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Functional Expression and Characterization of the Epstein-Barr Virus DNA Polymerase Catalytic Subunit

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A recombinant baculovirus containing the complete sequence for the Epstein-Barr virus (EBV) DNA polymerase catalytic subunit, BALF5 gene product, under the control of the baculovirus polyhedrin promoter was constructed. Insect cells infected with the recombinant virus produced a protein of 110 kDa, recognized by anti-BALF5 protein-specific polyclonal antibody. The expressed EBV DNA polymerase catalytic polypeptide was purified from the cytosolic fraction of the recombinant virus-infected insect cells. The purified protein exhibited both DNA polymerase and 3′-to-5′ exonuclease activities, which were neutralized by the anti-BALF5 protein-specific antibody. These results indicate that the 3′-to-5′ exonuclease activity associated with the EBV DNA polymerase (T. Tsurumi, Virology 182:376–381, 1991) is an inherent feature of the polymerase catalytic polypeptide. The DNA polymerase and the exonuclease activities of the EBV DNA polymerase catalytic subunit were sensitive to ammonium sulfate in contrast to those of the polymerase complex purified from EBV-producing lymphoblastoid cells, which were stimulated by salt. Furthermore, the template-primer preference for the polymerase catalytic subunit was different from that for the polymerase complex. These observations strongly suggest that the presence of EBV DNA polymerase accessory protein, BMRF1 gene product, does influence the enzymatic properties of EBV DNA polymerase catalytic subunit.

Epstein-Barr virus (EBV) is a human herpesvirus which is a causative agent of infectious mononucleosis. The EBV genome consists of a linear double-stranded DNA, which is 172 kb in length and encodes approximately 84 proteins (1). EBV infects and immortalizes B lymphocytes in vitro. The EBV has both a latent state and a lytic replicative cycle. During the latent phase of the EBV life cycle, the EBV genome is maintained as a circular plasmid molecule synthesized by host DNA polymerases (Pols) in the nuclei of the immortalized cells. ori P, one replication origin of EBV, mediates this type of replication (29). However, after induction of the lytic phase of viral replication, EBV replication proteins are induced and the EBV genome is amplified 100- to 1,000-fold. The replication product is a head-to-tail concatemer, which is synthesized by the EBV DNA Pol via a rolling-circle mechanism initiated from the other replication origin, ori Lyt (8). A number of features of EBV DNA replication make it an attractive model system for the study of eukaryotic DNA replication.

Recently, it has been demonstrated that EBV encodes at least seven viral genes that are essential for ori Lyt-dependent DNA replication (6). These genes and their functions which are known or predicted on the basis of sequence comparison with herpes simplex virus (HSV) type 1 are BALF5, the DNA Pol catalytic subunit; BMRF1, the DNA Pol accessory subunit; BALF2, the single-stranded DNA-binding protein; BBLF4 and BSLF1, the helicase and primase; BBLF2/3, a potential homolog of the third component of the helicase-primase complex; and genes encoded by the EBV *Sal*I-F fragment, of unknown function. Our goal is to understand the enzymatic processes during the lytic phase of EBV DNA replication.

So far, we have focused our efforts on the study of EBV DNA Pol. The EBV DNA Pol has been purified to varying degrees from cultured EBV-infected cells and characterized (11, 16, 24). The 110 kDa of the EBV DNA Pol catalytic polypeptide copurifies with the BMRF1 gene product. The neutralization of the EBV DNA Pol activity by monoclonal antibody to the BMRF1 protein (2) and the low activity in DNA Pol fraction lacking the BMRF1 protein (12) strongly suggest that the EBV DNA Pol catalytic subunit forms a tight complex with the BMRF1 protein in the EBV-infected cells to function as EBV Pol holoenzyme. The EBV DNA Pol activity is stimulated by ammonium sulfate when activated DNA is used as template-primer and is inhibited by aphidicolin or phosphonoacetic acid (PAA) (11, 24, 25). Poly(dC) · oligo(dG) is a good template-primer for the EBV DNA Pol (11, 25). Furthermore, the EBV DNA Pol can efficiently extend both RNA and DNA primers on the template DNA without ATP hydrolysis and exhibits strikingly high processivity, which is a desirable feature in the synthesis of multiple copies of the EBV genome in rolling-circle DNA replication (25). More recently, 3′-to-5′ exonuclease activity has been demonstrated to be associated with the purified EBV DNA Pol, which liberates 5′-deoxynucleoside monophosphates from primer termini (24, 26). The exonuclease activity is also stimulated by ammonium sulfate and is proposed to have a proofreading function as it preferentially excises terminally mismatched nucleotides incorporated at the primer terminus. These enzymatic properties are common to DNA Pols in the herpesvirus family.

In investigations of the molecular basis of protein-protein interactions between the subunits of the EBV DNA Pol holoenzyme, it is very useful to develop the overexpression and purification systems of the individual components and to characterize them. EBV DNA Pol accessory subunit, BMRF1 gene product, has recently been expressed, purified,

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and characterized (27). The accessory protein exhibited higher binding affinity for double-stranded DNA but had neither DNA Pol nor exonuclease activities. Here we report the overexpression of EBV DNA Pol catalytic subunit in the recombinant baculovirus-infected insect cells in order to get sufficient amounts of functionally active BALF5 gene product without cross-contamination of the EBV DNA Pol accessory subunit. The purification and the enzymatic properties of the catalytic EBV DNA Pol are described.

MATERIALS AND METHODS

Materials. [^{3}H]TTP (50 Ci/mmol), [1',2'-^{3}H]dGTP (50 Ci/mmol), [1',2',2,8-^{3}H]dATP (100 Ci/mmol), and unlabeled deoxynucleoside 5'-triphosphates were from Amersham Corp. Unlabeled polynucleotides [poly(rA), poly(rU), poly(dA), and poly(dC)] and oligonucleotides [$(dT)_{12-18}$, $(dG)_{12-18}$, $(dA)_{12-18}$, and $(rA)_{12-18}$] were from Pharmacia LKB Biotechnology Inc. $(dT)_{6,000}$ was from the Midland Certified Reagent Co. Heparin agarose and single-stranded DNA agarose were from Bethesda Research Laboratories. Phagemid pBS(+) was from Stratagene. The pVL1392 transfer vector was a gift from Max D. Summers (Texas A&M University).

DNA substrate. Activated calf thymus DNA was prepared as described previously (25).

Template-primers used in the template-primer preference assay were prepared as follows. An oligonucleotide was hybridized at a ratio of 1:2 (wt/wt) to the complementary polynucleotide in buffer B (20 mM Tris-HCl [pH 8.0], 5 mM $MgCl_2$, and 0.3 M NaCl) by incubation at 70°C for 20 min, slow cooling to room temperature over 1 h, and further incubation for 1 h at 30°C.

3'-terminally labeled activated calf thymus DNA was prepared by incubating activated DNA with the Klenow fragment of *Escherichia coli* Pol I in the presence of a single nucleotide substrate, [^{3}H]dGTP. The reaction mixture contained 50 mM Tris-HCl (pH 8.0), 6 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 160 μg of activated DNA per ml, 6 μM [^{3}H]dGTP (50 Ci/mmol), 0.8 mM 5'-AMP, and 40 U of *E. coli* Pol I Klenow fragment per ml. After 30 min of incubation at room temperature, the reaction was stopped by the addition of 100 mM EDTA and 0.5% sodium dodecyl sulfate (SDS) and deproteinized with phenol-chloroform. Activated DNA · [^{3}H]dGMP was separated from unincorporated nucleotides by centrifugation through a spun column of Chroma Spin-30 (Clontech Laboratory, Inc.). The specific activity of 3'-terminally labeled activated DNA · [^{3}H]dGMP was 144,000 cpm/μg.

Construction of recombinant baculovirus. A cosmid clone containing the EBV (strain Akata) DNA Pol open reading frame (a gift from K. Takada, Yamaguchi University) (23) was used to prepare a recombinant plasmid that would permit overexpression of the EBV DNA Pol catalytic subunit, BALF5 protein. Two overlapping EBV DNA Pol gene clones, pEBpolU and pEBpolL, were prepared (Fig. 1). The plasmid pEBpolU contains the *Xma*III (blunt)-*Xma*III (blunt) fragment including the upper part of the EBV Pol open reading frame at the *Hin*cII site of the pBS(+) vector. The plasmid pEBpolL contains the *Sal*I-*Dra*I (blunt) fragment including the lower part of the EBV Pol open reading frame between the *Sal*I and *Sma*I sites of the pBS(+) vector. To generate pEBPol containing the full-length EBV Pol gene, the *Sal*I-*Pvu*I (blunt) fragment of pEBPolL was ligated between the *Hin*dIII (blunt) and *Sal*I sites of pEBPolU. The DNA fragment containing a full-length copy of the BALF5

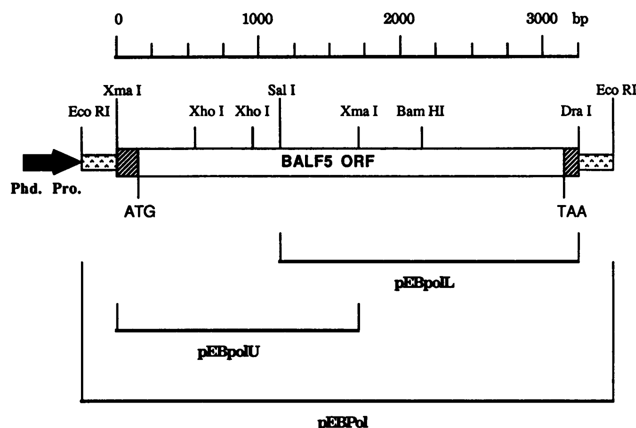

FIG. 1. Schematic representation of the EBV DNA Pol gene of the recombinant baculovirus vector pVL1392/BALF5. The diagram shows a restriction map of the EBV DNA Pol region of pVL1392/BALF5. Open region, BALF5 open reading frame; hatched regions, upstream and downstream regions of BALF5 open reading frame; dotted region, multiple cloning site of pBS(+) DNA; black region, polyhedrin promoter of pVL1392. Details of the construction of the recombinant transfer vector are given in Materials and Methods.

gene was isolated from the plasmid pEBPol by digestion with *Eco*RI and inserted into the *Eco*RI site of the baculovirus transfer vector, pVL1392 (18), to generate a recombinant plasmid, pVL1392/BALF5. The correct orientation was verified by restriction-mapping analyses. The recombinant plasmid pVL1392/BALF5 was transformed into *E. coli* HB101 and isolated. The pVL1392/BALF5 transfer vector (60 μg) was cotransfected with the *Sau*I-digested linear baculovirus DNA AcRP23*LacZ* (1 μg) (21) into *Spodoptera frugiperda* cells (Sf9 cells) as described by Kitts et al. (13). Viruses produced from the transfected cells were used to infect Sf9 cells, and the plaques were stained with 50 μg of neutral red per ml and 250 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) per ml at 3 days postinfection. The recombinant baculovirus plaques were distinguished from the original type of baculovirus plaques by the lack of blue in the infected cells. The recombinant baculovirus, AcBALF5, was purified by five rounds of plaque purification.

Preparation of infected cell extracts. Sf9 cells infected with the recombinant baculovirus AcBALF5 at a multiplicity of infection of 1 were harvested at 36 h postinfection and centrifuged at 1,400 × *g* for 10 min. The cells were washed in ice-cold phosphate-buffered saline (PBS) and recentrifuged as described above. The cell pellet was resuspended in ice-cold hypotonic buffer [20 mM Tris-HCl (pH 7.6), 5 mM EDTA, 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), 1 mM DTT, 1 mM PMSF, 4 μg of leupeptin per ml, and 4 μg of pepstatin A per ml] for 30 min at 0°C and then subjected to Dounce homogenization. Nuclei were removed from the lysate by centrifugation at 1,500 × *g* for 10 min at 4°C. The cytosolic extract was frozen in liquid nitrogen and stored at −80°C until it was to be used.

Purification of the EBV DNA Pol catalytic subunit. Unless otherwise noted, all steps were performed at 4°C. The cytosolic extract was quickly thawed at 37°C and applied to a column of heparin-agarose (3.0-ml bed volume) that had been equilibrated with buffer A (25% glycerol, 20 mM Tris-HCl [pH 7.6], 1 mM EDTA, 1 mM EGTA, 1 mM DTT,

1 mM phenylmethylsulfonyl fluoride, 4 μg of leupeptin per ml, and 4 μg of pepstatin A per ml) containing 0.1 M NaCl. The column was washed with 20 ml of the same buffer and eluted with a 36-ml linear gradient from 0.1 to 1.0 M NaCl in buffer A. Fractions (0.6 ml) were assayed for DNA Pol activity, and some aliquots were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) (8% polyacrylamide) and stained with silver or analyzed by Western blot (immunoblot) with anti-BALF5 protein-specific antibody. Peak fractions were pooled and dialyzed overnight against buffer A containing 0.1 M NaCl.

The dialysate was applied to a column of single-stranded DNA agarose (1-ml bed volume) equilibrated with buffer A containing 0.1 M NaCl. The column was washed with 6 ml of the same buffer, and a linear gradient (22 ml) from 0.1 to 0.8 M NaCl in buffer A was applied. Fractions (0.36 ml) were assayed for DNA Pol activity and subjected to SDS-PAGE (8% polyacrylamide) and stained with silver. Peak fractions were pooled and dialyzed overnight against buffer A containing 0.1 M NaCl.

The dialysate was applied to a column of heparin-agarose (0.4 ml) equilibrated with buffer A containing 0.1 M NaCl. The column was washed with 3 ml of the same buffer and eluted with an 8-ml linear gradient from 0.1 to 0.6 M NaCl in buffer A. Fractions (0.2 ml) were assayed for DNA Pol activity. Peak fractions were pooled, concentrated by centrifugation in a Centricon 30 microconcentrator, and stored at −80°C. The specific activity of the EBV DNA Pol catalytic subunit was 6,190,000 U/mg.

Preparation of EBV DNA Pol from EBV-producing lymphoblastoid cells. EBV DNA Pol complex was purified from B95-8 cells treated with phorbol 12-myristate 13-acetate and sodium *n*-butyrate as described previously (24). The specific activity of the purified EBV DNA Pol was 1,317,000 U/mg, which was corrected by the definition of this study.

Enzyme assays. DNA Pol activity was routinely assayed in the absence of ammonium sulfate. Each reaction mixture (25 μl) contained 50 mM Tris-HCl (pH 8.0); 10% glycerol; 6 mM $MgCl_2$; 100 μg of bovine serum albumin (BSA) per ml; 80 μg of activated calf thymus DNA per ml; 1 mM DTT; 25 μM (each) dATP, dCTP, and TTP; and 10 μM [^{3}H]dGTP (900 cpm/pmol). The reaction was started by the addition of the enzyme fraction, and incubation was for 20 min at 35°C. One unit of enzyme activity was defined as the amount required for incorporation of 1 pmol of deoxynucleoside monophosphates into acid-insoluble material in 60 min at 35°C.

The 3′-to-5′ exonuclease activity was assayed by measuring the release of [^{3}H]dGMP from 3′-terminally labeled activated DNA · [^{3}H]dGMP (144,000 cpm/μg) as described previously (26). Each reaction mixture (25 μl) contained 50 mM Tris-HCl (pH 8.0), 6 mM $MgCl_2$, 1 mM DTT, 100 μg of BSA per ml, and 3 μg of activated DNA · [^{3}H]dGMP per ml. The reaction was started by the addition of the enzyme, incubated for 5 min at 35°C, and stopped by the addition of 10 μl of 2 mg of sonicated salmon sperm DNA per ml containing 0.01 M sodium pyrophosphate and 100 μl of 100% (wt/vol) trichloroacetic acid. The reaction mixture was left on ice for 10 min and centrifuged at 11,000 × *g* for 10 min. One hundred microliters of the supernatant was added to 1.5 ml of ACS II (NEN), and then the radioactivity was counted.

Antibodies. An anti-BALF5 protein-specific antibody was produced against a fusion protein consisting of T7 phage φ10 protein (280 amino acids) and a truncated region of EBV BALF5 protein containing a 3′-to-5′ exonuclease domain and a nucleotide binding domain (361 amino acids) as follows. The *Sal*I-*Bam*HI fragment from pEBPol was inserted in frame between the *Sal*I and *Bam*HI sites of the expression vector pGEMEX2 (Promega). The recombinant plasmid pGEMEX2/BALF5 was transformed into BL21 (DE3), an *E. coli* cell line which expresses T7 RNA Pol. The BALF5 fusion protein was expressed in *E. coli* harboring pGE MEX2/BALF5 by induction with 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG). The *E. coli* cell pellet was collected and suspended in 10 volumes of lysis buffer (10 mM sodium phosphate [pH 7.5], 30 mM NaCl, 0.25% Tween 20, 10 mM 2-mercaptoethanol, 10 mM EDTA, 10 mM EGTA) and lysed by freeze-thawing and sonication. After centrifugation at 12,000 × *g* for 30 min, the cell pellet was resuspended with sample buffer for SDS-PAGE. The soluble sample was then subjected to SDS-PAGE. The band of BALF5 fusion protein was excised, and the protein was extracted with a buffer containing 25 mM Tris, 195 mM glycine, and 0.1% SDS. The purity of the obtained BALF5 fusion protein was more than 90% as judged by SDS-PAGE. A Japanese White rabbit was immunized subcutaneously by a standard protocol (9) with the purified fusion protein seven times at biweekly intervals. Immunoglobulin G (IgG) of the immunized rabbit serum was purified through the affinity column coupled with the purified BALF5 fusion protein as described previously (9). The protein concentration of the anti-BALF5 protein-specific antibody was 200 μg/ml in PBS.

An anti-BBLF4 protein-specific antibody was raised against BBLF4 carboxy-terminal oligopeptides (20 amino acids) coupled to keyhole limpet hemacyanin. Preparation for the anti-BBLF4 protein-specific IgG was performed according to the same procedure as that for the anti-BALF5 protein-specific antibody. The protein concentration of the anti-BBLF4-specific IgG was 1 mg/ml in PBS.

RESULTS

Expression of the EBV DNA Pol catalytic subunit in Sf9 cells. Monolayers of Sf9 cells were mock infected or infected at a multiplicity of infection of 5 PFU per cell with AcRP23*LacZ* or the recombinant baculovirus AcBALF5. The protein products were resolved by SDS-PAGE (8% polyacrylamide) (Fig. 2A). The AcRP23*LacZ*-infected insect cells expressed large amounts of β-galactosidase with a molecular weight of 116,000 (Fig. 2A, lane 2). By comparison with the controls (mock-infected and AcRP23*LacZ*-infected cells), a 110-kDa polypeptide was detected in the AcBALF5-infected cells. The expressed protein was shown to be EBV BALF5 gene product by its reaction with anti-BALF5 protein-specific antibody during Western immunoblot analysis (Fig. 2B). The expressed BALF5 gene product made up about 20% of the total cellular proteins.

Purification of the EBV DNA Pol catalytic subunit expressed in Sf9 cells. A monolayer culture of Sf9 cells was infected with the recombinant baculovirus AcBALF5. At 36 h postinfection, the cells were harvested, suspended in hypotonic buffer for 30 min at 0°C, and then subjected to Dounce homogenization. A large part of the expressed EBV DNA Pol catalytic subunit was transported to the nuclei of the infected Sf9 cells, but it was almost insoluble. No DNA Pol activity was associated with the insoluble material (data not shown). However, the cytosolic fraction contained a small amount of soluble enzyme as judged by Western blot analysis with anti-BALF5 protein-specific antibody. Thus, the cytosolic fraction was loaded onto a heparin-agarose column and eluted with a linear gradient from 0.1 to 1 M NaCl in buffer A. The recombinant BALF5 gene product of 110 kDa eluted at 0.36 M NaCl as judged by silver staining of

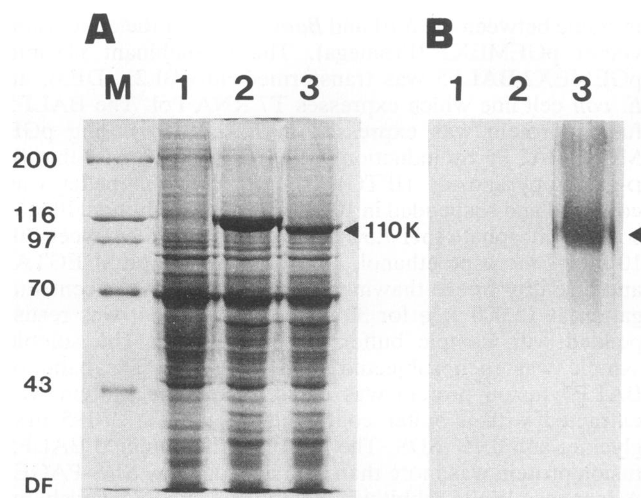

FIG. 2. Expression of the BALF5 gene product in Sf9 cells. Sf9 cells were mock infected (lanes 1) or infected with AcRP23*LacZ* (lanes 2) (20) or the recombinant baculovirus AcBALF5 (lanes 3) and harvested at 3 days postinfection. Proteins recovered from the cell extracts were resolved by SDS-PAGE (8% polyacrylamide) and stained with Coomassie blue (A) or analyzed by Western blot (B) with anti-BALF5 protein-specific antibody. The positions of the molecular weight standards (lane M) (in thousands) are indicated at the left of panel A. The position of the BALF5 gene product is indicated at the right of each panel (closed triangle).

SDS-PAGE and Western blot analyses (data not shown). DNA Pol activity was complicated by the presence of cellular, baculovirus, and the expressed EBV enzymes in a single infected cell.

The peak fractions were loaded onto a single-stranded DNA agarose column and eluted with a linear gradient from 0.1 to 0.8 M NaCl in buffer A. Single-stranded DNA agarose chromatography yielded two peaks of DNA Pol activity. The first major peak of DNA Pol activity, which eluted at 0.18 M NaCl, was coincident with the expressed EBV gene product as judged by silver staining of SDS-PAGE and Western blot analyses (data not shown). The DNA Pol activity of the second minor peak, which eluted at 0.3 M NaCl, was not coincident with the expressed BALF5 protein. Since the activity was stimulated threefold in the presence of 80 mM ammonium sulfate, it most likely reflected a baculovirus DNA Pol activity (20).

The peak fractions of the expressed EBV enzyme were again loaded onto a heparin-agarose column and eluted with a linear gradient from 0.1 to 0.6 M NaCl in buffer A. As shown in Fig. 3, the peak of DNA Pol activity eluted at 0.36 M NaCl, and the silver-stained polyacrylamide gel of the fractions containing the Pol activity showed a single distinct band that had an intensity coincident with that of the enzymatic activity (Fig. 3). The band migrated with an apparent molecular mass of 110 kDa, a value that is close to the expected molecular mass of the protein encoded by the EBV BALF5 (113 kDa) gene and was identical to the mobility of the corresponding subunit of the enzyme isolated from chemically induced EBV-infected cells (11, 24). Western blot analysis clearly showed that the purified enzyme is in fact an EBV BALF5 gene product (Fig. 3C). The summary of the purification is given in Table 1.

Detection of a 3′-to-5′ exonuclease activity. The 3′-to-5′ exonuclease activity is known to be associated with the EBV DNA Pol purified from chemically induced EBV-infected cells (24). To test whether the single subunit of the EBV DNA Pol has the 3′-to-5′ exonuclease activity, the activity was assayed for each fraction. The exonuclease activity was precisely coincident with the 110-kDa protein band of the expressed EBV DNA Pol catalytic subunit on the second step of chromatography of single-stranded DNA agarose (data not shown) and the last step of chromatography of heparin agarose (Fig. 3). The 3′-to-5′ exonuclease activity was assayed by using 3′-terminally labeled activated DNA · [^{3}H]dGMP as substrate. The reaction product was dGMP determined by polyethyleneimine thin-layer chromatography (data not shown). The 5′-to-3′ exonuclease activity was also assayed under the same assay condition as the 3′-to-5′ exonuclease activity except with 5′-terminally labeled 5′-^{32}P-poly(dA)$_{255}$ · poly(dT)$_{6,000}$ as substrate. However, the 5′-to-3′ exonuclease activity was not detected under the condition (data not shown). These observations indicate that the EBV DNA Pol catalytic subunit hydrolyzed 3′-terminal nucleotide. Thus, we conclude that the 3′-to-5′ exonuclease activity associated with the EBV DNA Pol is an intrinsic feature of the EBV BALF5 gene product.

Inhibition of DNA Pol and 3′-to-5′ exonuclease activities by an antibody raised against BALF5 fusion protein. An anti-BALF5 protein-specific antibody was raised against a fusion protein consisting of T7 ϕ10 protein and a truncated region of the BALF5 protein including the 3′-to-5′ exonuclease and a part of nucleotide binding domains. The antibody was tested for its ability to neutralize the Pol and the 3′-to-5′ exonuclease activities. The purified catalytic subunit of EBV DNA Pol was incubated with the antibody at 0°C for 30 min and then assayed for these activities. As shown in Table 2, the antibody inhibited both the Pol and the exonuclease activities to the same degree, whereas anti-BBLF4 protein-specific antibody did not. These results are consistent with the view that both DNA Pol and 3′-to-5′ exonuclease activities are present on the same polypeptide.

Enzymatic properties of the single subunit of the EBV DNA Pol. (i) Reactivity to aphidicolin and PAA. EBV DNA Pol is distinct from DNA Pol α for its hypersensitivity to the pyrophosphate analog, PAA. Another compound, aphidicolin, a potent DNA synthesis inhibitor in vivo, inhibits all α-like DNA Pols in vitro (15). The EBV DNA Pol purified from EBV-producing cells is known to be sensitive to both PAA and aphidicolin (11, 25). The single subunit of the EBV DNA Pol was assayed for DNA Pol activity in the presence of increasing concentrations of PAA or aphidicolin (Fig. 4). The single subunit of the EBV DNA Pol was also sensitive to these compounds.

(ii) Salt sensitivity. It is well known that the Pol activity associated with DNA Pols in the herpesvirus family is stimulated by salt when activated DNA is used as template-primer in the Pol assay (11, 14, 24). To know the salt effect on the DNA Pol and the 3′-to-5′ exonuclease activities of the EBV DNA Pol catalytic subunit, both activities were assayed in the presence of increasing concentrations of ammonium sulfate (Fig. 5). Both the Pol and the 3′-to-5′ exonuclease activities were inhibited by the salt in a dose-dependent manner, whereas both activities associated with the enzyme purified from EBV-producing lymphocytes were stimulated by the salt.

(iii) Template-primer preference. One of the conspicuous enzymatic properties of herpesvirus DNA Pols is template-primer preference for poly(dC) · oligo(dG) (11, 25). The

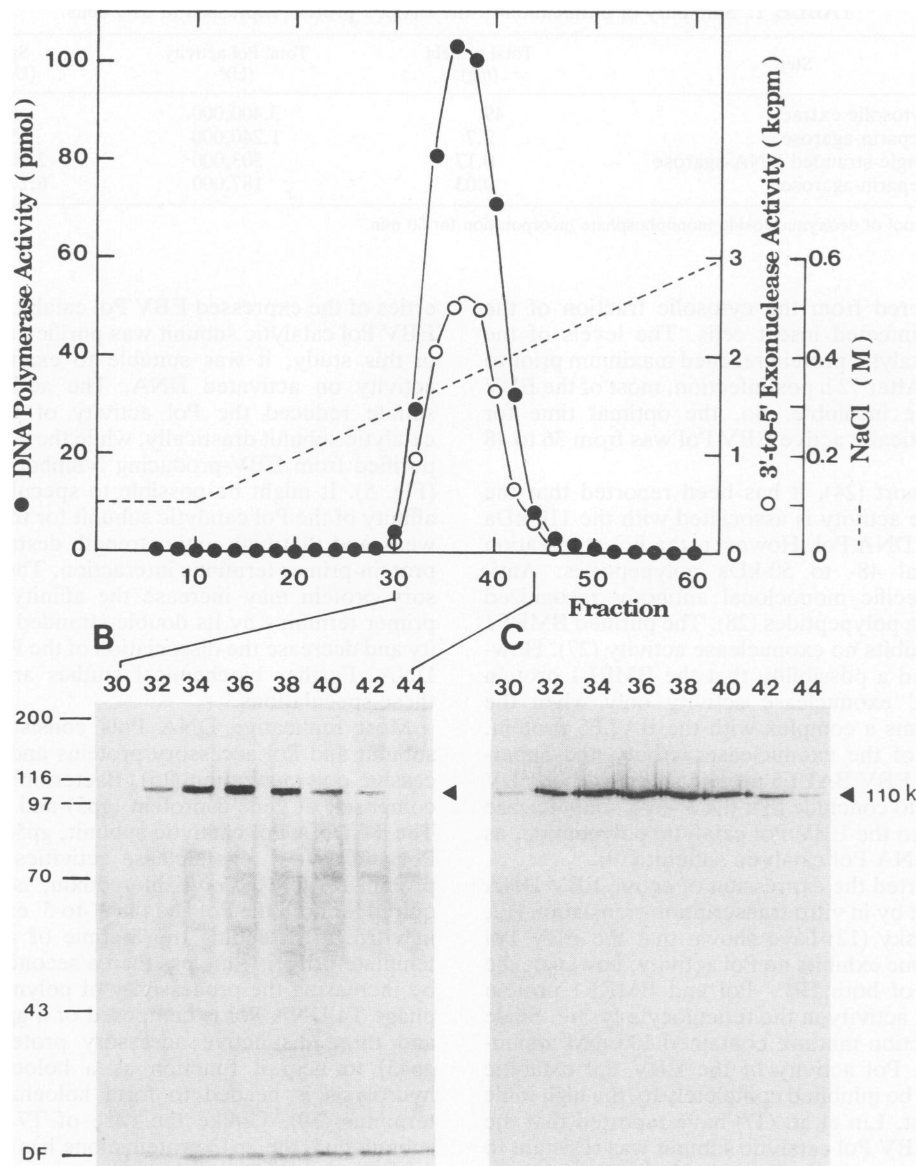

FIG. 3. The last step in chromatography of the heparin-agarose column of the BALF5 Pol catalytic protein. The peak fractions of the single-stranded DNA agarose column chromatography were loaded onto a heparin-agarose column and eluted with a linear gradient from 0.1 to 0.6 M NaCl in buffer A. Fractions were assayed for either DNA Pol or 3'-to-5' exonuclease activity. Results are expressed as picomoles of dGMP incorporated in 20 min (DNA Pol activity), or counts per minute of released dGMP in 5 min (3'-to-5' exonuclease activity). Aliquots of the indicated fractions from the chromatography were subjected to SDS-PAGE (8% polyacrylamide) and stained with silver (B) or analyzed by Western blot with anti-BALF5 protein-specific antibody (C). The positions of the molecular weight standards (in thousands) are indicated at the left of panel B. The positions of the BALF5 protein are indicated at the right of panels B and C (closed triangles).

EBV DNA Pol purified from EBV-producing cells utilized poly(dC) · oligo(dG) 29-fold more efficiently than activated DNA as template-primer, while the single subunit of the EBV DNA Pol utilized it poorly (Table 3). However, poly(rA) · oligo(dT), poly(rU) · oligo(dA), and poly(dT) were not utilized at all as well. Thus, the absence of the BMRF1 Pol accessory protein drastically changed the template-primer preference. The BMRF1 protein may have a nucleotide sequence specificity for its double-stranded DNA binding and influence the Pol and primer-terminus interaction.

DISCUSSION

We have expressed the EBV DNA Pol catalytic subunit in insect cells by using the baculovirus system. Up to 20% of the total cellular proteins produced in the infected insect cells is the recombinant EBV DNA Pol polypeptide. Unfortunately, almost-expressed EBV Pol protein remained as an insoluble fraction, which was enzymatically inactive, as was observed for the expression of human cytomegalovirus and varicella-zoster virus DNA Pols in insect cells (5). However, the soluble and enzymatically active EBV DNA Pol catalytic

TABLE 1. Summary of purification of the BALF5 protein expressed in SF9 cells

Fraction no.	Step	Total protein (mg)	Total Pol activity (U)[a]	Sp act (U/mg)[a]	Yield (%)
I	Cytosolic extract	49	3,400,000	69,400	100
II	Heparin-agarose	2.7	1,240,000	459,000	36
III	Single-stranded DNA-agarose	0.17	503,000	2,960,000	15
IV	Heparin-agarose	0.03	187,000	6,170,000	5

[a] 1 U is defined as 1 pmol of deoxynucleoside monophosphate incorporation for 60 min.

subunit was recovered from the cytosolic fraction of the recombinant virus-infected insect cells. The levels of the soluble EBV Pol catalytic protein reached maximum prior to 48 h postinfection. After 72 h postinfection, most of the EBV Pol protein became insoluble. So, the optimal time for isolation of enzymatically active EBV Pol was from 36 to 48 h postinfection.

In a previous report (24), it has been reported that the 3′-to-5′ exonuclease activity is associated with the 110-kDa subunit of the EBV DNA Pol. However, the Pol preparation contained additional 48- to 50-kDa polypeptides. Anti-BMRF1 protein-specific monoclonal antibody recognized these 50- and 48-kDa polypeptides (28). The purified BMRF1 protein by itself exhibits no exonuclease activity (27). However, there remained a possibility that the BMRF1 protein can exhibit 3′-to-5′ exonuclease activity only when the BMRF1 protein forms a complex with the BALF5 protein. The copurification of the exonuclease activity and apparently homogeneous EBV BALF5 protein lacking other EBV proteins permits us to conclude that the 3′-to-5′ exonuclease activity is intrinsic to the EBV Pol catalytic polypeptide, as is true with HSV DNA Pol catalytic subunit (19).

Others have reported the expression of active EBV DNA Pol catalytic subunit by in vitro transcription-translation (12, 17). Kiehl and Dorsky (12) have shown that the EBV Pol catalytic subunit alone exhibits no Pol activity; however, the expression system of both EBV Pol and BMRF1 protein generated DNA Pol activity in the reticulocyte lysate. Since their Pol assay reaction mixture contained 100 mM ammonium sulfate, DNA Pol activity of the EBV Pol catalytic subunit alone might be inhibited completely by the high ionic strength. In contrast, Lin et al. (17) have reported that the Pol activity of the EBV Pol catalytic subunit was resistant to ammonium sulfate; however, salt-stimulated Pol activity was not observed. The discrepancy between them is not known with certainty. The reticulocyte lysate contains cellular DNA Pols and many other proteins. It is possible to speculate that those factors may mask the enzymatic properties of the expressed EBV Pol catalytic subunit. Since the EBV Pol catalytic subunit was purified to near homogeneity in this study, it was suitable to examine for its specific activity on activated DNA. The addition of ammonium sulfate reduced the Pol activity of the EBV DNA Pol catalytic subunit drastically, while the activity of the enzyme purified from EBV-producing lymphocytes was stimulated (Fig. 5). It might be possible to speculate that the binding affinity of the Pol catalytic subunit for the primer terminus is weak and that high ionic strength destabilizes the catalytic protein-primer terminus interaction. The BMRF1 Pol accessory protein may increase the affinity of the Pol for the primer terminus by its double-stranded DNA binding activity and decrease the dissociation of the Pol from the template DNA. Further biochemical studies are needed to clarify these speculations.

Most replicative DNA Pols consist of a Pol catalytic subunit and Pol accessory proteins and display highly processive polymerization (15). Bacteriophage T7 DNA Pol is composed of gene 5 protein (gp5) and *E. coli* thioredoxin. The T7 DNA Pol catalytic subunit, gp5, alone has very low Pol and 3′-to-5′ exonuclease activities (22). An accessory protein to gp5, *E. coli* thioredoxin, is near-absolutely required for both the Pol and the 3′-to-5′ exonuclease activities in vitro by extending the lifetime of a gp5 complex with template-primer from less than a second to about 5 min and by increasing the processivity of polymerization. Bacteriophage T4 DNA Pol is composed of a gp43 Pol core protein and three distinctive accessory proteins (gp44-gp62 and gp45) to help it function as a holoenzyme, while ATP hydrolysis is needed to form holoenzyme at the primer terminus (30). Unlike the case of T7 DNA Pol catalytic subunit gp5, the gp43 protein alone has high Pol and 3′-to-5′

TABLE 2. Effect of anti-BALF5 protein-specific antibody on the Pol and the 3′-to-5′ exonuclease activities of the EBV DNA Pol catalytic subunit[a]

Additive	DNA Pol activity		3′-to-5′ exonuclease activity	
	pmol	%	cpm	%
BSA	84 ± 4	100	1,826 ± 88	100
Anti-BALF5 IgG	50 ± 5	54	785 ± 78	43
Anti-BBLF4 IgG	91 ± 7	109	1,862 ± 63	102

[a] Prior to the assay, 8 μl of the purified BALF5 protein was incubated with 8 μl of anti-BALF5 protein-specific antibody (200 μg/ml), anti-BBLF4 protein-specific antibody (1 mg/ml), or BSA (200 μg/ml) at 0°C for 30 min. The Pol and the exonuclease activities were then determined as described in Materials and Methods. Results are the means ± standard errors of the mean (n = 3).

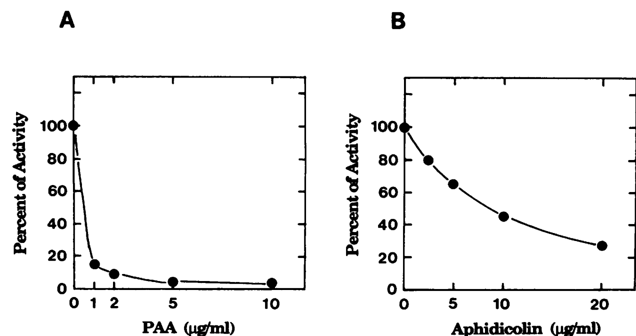

FIG. 4. Effect of PAA (A) and aphidicolin (B) on the Pol activity of the EBV DNA Pol catalytic subunit. The DNA Pol activity was assayed on activated DNA template as described in Materials and Methods, supplemented with PAA or aphidicolin to the final concentrations indicated. The 100% value of the EBV DNA Pol catalytic subunit was 60 pmol of deoxynucleoside monophosphates incorporated per 20 min.

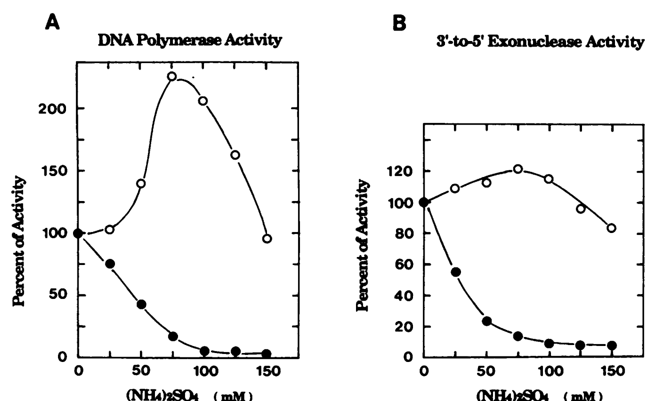

FIG. 5. Effect of ammonium sulfate on the Pol and the 3'-to-5' exonuclease activities of the EBV DNA Pol catalytic subunit (●) and the EBV DNA Pol purified from EBV-producing lymphocytes (○). (A) The DNA Pol activity was assayed by using activated DNA as substrate as described in Materials and Methods except that ammonium sulfate concentrations were varied as indicated. The activity is expressed as the percentage of the activity obtained in the absence of ammonium sulfate. (B) The 3'-to-5' exonuclease activity was assayed by using activated DNA · [^{3}H]dGMP as substrate as described in Materials and Methods except that ammonium sulfate concentrations were varied as indicated. The activity is expressed as the percentage of the activity obtained in the absence of ammonium sulfate.

exonuclease activities. The accessory protein complex stimulates the processivity of the gp43 protein. Human DNA Pol α is made up of a cluster of a 180-kDa Pol catalytic subunit, a 70-kDa phosphoprotein of unknown function, and two polypeptides of 55 and 49 kDa with primase activity (15). The DNA Pol α catalytic subunit has been functionally expressed by a recombinant baculovirus in insect cells (3). The catalytic polypeptide exhibits Pol activity but has no 3'-to-5' exonuclease activity. The kinetic parameters of Pol catalysis, sensitivity to inhibitors, and processivity of the Pol catalytic subunit are identical to that observed with the four-subunit Pol α-primase complex. HSV type 1 DNA Pol also consists of UL30 Pol catalytic subunit and UL42 Pol accessory subunit (4). The HSV DNA Pol catalytic subunit has been also overexpressed in the baculovirus system (10, 19). The HSV Pol is resistant to salt although salt-stimulated

TABLE 3. Template-primer preferences for the catalytic subunit or the complex form of the EBV DNA Pol[a]

Template-primer	EBV DNA Pol (%)	
	Catalytic subunit	Pol complex
Activated DNA	100	100
Poly(dC) · oligo(dG)	48	2,900
Poly(dT) · oligo(rA)	190	670
Poly(rU) · oligo(dA)	<1	<1
Poly(rA) · oligo(dT)	<1	<1
Poly(dT)	<1	<1

[a] All DNA Pol assays contained 50 mM Tris-HCl (pH 8.0), 6 mM $MgCl_2$, 1 mM DTT, 100 μg of BSA per ml, 10% glycerol, 40 μM ^{3}H-deoxynucleoside triphosphate complementary to the template, and the indicated template-primer. Reactions were performed for 20 min at 35°C. All templates and primers were present at 80 and 40 μg/ml, respectively. The template-primer concentration in reaction with activated DNA or poly(dT) was 80 μg/ml. All assays were performed in triplicate, and the data represent the mean values relative to that with activated DNA.

Pol activity was not observed (19). Furthermore, its chromatographic behaviors are similar to those for the enzyme purified from HSV-infected cells (19). Although UL42 Pol accessory protein does not change the HSV DNA Pol activity on activated DNA template, it stimulates the processivity of the HSV Pol catalytic subunit on singly primed M13 single-stranded DNA template (7, 10, 19). In the case of EBV DNA Pol, the EBV Pol catalytic subunit alone exhibited both the DNA Pol and the 3'-to-5' exonuclease activities in the absence of BMRF1 Pol accessory protein. However, the EBV DNA Pol catalytic subunit was distinguished from the enzyme purified from EBV-producing cells in salt sensitivity, template-primer preference, and chromatographic properties (24). The functional expression of the EBV Pol catalytic subunit provides us with an amenable system for pursuing structure-function studies. For example, it is very interesting to know how many nucleotides the EBV Pol catalytic subunit alone polymerizes and how the BMRF1 Pol accessory protein changes the enzymatic activity of the Pol catalytic subunit. In vitro study of the interactions between the BALF5 Pol catalytic protein and the BMRF1 Pol accessory protein with regard to the binding affinity for the primer terminus and the processivity is now in progress.

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