitable for enzyme purification.

Purification of the EBV DNA polymerase. All steps in the EBV DNA polymerase purification were carried out at 4°C unless otherwise indicated. Frozen cells were thawed in four volumes of PET buffer (150 mM KCl, 1 mM EDTA, 20 mM Tris-hydrochloride [pH 7.2] [25°C], 10 mM mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride, 0.1 μg of Pepstatin A per ml). The pH of the extract was checked and adjusted to 7.2 (on ice). The extract was clarified by centrifugation at 20,000 rpm for 1 h in a Sorvall SS 34 rotor. The supernatant was carefully aspirated, avoiding lipids and loosely packed material above the pellet.

The extract was loaded onto a 50-ml column of double-stranded DNA-cellulose at a flow rate of 25 ml/h. The column had previously been equilibrated with KPE buffer (150 mM KCl, 1 mM EDTA, 20 mM potassium phosphate buffer [pH 7.2], 0.5 mM phenylmethylsulfonyl fluoride, 0.1 μg of Pepstatin A per ml, and 20% glycerol). After the column was washed with KPE buffer, bound material was eluted with a 300-ml linear gradient from 150 to 600 mM KCl in KPE buffer. Fractions of 5 ml were collected and assayed for enzyme activity. Those containing the viral DNA polymerase were pooled and extensively dialyzed against KPE buffer containing 100 mM KCl.

The dialyzed viral DNA polymerase from DNA cellulose was loaded onto a 5-ml column of phosphocellulose at a flow rate of 10 ml/h. Bound proteins were eluted with a 40-ml linear gradient, 100 to 600 mM KCl in KPE buffer. The viral DNA polymerase eluted as a sharp peak at 280 mM KCl. The fractions containing the enzyme were immediately pooled, made 400 mM in KCl, and applied to a 1-ml column of Blue Sepharose. The viral DNA polymerase did not bind to the column, whereas most of the proteins did. The DNA polymerase recovered from the column flow-through fractions was pooled, concentrated to ca. 1 ml, and made 50% in glycerol. When stored at −20°C, the enzyme activity was stable for several months.

Gel filtration on Sephacryl S-300. Cells (1 g) were sonicated in PET buffer containing 500 mM KCl, centrifuged, and applied to a 45-ml column of packed Sephacryl S-300 equilibrated with KPE buffer containing 400 mM KCl. Then 1 ml of the extract was applied at a flow rate of 6 ml/h. Fractions of 0.3 ml were collected. Catalase (232,000), aldolase (158,000), BSA (69,000), and ovalbumin (45,000) were used as molecular weight markers.

Assays for DNA polymerase and nuclease activity. The standard DNA polymerase assay system contained, in a final volume of 200 μl, 50 mM Tris-hydrochloride (pH 8.0), 6 mM $MgCl_2$, 0.1 mM EDTA, 10 mM mercaptoethanol, 50 μg of activated calf thymus DNA, 80 μg of heat-inactivated BSA, 0.1 mM each of dCTP, dGTP, and dATP, and 0.01 mM [^{3}H]TTP. The specific activity of the [^{3}H]TTP was 330 cpm/pmol. To permit differentiation between viral and cellular DNA polymerase activities, all fractions were assayed in both the presence and absence of 100 mM ammonium sulfate (7, 9, 26). The incorporation of [^{3}H]TMP in the presence of salts was regarded as viral. Incubations were performed at 37°C for 30 min. To terminate incorporation, the tubes were placed on ice and made 10% in trichloroacetic acid. The precipitates were collected on Whatman GF/C fiber glass filters and dried. The filters were counted in an LKB Rackbeta scintillation counter (LKB Instruments Inc.). All assays were done in duplicate. Calf thymus DNA polymerase alpha, used as a control in the experiments described in Tables 2 and 3, was assayed as described previously (9).

Assays for DNase were done with DNA of P3HR-1 cells labeled with [^{3}H]thymidine to a specific activity of 24,000 cpm/μg. Each assay of 200 μl contained 2 μg of labeled DNA. Concentrations of Tris-hydrochloride, $MgCl_2$, EDTA, mercaptoethanol, and BSA were as in the DNA polymerase assay. Incubations were carried out for 30 min at 37°C. Digestion was terminated by the addition of trichloroacetic acid, and the remaining radioactivity was determined as described above. DNase activity is expressed as the percentage of added [^{3}H]DNA solubilized.

Immunoblotting. Proteins were transferred electrophoretically to nitrocellulose filters (22). After transfer, additional binding sites were blocked by immersion in 2% BSA for 30 min at 37°C. The filters were incubated in serum (15) diluted 1 to 20 in phosphate-buffered saline containing 0.05% Tween 20. The filters were washed twice and incubated with protein A conjugated with alkaline phosphatase (30 min at room temperature). After three washes, the filter was immersed in the developer containing 100 mM Tris-hydrochloride (pH 8.6), 5 mM $MgCl_2$, 1 mg of α-naphthylphosphate per ml, and 2.5 mg of Fast Red per ml. The enzyme reaction was terminated by washing the filter in distilled water, and the filter was air dried.

Determination of amino acid compositions. The major 110K polypeptide obtained after Blue Sepharose was identified after electrophoresis in 7% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The band was excised from the stained gel, cut into small pieces, and immersed in 20 volumes of 100 mM Tris-hydrochloride (pH

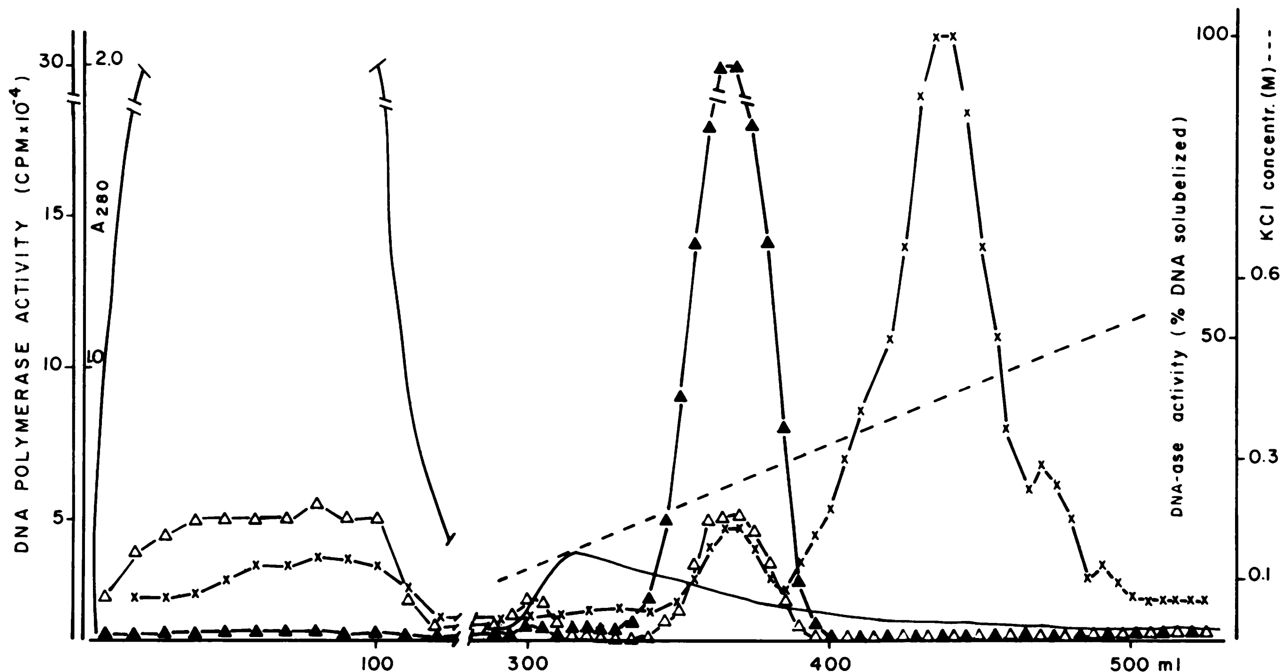

FIG. 2. DNA-cellulose chromatography of extracts from induced P3HR-1 cells. Fractions were assayed for nuclease activity (X) and DNA polymerase activity in the presence (▲) and absence (△) of ammonium sulfate.

7.5) and 1% SDS. The protein was eluted by stirring for 72 h at room temperature. Acrylamide was removed by filtration, and the protein was precipitated by the addition of 10% trichloroacetic acid in the cold. After centrifugation, the protein pellet was washed with ether, dissolved in formic acid, and transferred to a hydrolysis tube. Hydrolysis was for 24 h at 110°C with 6 M HCl and 0.5% phenol in evacuated tubes. Amino acids obtained were determined in a Beckman 121 M analyzer.

Miscellaneous methods. Preparation of activated calf thymus DNA and UV-irradiated DNA cellulose has been described previously (20, 25). Determinations of protein concentrations were made with BSA as standard (4). Cellular alpha and beta DNA polymerases were extracted from Raji cells and separated by chromatography on DEAE-Sephacel. The beta polymerase was found in the unbound fraction. It was resistant to inactivation by NEM and had a preference for synthetic templates, as expected (18). Polymerase alpha was eluted with a linear gradient from 40 to 400 mM KCl in Tris-hydrochloride (pH 8.0). SDS gel electrophoresis (19) was carried out with molecular weight markers 200,000 (myosin), 116,500 (beta-galactosidase), 92,500 (phosphorylase B), 66,200 (BSA), and 45,000 (ovalbumin). Electrophoretic transfer of proteins to nitrocellulose, conditions for immunoblotting, and the serum used for immunoblotting and immunoprecipitation have been described previously (15, 22).

RESULTS

Induction of the EBV DNA polymerase in *n*-butyrate-treated cells. For a determination of the optimal time of harvest for purification, the induction of the salt-stimulated DNA polymerase was monitored in crude extracts of cells harvested at various times after addition of the inducer (Fig. 1). Significant quantities of the salt-stimulated DNA polymerase were detected 18 h after addition of the inducer. Maximal activity was obtained 24 to 30 h postinduction. After 30 h, the enzyme activity declined rapidly. The decrease of polymerase activity was paralleled by a rapid increase of dead cells. Little or no salt-stimulated DNA polymerase activity was detected in uninduced cells. The level in uninduced P3HR-1 cells was nearly identical to that in virus-negative cell lines such as Bjab and Ramos (data not shown). The time course of nuclease induction followed a pattern similar to that of the viral DNA polymerase (data not shown).

Extraction and purification of the viral DNA polymerase. Induced cells were harvested between 24 and 28 h after induction. Several extraction procedures were explored, including the high salt extraction described by Powell and Purifoy (28). Extraction by sonication followed by addition of 500 mM KCl resulted in solubilization of more than 90% of the enzyme activity. However, this procedure resulted in extensive solubilization of DNA which could not be removed by precipitation with streptomycin sulfate or polyetyleneimine without substantial loss of enzyme activity. Exposure of the enzyme to salt concentrations higher than 700 mM KCl led to rapid loss of enzyme activity, as did exposure to pH above 7.6. Thawing of cells in isotonic salt at a pH close to neutral appeared to be most compatible with the preservation of active enzyme, and ca. 80% of the salt-stimulated DNA polymerase was solubilized.

Figure 2 shows the purification of the viral DNA polymerase from induced P3HR-1 cells by chromatography on double-stranded DNA-cellulose. The viral DNA polymerase eluted as a sharp peak at 280 mM KCl. Some nuclease activity was associated with the polymerase, but most of it eluted between 300 and 450 mM KCl. Neither the viral DNA polymerase nor the major nuclease was detected upon chromatography of extracts from uninduced cells. Small quantities of DNA polymerase inhibited by salt were eluted in the first fractions of the salt gradient. This activity was also observed upon chromatography of uninduced cells and was therefore not regarded as associated with the viral

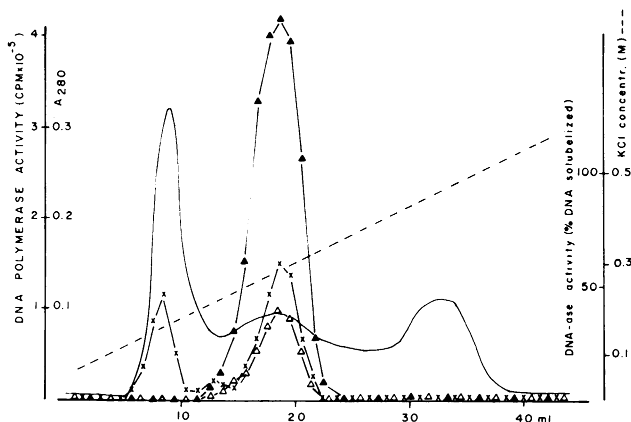

FIG. 3. Phosphocellulose chromatography of the DNA-cellulose-purified DNA polymerase. Symbols are the same as those in Fig. 2.

cycle. As our results indicated that both the salt-stimulated DNA polymerase and the major nuclease observed by chromatography on DNA cellulose were associated with the viral cycle in P3HR-1 cells, these activities will be designated the viral DNA polymerase and the viral nuclease, respectively.

Further purification of the viral DNA polymerase was obtained by chromatography on phosphocellulose (Fig. 3). Analysis of the separation by phosphocellulose revealed a single peak of DNA polymerase activity eluting at ca. 280 mM KCl. Assay for DNA polymerase in the absence of salt did not reveal additional peaks. Most of the nuclease activity remained associated with the viral DNA polymerase. Some nuclease activity eluted at lower ionic strength.

The DNA polymerase from the phosphocellulose step was obtained in low protein concentration and was unstable upon storage or dialysis to low ionic strength. Thus, additional purification was difficult with most conventional procedures, such as ion-exchange chromatography. Pilot-scale experiments with labeled viral DNA-binding proteins (29) indicated that most of these polypeptides were bound to Blue Sepharose in the presence of 400 mM KCl. One protein (here designated 110K) failed to bind this ionic strength. Other pilot experiments with DNA cellulose-purified EBV DNA polymerase indicated that this enzyme had no affinity for Blue Sepharose at 400 mM KCl. For these reasons, the pooled fractions from phosphocellulose were adjusted to 400 mM KCl. All of the enzyme activity was present in the flow-through fractions. Since this experiment could be performed in less than 30 min, the final purification step could be performed with high yield and usually with three- to fourfold purification. The proteins bound to the column and eluted with increasing salt concentration did not contain detectable DNA polymerase activity.

TABLE 1. Purification of EBV DNA polymerase from induced P3HR-1 cells

Source	Total protein (mg)	Total activity (U)[a]	Sp act (U/mg)	Recovery (%)	Purification (fold)
Cell extract	558	12,240	21.9	100	1
DNA-cellulose	3.27	6,529	1,997	53.3	91
Phosphocellulose	0.65	5,459	8,398	44.6	383
Blue Sepharose	0.17	5,104	30,024	41.7	1,371

[a] One unit is defined as the amount of enzyme resulting in the incorporation of 1 nmol of [^{3}H]TMP under standard assay conditions.

By a three-step procedure (Table 1), an apparent purification of 1,370-fold was obtained with a recovery of ca. 42%. The most critical step was the DNA cellulose purification in which the recovery varied between 10 and 60%. This variability of yield might be associated with the presence of variable amounts of DNA in the extract and DNA cellulose eluate. It is likely that this DNA caused aggregation in the subsequent dialysis step. The low protein concentration might also contribute to the destabilization of the DNA polymerase activity. Low recovery in the first step made the EBV DNA polymerase refractory to further purification.

Determination of the molecular size of the EBV DNA polymerase. Efforts to determine the size of the purified enzyme by gel filtration and by sedimentation in sucrose were inconclusive due to the poor stability of the enzyme in dilute form. In contrast, gel filtration of crude enzyme in the presence of 400 mM KCl could be performed with good recovery (Fig. 4). The gel filtration on Sephacryl S-300 indicated an apparent molecular weight of 185,000 for the viral DNA polymerase and 70,000 for the viral nuclease. Attempts to perform the gel filtration in low salt (150 mM KCl) did not produce reliable data, probably due to aggregation of the enzyme. Sedimentation of the enzyme in crude form or after DNA cellulose purification was inconclusive since recoveries of the enzyme activity were less than 1%. The sedimentation coefficient thus could not be determined.

Polypeptides from the three purification steps of the EBV DNA polymerase were separated by SDS-PAGE (Fig. 5). The samples were analyzed in parallel with an immunoprecipitate from [^{35}S]methionine-labeled induced P3HR-1 cells (15) to permit comparison of the electrophoretic mobilities of the purified proteins and the viral polypeptides detected by anti-EBV serum. Each of the three fractions and the immunoprecipitate were also transferred to nitrocellulose (Fig. 5, lanes D to G) for detection of viral polypeptides by immunoblotting. In the Coomassie-stained gel (Fig. 5, lanes A to C), three major proteins remained after the third purification step. The most abundant polypeptide of the viral DNA polymerase preparation comigrated with the viral 110K polypeptide detected by immunoprecipitation. We have shown previously that this polypeptide binds to DNA

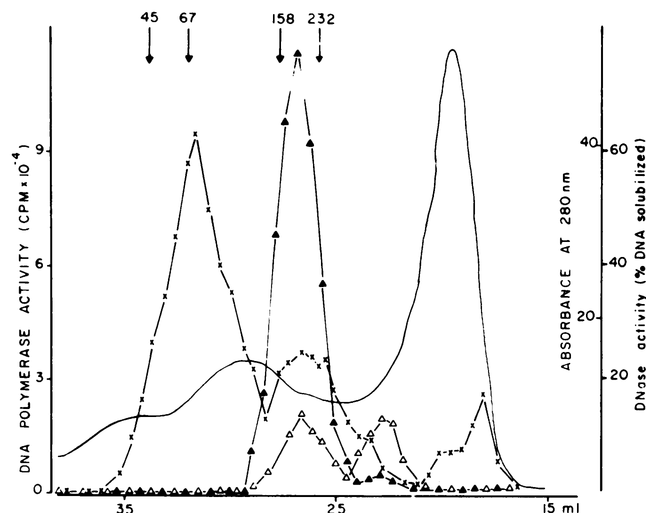

FIG. 4. Gel filtration on Sephacryl S-300 of extract of induced P3HR-1 cells. Symbols are the same as in Fig. 2 and 3. Markers are listed in the text.

(29). A less abundant polypeptide of the final purification step had an apparent molecular weight of 50,000. This polypeptide also comigrated with an immunoprecipitated viral polypeptide. The immunoblotting experiment identified the 110K polypeptide as viral, and it was readily detected in all three purification steps. The other polypeptides of the DNA polymerase preparation were not reactive in immunoblotting.

In the first two purification steps, the major immunoreactive polypeptide had an apparent molecular weight of ca. 80,000. This polypeptide probably represents the nuclear antigen EBNA (33) as it was also detected in uninduced cells and had high affinity for Blue Sepharose (33). This antigen was readily detected by immunoblotting, and the polypeptide also transferred well to the nitrocellulose. In contrast, the 110K and 135K polypeptides transferred poorly, and their detection by immunoblotting was difficult. We therefore cannot exclude that some of the minor high-molecular-weight polypeptides of the enzyme preparation are viral but evaded detection by immunoblotting.

The purified EBV DNA polymerase was analyzed by electrophoresis on 12% polyacrylamide. Proteins eluted from Blue Sepharose (400 to 1,000 mM KCl) were analyzed in parallel (Fig. 6). The latter preparation had no DNA polymerase activity and did not contain the 110K viral protein which was the predominant component of the purified DNA polymerase (Table 1). The remaining two proteins of the polymerase preparation (66K and 51K) were detected in the Blue Sepharose fractions lacking enzyme activity. Thus, of the proteins of the purified enzyme, only the 110K protein correlated with the viral DNA polymerase activity.

Attempts to identify the catalytic subunit of the viral DNA polymerase were made by the in situ detection method (32).

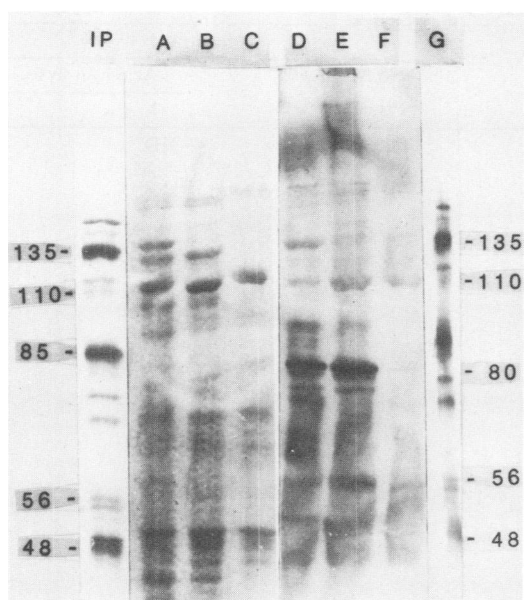

FIG. 5. SDS-polyacrylamide gel electrophoresis in 7% polyacrylamide of polypeptides from the three purification steps: DNA-cellulose (lanes A and D), phosphocellulose (B and E), and Blue Sepharose (C and F). Lanes A through C were stained with Coomassie brilliant blue. IP, Immunoprecipitate of viral proteins from induced cells labeled with [^{35}S]methionine. The same serum was used for immunoblotting (lanes D through F). Lane G is an immunoprecipitate transferred to nitrocellulose.

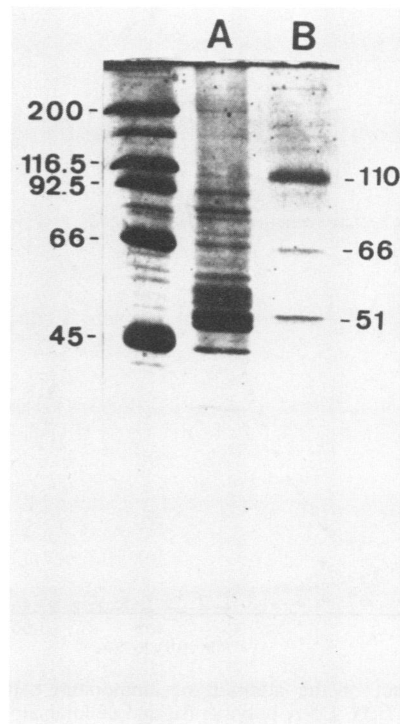

FIG. 6. SDS-polyacrylamide gel electrophoresis in 12% polyacrylamide of (A) Blue Sepharose binding proteins eluted stepwise with 0.4 to 1.0 M KCl (these fractions were not associated with any DNA polymerase activity) and (B) EBV DNA polymerase from the Blue Sepharose flow-through fraction.

Although this method readily detected different cellular enzymes in uninduced cells, no novel species were detected in the cells after induction of the viral cycle. EBV DNA polymerase from any of the three purification steps failed to produce specific bands (data not shown).

Properties of the purified DNA polymerase. The purified EBV DNA polymerase was strongly stimulated by the addition of ammonium sulfate to the assay system, whereas the cellular alpha and beta DNA polymerases purified from Raji cells were strongly inhibited under identical conditions (Fig. 7). The purified enzyme failed to utilize unnicked DNA as a template, whereas nicked (activated) DNA was an efficient template (Table 2). Among the synthetic templates tested, the purified enzyme had a strong preference for $(dC)_n$ $(dG)_{12-18}$. This template was six times more efficient than activated DNA, but both $(dA)_n$ $(dT)_{12-18}$ and $(dT)_n$ $(rA)_{12-18}$ were as efficiently copied as activated DNA. The RNA template $(rA)_n$ $(dT)_{12-18}$ could not be utilized at all. Control experiments with DNA polymerase alpha from calf thymus showed a distinctly different pattern. The cellular enzyme had a strong preference for RNA-primed template $(dT)_n$ $(rA)_{12-18}$ but utilized the template $(dC)_n$ $(dG)_{12-18}$ significantly less effectively than did the EBV DNA polymerase. Similarly, the alpha polymerase failed to copy the RNA template.

In Table 3, the EBV DNA polymerase and the calf thymus DNA polymerase alpha were compared further with respect to four agents with known effects on certain DNA polymerases. The cellular enzyme was over 30 times more sensitive to inactivation by *N*-ethylmaleimide. For the two PP_i analogs PFA and PAA, the sensitivity pattern was reversed, and the DNA polymerase alpha required an 8.0 μM concentration for 50% inhibition. The EBV DNA polymerase was inhibited to 50% already at 10-fold-lower concentrations.

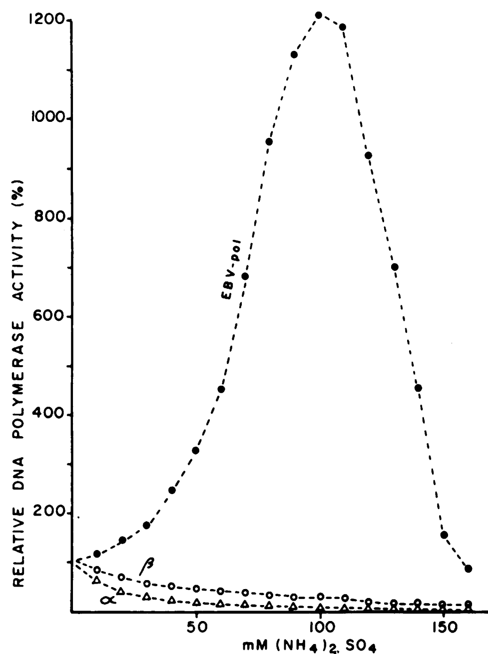

FIG. 7. Effect of the addition of ammonium sulfate on the activity of the EBV DNA polymerase and cellular alpha and beta DNA polymerases.

The two enzymes had similar sensitivities to treatment with aphidicholin, a tetracyclic diterpenoid.

DISCUSSION

The viral DNA polymerases in cells infected by HSV type 1 or 2 are characterized by their sensitivity to PP_i analogs and to stimulation by salts. In addition, the HSV DNA polymerases bind tightly to DNA in vitro. These three properties clearly distinguish the HSV enzymes from the DNA polymerases of uninfected cells (17, 23, 26, 28).

The salt-stimulated DNA polymerase isolated from virus-producing P3HR-1 cells constitutes more than 50% of the DNA polymerase in these cells and correlates with the productive cycle of the virus. Chromatography of the salt-stimulated DNA polymerase on DNA-cellulose readily separates it from its cellular counterparts, and in the subsequent purification steps the enzyme appears to be homogeneous with respect to contaminating DNA polymerases. The EBV DNA polymerase has a sensitivity to PAA and PFA similar to that of the HSV DNA polymerase (17, 26). With respect to the preference for synthetic template primers, the EBV DNA polymerase also has the characteristics of the HSV DNA polymerase. These findings clearly suggest that the EBV DNA polymerase is an HSV-type DNA polymerase. This conclusion is in agreement with several previous reports (2, 5, 7, 10, 25) and is in line with the observation that PAA inhibits EBV replication (34). The EBV DNA polymerase also has recently been shown to be genetically related to the HSV DNA polymerase (G. F. Hatfull, B. G. Barrell, J. Quinn, and D. McGeoch, manuscript in preparation). These authors have identified a region of the EBV genome consisting of a 3-kilobase open reading frame (in the *Eco*RI C fragment) which reveals striking homology with the HSV type 1 DNA polymerase gene. The molecular weight of the predicted protein (113,400) and its amino acid composition were in good agreement with that of the 110K protein shown

TABLE 2. Template primer preference of cellular alpha and EBV DNA polymerase

Template primer[a]	Substrate	DNA polymerase activity[a] α	EBV
Native DNA	[^{3}H]dNTP	0	0
Activated DNA	[^{3}H]dNTP	100	100
$(dA)_n(dT)_{12-18}$	[^{3}H]dTTP	162	106
$(dC)_n(dG)_{12-18}$	[^{3}H]dGTP	189	643
$(dT)_n(rA)_{12-18}$	[^{3}H]dATP	1,138	102
$(rA)_n(dT)_{12-18}$	[^{3}H]dTTP	0	0

[a] Each template primer was used at a concentration of 50 μg/ml.
[b] For DNA polymerase alpha and the EBV DNA polymerase, 100% of activity represents 124 and 46 pmol of [^{3}H]NTP incorporated, respectively.

TABLE 3. Effect of various inhibitors on the activity of cellular DNA polymerase alpha and the EBV DNA polymerase

Inhibitor	ID_{50} (μM)[a] DNA polymerase α	EBV DNA polymerase
PFA	8.0	0.5
PAA	8.0	0.8
Aphidicolin	26	11
NEM[b]	50	1,700

[a] ID_{50}, Concentration giving 50% inhibition of enzyme activity.
[b] NEM, *N*-Ethylmaleimide, tested in the absence of β-mercaptoethanol.

TABLE 4. Amino acid composition of the predicted 113.4K protein of the DNA polymerase gene and comparison with the 110K polypeptide associated with the EBV DNA polymerase[a]

Amino acid	Molar ratios (%) Predicted 113.4K		Acid hydrolysis 110K I	II
Cys	2.85		ND[b]	ND
Asn	2.56			
		Asx 8.26	8.2	9.0
Asp	5.71			
Thr	4.82		4.7	4.8
Ser	5.61		6.9	6.6
Gln	3.74			
		Glx 9.75	11.9	11.7
Glu	5.91			
Pro	5.12		4.7	6.0
Gly	6.89		10.4	8.7
Ala	8.86		8.8	9.2
Val	7.68		6.4	6.4
Met	1.48		1.1	1.4
Ile	4.63		3.9	4.0
Leu	10.33		9.7	10.3
Tyr	3.84		3.4	3.8
Phe	4.72		4.6	4.7
Trp	0.89		0.5	ND
Lys	4.72		5.3	4.8
His	2.66		2.6	2.4
Arg	6.89		7.0	6.2

[a] The analysis of the 110K protein was made on two separate preparations and with a single time of hydrolysis (24 h). The amino acid composition of the predicted protein (101.5 amino acids) was determined by the use of a DNA sequence analysis program (M. Nilsson and G. O. Klein, submitted for publication). The complete nucleotide sequence of the EBV genome was made available by B. Barrell and P. Farrell, Cambridge, United Kingdom.
[b] ND, Not detected.

here to be associated with the purified EBV DNA polymerase (Table 4).

Although our data clearly show that the EBV DNA polymerase is similar to the HSV enzyme, the chromatographic properties appear to be distinctly different. Normally, the first step in the purification of the HSV DNA polymerase is chromatography on DEAE-cellulose (23, 24, 26, 28). Under similar conditions (Tris-hydrochloride [pH 8.0], 40 mM KCl), the EBV enzyme fails to bind to DEAE-Sephacel. At lower pH (phosphate buffer [pH 6.5], 100 mM KCl), the EBV DNA polymerase binds to the cationic exchanger Mono S (Pharmacia). This might indicate that the EBV DNA polymerase has a higher positive charge than the HSV enzyme. We have previously shown that the EBV DNA polymerase is eluted from a chromatofocusing column at pH 8 (32).

It was not possible to obtain further purification of the enzyme since such attempts invariably resulted in excessive loss of enzyme activity. The predominant protein of the third purification step (110K) was not found in the Blue Sepharose-bound fraction, which did not have any DNA polymerase activity. The 66K and 51K proteins were both present in the enzyme-inactive fractions, and the latter two proteins therefore were probably unrelated to the catalytic function of the EBV DNA polymerase. The unexpected discrepancy between the size determinations by gel filtration (185K) and SDS-PAGE (110K) might be explained if the native enzyme is very asymmetrical or if the enzyme contains additional subunits. Replicative DNA polymerases have been shown to consist of a single, catalytically active subunit (13). The HSV DNA polymerase and the equine herpesvirus DNA polymerases do not seem to be exceptions in this respect (3, 28).

The availability of the primary structure of the EBV DNA polymerase (Hatfull et al., in preparation) will make it possible to identify the protein associated with the enzymatic activity by using antibodies of predetermined specificity, as has been done for the identification of EBNA (8). This approach also will clearly be of help in determining whether the nuclease activity which copurifies with the polymerase is an integral part of the DNA polymerase or whether the nuclease activity is associated with a separate protein.

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LITERATURE CITED

1. **Allaudeen, H. S., and J. R. Bertino.** 1978. Isolation of a herpesvirus-specific DNA polymerase from tissue of an American patient with Burkitt lymphoma. Proc. Natl. Acad. Sci. U.S.A. **75**:4504–4508.
2. **Allaudeen, H. S., and G. Rani.** 1982. Cellular and Epstein-Barr virus specific DNA polymerase in virus-producing Burkitt's lymphoma cell lines. Nucleic Acids Res. **10**:2453–2465.
3. **Allen, P. A., D. J. O'Callaghan, and C. C. Randal.** 1977. Purification and characterization of equine herpesvirus-induced DNA polymerase. Virology **76**:395–408.
4. **Bradford, M. M.** 1976. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal. Biochem. **72**:248–254.
5. **Cheng, Y. C., J. Y. Chen, P. J. Hoffmann, and R. Glaser.** 1980. Studies on the activity of DNase associated with the replication of the Epstein-Barr virus. Virology **100**:334–338.
6. **Clough, W.** 1979. Deoxyribonuclease activity found in Epstein-Barr virus producing lymphoblastoid cells. Biochemistry **18**:4517–4521.
7. **Datta, A. K., R. Y. Feighny, and J. S. Pagano.** 1980. Induction of Epstein-Barr virus-associated DNA polymerase by 12-*O*-tetradecanoyl-phorbol-13-acetate. Purification and characterization. J. Biol. Chem. **255**:5120–5125.
8. **Dillner, J., L. Sternås, B. Kallin, H. Alexander, B. Ehlin-Henriksson, H. Jörnvall, G. Klein, and R. Lerner.** 1984. Antibodies against a synthetic peptide identify the Epstein-Barr virus-determined nuclear antigen (EBNA). Proc. Natl. Acad. Sci. U.S.A. **81**:4652–4656.
9. **Eriksson, B., A. Larson, E. Helgestrand, N. G. Johansson, and B. Öberg.** 1980. Pyrophosphate analogues as inhibitors of herpes simplex virus type 1 DNA polymerase. Biochim. Biophys. Acta **607**:53–64.
10. **Feighny, R. J., B. E. Henry II, A. K. Datta, and J. S. Pagano.** 1980. Induction of DNA polymerase activity after superinfection of Raji cells with Epstein-Barr virus. Virology **107**:415–423.
11. **Goodman, S. R., C. Prezyna, and W. C. Benz.** 1978. Two Epstein-Barr virus associated DNA polymerase activities. J. Biol. Chem. **253**:8617–8628.
12. **Hoffmann, P. J., and Y. C. Cheng.** 1978. The deoxyribonuclease induced after infection of KB cells by herpes simplex virus type 1 or type 2. J. Biol. Chem. **253**:3557–3562.
13. **Hübscher, U., A. Spanos, W. Albert, F. Grummt, and G. R. Banks.** 1981. Evidence that a high molecular weight replicative DNA polymerase is conserved during evolution. Proc. Natl. Acad. Sci. U.S.A. **78**:6771–6775.
14. **Hummel, M., and E. Kieff.** 1982. Mapping of polypeptides encoded by the Epstein-Barr virus genome in productive infection. Proc. Natl. Acad. Sci. U.S.A. **79**:5698–5702.
15. **Kallin, B., and G. Klein.** 1983. Epstein-Barr virus carried by Raji cells: a mutant in early functions? Intervirology **19**:47–51.
16. **Kallin, B., J. Luka, and G. Klein.** 1979. Immunochemical characterization of Epstein-Barr virus-associated early and late antigens in *n*-butyrate-treated P3HR-1 cells. J. Virol. **32**:710–716.
17. **Knopf, K. W.** 1979. Properties of herpes simplex virus DNA polymerase and characterization of its associated exonuclease activity. Eur. J. Biochem. **98**:231–244.
18. **Kornberg, A.** 1980. DNA replication. W. E. Freeman and Co., San Francisco.
19. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
20. **Litman, R. M.** 1968. A deoxyribonucleic acid polymerase from Micrococcus luteus (Micrococcus lysodekticus) isolated on deoxyribonucleic acid-cellulose. J. Biol. Chem. **243**:6222–6233.
21. **Luka, J., B. Kallin, and G. Klein.** 1979. Induction of Epstein-Barr virus (EBV) cycle in latently infected cells by n-butyrate. Virology **94**:228–231.
22. **Luka, J., E. Sternås, H. Jörnvall, G. Klein, and R. Lerner.** 1983. Antibodies of predetermined specificity for the NH_2 terminus of a cellular protein p53 react with the native molecule. Proc. Natl. Acad. Sci. U.S.A. **80**:1199–1203.
23. **Mar, E.-C., and E.-S. Huang.** 1979. Comparative study of herpes group virus-induced DNA polymerases. Intervirology **12**:73–83.
24. **Morrison, J. M., and H. M. Keir.** 1968. A new DNA-exonuclease in cells infected with herpes virus: partial purification and properties of the enzyme. J. Gen. Virol. **3**:337–347.
25. **Ooka, T., G. Lenoir, and J. Daillie.** 1979. Characterization of an Epstein-Barr virus-induced DNA polymerase. J. Virol. **29**:1–10.
26. **Ostrander, M., and Y. C. Cheng.** 1980. Properties of herpes simplex virus type 1 and type 2 DNA polymerase. Biochim. Biophys. Acta **609**:232–245.
27. **Pearson, G. R., B. Vroman, B. Chase, T. Sculley, M. Hummel, and E. Kieff.** 1983. Identification of polypeptide components of the Epstein-Barr virus early antigen complex with monoclonal

antibodies. J. Virol. **47**:193–201.

28. **Powell, K. L., and D. J. M. Purifoy.** 1977. Nonstructural proteins of herpes simplex virus. I. Purification of the induced DNA polymerase. J. Virol. **24**:618–626.
29. **Roubal, J., B. Kallin, J. Luka, and G. Klein.** 1981. Early DNA-binding polypeptides of Epstein-Barr virus. Virology **113**:285–292.
30. **Schneider, E. L., E. J. Stanbridge, and C. J. Epstein.** 1974. Incorporation of ^{3}H uridine and ^{3}H uracil into RNA. A simple technique for the detection of mycoplasma contamination of cultured cells. Exp. Cell Res. **84**:311–318.
31. **Spanos, A., S. G. Sedgwick, G. T. Yarraton, U. Hübscher, and G. E. Banks.** 1981. Detection of the catalytic activities of DNA polymerases and their associated exonucleases following SDS polyacrylamide gel electrophoresos. Nucleic Acids Res. **8**:1825–1839.
32. **Sternås, L., J. Luka, B. Kallin, B. F. Vestergaard, and G. Klein.** 1982. Detection and characterization of EBV antigens by micro-ELISA and chromatofocusing. J. Virol. Methods **4**:229–240.
33. **Strnad, B. C., B. C. Schuster, R. F. Hopkins, R. H. Neubauer, and H. Rabin.** 1981. Identification of an Epstein-Barr virus nuclear antigen by fluoroimmunoelectrophoresis and radioimmunoelectrophoresis. J. Virol. **38**:996–1004.
34. **Summers, W. C., and G. Klein.** 1976. Inhibition of Epstein-Barr virus DNA synthesis and late gene expression by phosphonoacetic acid. J. Virol. **18**:151–155.