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Purification and Characterization of the DNA-Binding Activity of the Epstein-Barr Virus DNA Polymerase Accessory Protein BMRF1 Gene Products, as Expressed in Insect Cells by Using the Baculovirus System

TATSUYA TSURUMI

Laboratory of Virology, Research Institute for Disease Mechanism and Control, Nagoya University School of Medicine, 65 Tsuruma-cho, Showa-ku, Nagoya 466, Japan

Received 13 July 1992/Accepted 3 November 1992

A recombinant baculovirus containing the complete sequence for the Epstein-Barr virus (EBV) BMRF1 gene product, the EBV DNA polymerase accessory protein, under the control of the polyhedrin promoter was constructed. Insect cells infected with the recombinant virus produced two phosphoproteins of 52 and 50 kDa and one unphosphorylated protein of 48 kDa, recognized by anti-BMRF1 protein-specific monoclonal antibody. The major protein bands were 50 and 48 kDa. The expressed BMRF1 gene products were purified to near homogeneity from the nuclear extract of the recombinant baculovirus-infected insect cells by double-stranded DNA–cellulose column chromatography followed by heparin-agarose column chromatography. The purified BMRF1 gene products exhibited higher binding affinity for double-stranded DNA than for single-stranded DNA without ATP hydrolysis. The protein-DNA interaction did not necessarily require a primer terminus. The present system will open the way for the biochemical characterization of the EBV DNA polymerase accessory protein.

The genome of Epstein-Barr virus (EBV), a human lymphotropic herpesvirus, consists of a linear duplex DNA which is 172 kb long and encodes approximately 84 proteins (1). During the latent phase of the viral life cycle, the EBV genome is maintained as a circular plasmid molecule. However, after induction of the lytic phase of viral replication, the EBV DNA replication proteins are expressed 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 polymerase via a rolling-circle mechanism initiated from ori *Lyt* (10).

The EBV DNA polymerase, which exhibits highly processive replication and possesses 3′-to-5′ exonuclease activity (29–31), has been purified (15, 18, 29). The 110-kDa EBV DNA polymerase catalytic polypeptide copurifies with the 48- to 55-kDa nuclear phosphoproteins encoded by the BMRF1 open reading frame identified as a part of the early antigen diffuse (EA-D) component (15, 18). The BMRF1 gene products appear to function as EBV DNA polymerase accessory proteins because of the neutralization of the EBV DNA polymerase activity by monoclonal antibodies (MAbs) to the BMRF1 protein and the low activity in DNA polymerase fractions lacking the BMRF1 proteins (16, 18). In investigations of the molecular basis of protein-protein interactions between the subunits of the EBV DNA polymerase holoenzyme, the development of overexpression and purification systems of the individual components is very useful.

I have expressed EBV BMRF1 gene products in the recombinant baculovirus-infected insect cells in order to get sufficient amounts of functionally active BMRF1 gene products without cross-contamination of the EBV DNA polymerase catalytic subunit. The expressed proteins have been purified to near homogeneity, and the DNA-binding activity has been characterized in detail. The BMRF1 gene products exhibited double-stranded DNA (dsDNA)-binding activity without ATP hydrolysis, and the DNA binding did not require a primer terminus, unlike bacteriophage T4 polymerase accessory proteins, gene 44/62 proteins (14), and the eukaryotic DNA polymerase δ accessory protein, RF-C (27, 28). This system will enable the biochemical and structural studies of the EBV DNA polymerase accessory protein.

Construction of the recombinant baculovirus, AcBMRF1. B95-8 cells, cells of a virus-producing marmoset lymphoblastoid cell line immortalized with human EBV from a patient with mononucleosis, were treated with phorbol-12-myristate-13-acetate and sodium *n*-butyrate as described previously (29). After incubation for 48 h at 36°C, the culture medium was collected and extracellular virus particles were purified and treated with sodium dodecyl sulfate (SDS) and proteinase K as described previously (33). The EBV whole DNA was completely digested with *Bam*HI. After the *Bam*HI-digested EBV DNA fragments were inserted into the *Bam*HI site of pACYC184, the ligated recombinant plasmids were transformed into *Escherichia coli* HB101. Tetracycline-sensitive and chloramphenicol-resistant colonies were picked up. The recombinant plasmid containing the BMRF1 open reading frame, pEBBamHIM, was identified by the size of the inserted DNA and by restriction-mapping analyses (data not shown).

The recombinant plasmid pEBBamHIM was again transformed into *E. coli* GM33 (*dam dcm*) and then purified. The DNA fragment containing a full-length copy of the BMRF1 gene, EBV nucleotides 79894 to 81230, was isolated from plasmid pEBBamHIM by digestion with *Bcl*I and *Bgl*II and inserted into the *Bam*HI site of the baculovirus transfer vector pVL941 (19) to generate recombinant plasmid pVL941/BMRF1. The correct orientation was verified by restriction-mapping analyses.

The AcRP23*lacZ* baculovirus (23) contains the *lacZ* gene, which is under the control of the polyhedrin promoter, in

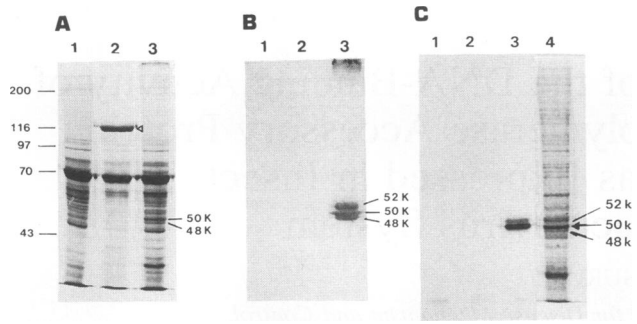

FIG. 1. Expression of the BMRF1 gene products in Sf9 cells. (A and B) Sf9 cells were mock infected (lanes 1) or infected with AcRP23*lacZ* (lanes 2) or the recombinant baculovirus AcBMRF1 (lanes 3) and harvested at 3 days postinfection. Proteins recovered from the cell extracts were resolved by SDS-PAGE (8% polyacrylamide) and stained with Coomassie blue (panel A) or analyzed by Western blot (panel B) with anti-BMRF1 protein-specific MAb 9240. The nitrocellulose blot was blocked with 10 mg of BSA per ml and reacted with anti-BMRF1 protein MAb 9240 (R3 mouse immunoglobulin G1 MAb to EBV EA-D [22, 26]; NEN/Dupont) for 1 h at room temperature. Bound antibody was detected by reacting the blot with peroxidase-conjugated sheep anti-mouse immunoglobulin G antibody (MBL, Inc.) and the immunostaining HPR kit IS-50B (Konica, Inc.). The positions of the molecular weight standards (in thousands) are indicated at the left of panel A. Positions of the BMRF1 gene products are indicated at the right of each panel. Open triangle, β-galactosidase. (C) Immunoprecipitation and phosphorylation of the recombinant BMRF1 proteins from the AcBMRF1-infected Sf9 cells. Lanes 1 to 3 are from an autoradiogram of immunoprecipitations from mock-infected, AcRP23*lacZ*-infected, and AcBMRF1-infected Sf9 cell nuclear extracts, respectively. Lane 4 represents a Coomassie blue-stained SDS-polyacrylamide gel of the nuclear extract from AcBMRF1-infected Sf9 cells.

place of the polyhedrin gene. The AcRP23*lacZ*-infected insect cells express large amounts of β-galactosidase. The restriction enzyme *Sau*I cuts the AcRP23*lacZ* baculovirus DNA at a single site within the *lacZ* gene. The pVL941/BMRF1 transfer vector (60 μg) was cotransfected with the *Sau*I-digested linear baculovirus DNA AcRP23*lacZ* (1 μg) into *Spodoptera frugiperda* cells (Sf9 cells) as described by Kitts et al. (17). Viruses produced from the transfected cells were infected with 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, AcBMRF1, was purified by three rounds of plaque purification.

Expression of the BMRF1 gene products in Sf9 cells. Monolayers of Sf9 cells were mock infected or infected at a multiplicity of infection of 1 PFU per cell with AcRP23*lacZ* or the recombinant baculovirus AcBMRF1. By comparison with the controls (mock-infected and AcRP23*lacZ*-infected cells), 52-, 50-, and 48-kDa proteins were detected in the AcBMRF1-infected cells. The expressed proteins were shown to be identified as EBV BMRF1 gene products (EA-D) by their reaction with anti-BMRF1 protein-specific MAb 9240 during Western immunoblot analysis (Fig. 1B). The 50-kDa protein was most abundant in the infected insect cells. Since very little 52-kDa protein was present, it was difficult to detect the protein band in SDS-PAGE with Coomassie blue staining (Fig. 1A). However, Western blot analysis clearly demonstrated the existence of the 52-kDa protein (Fig. 1B). As expected, no traces of β-galactosidase protein was detected in the AcBMRF1-infected insect cells. Although the levels of the BMRF1 protein did not approach that of β-galactosidase, the expressed BMRF1 gene products made up about 4% of the total cellular proteins (Table 1).

To determine the cellular location of the expressed recombinant BMRF1 gene products in the insect cells, indirect-immunofluorescence tests were performed on the AcBMRF1-infected Sf9 cells at 48 h postinfection (data not shown). Strong fluorescence was observed in the nuclei of the AcBMRF1-infected Sf9 cells. In contrast, no fluorescence was detected in mock-infected or AcRP23*lacZ*-infected cells.

To test whether the recombinant BMRF1 gene products are phosphorylated in the insect cells, AcBMRF1-infected Sf9 cells were incubated with $^{32}P_i$ in TNM-FH medium. At 48 h postinfection, 330 μCi of $^{32}P_i$ was added to 2×10^5 Sf9 cells in a 24-hole tissue culture plate in 250 μl of normal TNM-FH medium. Sf9 cells were harvested at 60 h postinfection and suspended in hypotonic buffer for 30 min at 0°C. The nuclei were prepared by hypotonic Dounce homogenization and suspended in 100 μl of buffer A containing 0.5 M KCl. An equal volume of lysis buffer (25 mM Tris-HCl [pH 7.4], 50 mM NaCl, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride) was added to the samples. The samples were sonicated and centrifuged for 20 min at 12,000 × *g*. The supernatants were incubated with 16 μl of anti-BMRF1 protein-specific MAb 9240 at 4°C and incubated with 15 μl of sheep anti-mouse immunoglobulin G antibody for 1 h at 4°C. Then 20 μl of 20% (vol/vol) protein A–Sepharose CL-4B (Pharmacia, Inc.) was added to the mixtures for 30 min at 4°C. The beads were washed four times with high NET buffer (50 mM Tris-HCl [pH 7.4], 0.5 M NaCl, 5 mM EDTA, 0.5% Nonidet P-40) and then twice with regular NET buffer (10 mM Tris-HCl [pH 7.4], 0.15 M NaCl, 5 mM EDTA, 0.05% Nonidet P-40), and then boiled in SDS-containing gel-loading buffer. After centrifugation, the supernatants were loaded onto an SDS–8% polyacrylamide gel. After polyacrylamide gel electrophoresis (PAGE), the gel was stained with Coomassie blue, destained, dried, and subjected to autoradiography. Immunoprecipitation of labeled nuclear extracts with anti-BMRF1

TABLE 1. Purification of the BMRF1 gene products expressed in *S. frugiperda* cells

Fraction	Step	Total protein (μg)	BMRF1 protein[a] (μg)	Purity (%)	Yield (%)
I	Total cell extract (cytosol + nuclei)	8,700	348	4	100
II	0.5 M KCl nuclear extract	2,400	346	14.4	99
III	dsDNA-cellulose	221	140	63.3	40
IV	Heparin-agarose	43	43	100	12

[a] The amounts of BMRF1 gene products were calculated from densitometric analyses of Coomassie blue-stained SDS-PAGE gels by the DVS3000 Image-Processing Scanner (Hamamatsu Photonic, Inc.).

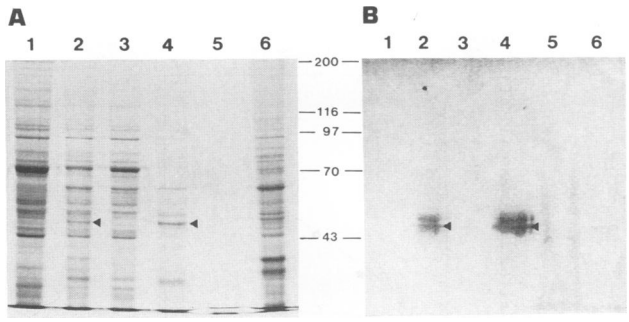

FIG. 2. Fractionation and solubilization of the BMRF1 gene products from the AcBMRF1-infected Sf9 cells. Sf9 cells were infected with the AcBMRF1 virus. At 72 h postinfection the cells were harvested, suspended in hypotonic buffer, and Dounce homogenized. The nuclei were suspended in buffer A containing 0.5 M KCl and centrifuged. The pellet was suspended in buffer A containing 1.7 M KCl and ultracentrifuged at 95,000 rpm in a Beckman TL100 rotor for 30 min at 4°C. The fractionated samples were subjected to SDS-PAGE (8% polyacrylamide) and stained with Coomassie blue (A) or analyzed by Western blot (B) with anti-BMRF1 protein-specific MAb 9240. Lanes: 1, mock-infected whole-cell lysate; 2, AcBMRF1-infected whole-cell lysate; 3, cytosol fraction of the infected cells; 4, medium-salt (0.5 M KCl) nuclear extract of the infected cells; 5, high-salt (1.7 M KCl) nuclear extract of the infected cells; 6, insoluble nuclear fraction of the infected cells. Solid triangles represent the positions of the 50-kDa BMRF1 gene products. The positions of the molecular weight standards (in thousands) are indicated.

protein-specific MAb 9240 demonstrated readily detectable phosphoproteins of 52 and 50 kDa from lysates of AcBMRF1-infected Sf9 cells labeled in the medium (Fig. 1C). Although the 48-kDa BMRF1 protein was also immunoprecipitated as judged by SDS-PAGE with Coomassie blue staining (data not shown), the 48-kDa protein was not phosphorylated (Fig. 1C).

Purification of the BMRF1 gene products expressed in Sf9 cells. To open the biochemical study of EBV DNA polymerase accessory protein, a purification procedure for the BMRF1 gene products expressed in Sf9 cells was developed.

(i) Preparation of the nuclear extract. A monolayer culture of Sf9 cells was infected with the recombinant baculovirus AcBMRF1. At 72 h postinfection, the cells were harvested, suspended in hypotonic buffer [20 mM Tris-HCl (pH 7.4), 5 mM EDTA, 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 4 μg of leupeptin per ml, 4 μg of pepstatin A per ml] for 30 min at 0°C and then subjected to Dounce homogenization. The nuclei were suspended in buffer A (25% glycerol, 20 mM Tris-HCl [pH 7.4], 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 4 μg of leupeptin per ml, 4 μg of pepstatin A per ml) containing 0.5 M KCl and centrifuged. Almost all the BMRF1 gene products (52, 50, and 48 kDa) expressed in Sf9 cells were solubilized from the nuclei by the medium-salt treatment as judged by Western blot analysis with anti-BMRF1 protein-specific MAb 9240 (Fig. 2). The nuclear extract was dialyzed overnight against buffer A containing 0.1 M NaCl (fraction II).

(ii) dsDNA-cellulose column chromatography. Fraction II was loaded onto a dsDNA-cellulose column equilibrated with buffer A containing 0.1 M NaCl. The dsDNA-cellulose column was washed with buffer A containing 0.1 M NaCl and eluted with a 24-ml linear gradient from 0.1 to 1 M NaCl in buffer A. As shown in Fig. 3, almost all the other proteins in the nuclear extract flowed through the dsDNA-cellulose column. The recombinant BMRF1 gene products of 52, 50, and 48 kDa eluted at 0.24 M NaCl. Peak fractions were pooled (fraction III).

(iii) Heparin-agarose column chromatography. Fraction III was applied to a column of heparin-agarose equilibrated with buffer A containing 0.1 M NaCl. The column was washed and eluted with a 16-ml linear gradient from 0.1 to 0.8 M NaCl in buffer A. The BMRF1 proteins eluted at 0.36 M NaCl and were purified to near homogeneity (data not shown). The peak fractions were collected. The pooled

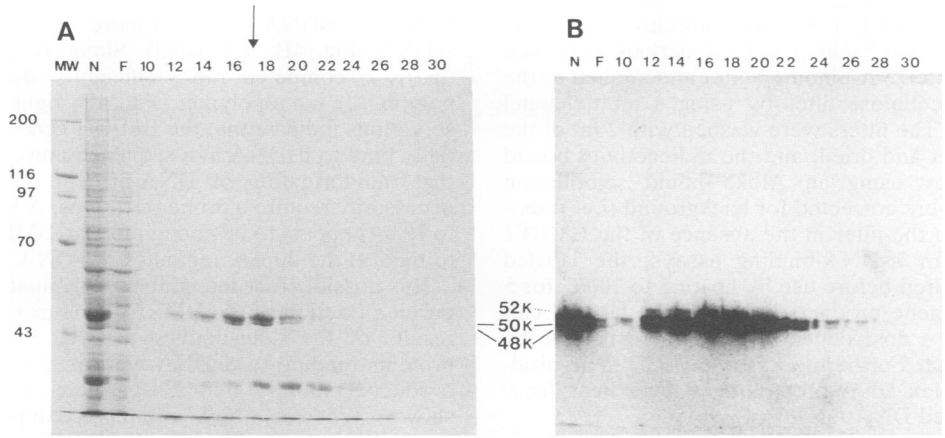

FIG. 3. dsDNA-cellulose column chromatography of the recombinant BMRF1 gene products. The nuclear extract of the AcBMRF1-infected Sf9 cells (fraction II) was dialyzed against buffer A containing 0.1 M NaCl and loaded onto a dsDNA-cellulose column (bed volume, 0.4 ml) equilibrated with buffer A containing 0.1 M NaCl. The dsDNA-cellulose column was washed with buffer A containing 0.1 M NaCl and eluted with a 24-ml linear gradient from 0.1 to 1.0 M NaCl in buffer A. Aliquots of the indicated fractions from the chromatography were subjected to SDS-PAGE (8% polyacrylamide) and stained with Coomassie blue (A) or analyzed by Western blot with anti-BMRF1 protein-specific MAb 9240 (B). The arrow denotes the peak position of the BMRF1 gene products. The lane labeled MW contains the molecular weight standards (numbers given in thousands); the lanes labeled N contain a nuclear extract of the AcBMRF1-infected Sf9 cells; the lanes labeled F contain the fraction that flowed through the dsDNA-cellulose column.

fraction (33 μg/ml) contained two major protein bands of 50 and 48 kDa and a minor band of 52 kDa.

The purification of the recombinant EBV BMRF1 gene products is summarized in Table 1. The yield was determined to represent 12% of the original quantity of the BMRF1 proteins produced in the infected insect cells. The peak fractions of the BMRF1 gene products were devoid of any detectable DNA polymerase or nuclease activity.

The recombinant BMRF1 gene products exhibit dsDNA-binding activity. It has been reported that pp58 and pp50 phosphoproteins expressed in the EBV-nonproducer cell line NC 37 bound dsDNA-cellulose column and eluted from the column by a buffer containing 0.3 M NaCl (24). These proteins react with MAb R3, which is identical to MAb 9240 (26). The BMRF1 gene products expressed in the AcB-MRF1-infected Sf9 cells consisted of two phosphoproteins of 52 and 50 kDa and an unphosphorylated protein of 48 kDa. All forms of the BMRF1 gene products bound the dsDNA-cellulose column and eluted at 0.24 M NaCl on a linear gradient from 0.1 to 1 M NaCl in buffer A (Fig. 3). This observation shows that the recombinant BMRF1 gene products, as well as the authentic proteins, exhibit dsDNA-binding activity.

To characterize the DNA-binding properties in detail, nitrocellulose filter-binding assays were performed by using the purified BMRF1 gene products (Fig. 4A). Formation of BMRF1 protein-DNA complexes was measured by using alkali-treated nitrocellulose filters (type HAWP; pore size, 0.45 μm; Millipore Corp.) as described previously (20). The filters were soaked in 0.5 M KOH for 20 min at room temperature, washed extensively with distilled water, washed with 100 mM Tris-HCl (pH 7.6), and stored in the same buffer at 4°C. Neither ssDNA nor duplex DNA is retained by the alkali-washed filters, but protein-DNA complexes are efficiently retained. The DNA substrate used was activated calf thymus DNA, which was labeled with [^{3}H]dGTP in the presence of dATP, dCTP, and dTTP with *E. coli* polymerase I large fragment. The standard assay mixture (50 μl) contained 0.1 μg of the labeled dsDNA (2.2×10^6 cpm/μg) and the indicated volumes of the purified BMRF1 gene products (33 μg/ml) or bovine serum albumin (BSA; 100 μg/ml) in DNA-binding buffer (20 mM Tris-HCl [pH 8.0], 5 mM $MgCl_2$, 20 mM NaCl, 1 mM dithiothreitol). The mixtures were incubated for 5 min at 30°C. Reactions were then diluted 10-fold in the DNA-binding buffer and applied to the alkali-treated nitrocellulose filter by using a multichannel filtration manifold. The filters were washed with 2 ml of the DNA-binding buffer and dried, and the radioactivity bound was determined by using an Aloka liquid scintillation counter. All data were corrected for background (i.e. radioactivity retained on the filter in the absence of the BMRF1 gene products). For ssDNA-binding assays, the labeled dsDNA was denatured before use by heating to 100°C for 5 min. The BMRF1 gene products bound to the labeled calf thymus dsDNA in a dose-dependent fashion, whereas the control BSA did not. Furthermore, the BMRF1 gene products bound to duplex DNA preferentially over heat-denatured single-stranded DNA (ssDNA).

The relative affinity of the BMRF1 gene products for a variety of nucleic acids was examined by a competitive filter-binding assay. A 25-ng portion of ^{3}H-labeled calf thymus dsDNA was mixed with 25, 50, 100, and 150 ng of the following unlabeled nucleic acids: linear λ dsDNA, circular M13mp18 ssDNA, poly(dC)$_{300}$ · oligo(dG)$_{12-18}$, and poly(dC)$_{300}$ (Fig. 4B). Then 4 μl of the purified BMRF1 gene products was added, and filter-binding assays were performed. λ dsDNA competed more efficiently than M13mp18 ssDNA (Fig. 4B, left panel). Similarly, poly(dC)$_{300}$ · oligo (dG)$_{12-18}$ competed more efficiently than single-stranded poly(dC)$_{300}$ homopolymer (Fig. 4B, right panel). These observations indicate that the BMRF1 gene products preferentially bind to dsDNA in a sequence-nonspecific manner and that the formation of DNA-protein complexes does not necessarily require a primer terminus. A length of at least 12 to 18 bp appears to be enough for the BMRF1 gene products to bind to the duplex region of the DNA.

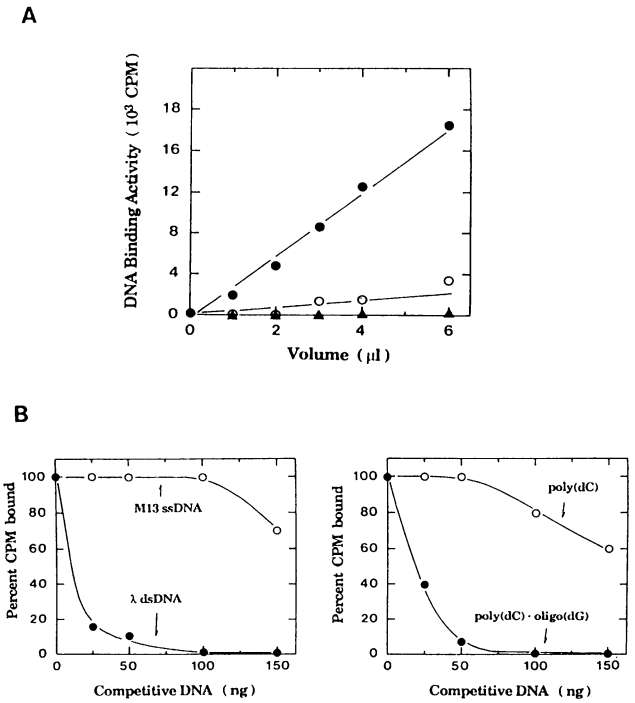

FIG. 4. (A) DNA-binding activity of the purified BMRF1 gene products. The BMRF1 gene products were tested for the ability to bind ^{3}H-labeled dsDNA (●) or ssDNA (○) in a nitrocellulose filter-binding assay. ▲, BSA (100 μg/ml) control. Points represent the average of triplicate filter-binding assays. (B) Competitive filter-binding assay. Filter-binding assays were performed in the presence of increasing amounts of unlabeled competing nucleic acids. The left panel shows linear λ dsDNA (●) and circular M13mp18 ssDNA (○); the right panel shows poly(dC)$_{300}$ · oligo(dG)$_{12-18}$ (●) and poly (dC)$_{300}$ (○). The standard assay mixture (50 μl) contained 4 μl of the purified BMRF1 gene products (33 μg/ml) and 25 ng of labeled calf thymus dsDNA (2.2×10^6 cpm/μg) and the indicated amounts of unlabeled competing nucleic acids. Points represent the average of duplicate assays.

It is possible that the minor contaminating proteins in the purified fraction of the BMRF1 gene products influence the results of the filter-binding assays. To confirm that the proteins binding to dsDNA were in fact the BMRF1 gene products, mobility shift assays were carried out (data not shown). When a 48-bp 5′-end-labeled duplex DNA fragment was mixed with the purified BMRF1 gene products, the labeled DNA displayed reduced mobility. The addition of anti-BMRF1 protein-specific MAb 10 min later produced a new complex of reduced mobility, whereas MAb 9240 alone did not alter the mobility of the DNA. These observations confirmed that the dsDNA binding is an inherent property of the protein and does not require a primer terminus region.

The other properties of the DNA-binding activity are

TABLE 2. Characteristics of the DNA-binding activity of the BMRF1 gene products

Change in reaction conditions	DNA binding (% of maximum)
None (complete)[a]	100
Omit Mg^{2+}	96
Omit DTT plus *N*-ethymaleimide (5 mM)	102
Preincubate at 42°C for 15 min	8
Add NaCl (200 mM)	18
Add NaCl (300 mM)	4
Add ATP (2 mM)	94
Add GTP (2 mM)	104

[a] Reaction conditions are given in the text. The standard assay mixture (50 μl) contained 4 μl of the purified BMRF1 gene products (33 μg/ml) and 100 ng of labeled calf thymus dsDNA (2.2×10^6 cpm/μg). Values represent the average of triplicate filter-binding assays.

summarized in Table 2. The dsDNA-binding activity of the BMRF1 gene products is maximal at pH 8.0. Mg^{2+} was not essential for the activity. At 5 mM, the thiol reagent *N*-ethylmaleimide had almost no inhibitory effect on the DNA-binding activity. However, the proteins incubated at 42°C for 15 min displayed no observed binding activity. The binding activity was significantly sensitive to changes in the salt concentrations. NaCl concentrations of over 200 mM inhibited the formation of DNA-BMRF1 protein complexes. The addition of 2 mM ATP did not stimulate the formation of the DNA-protein complex, unlike the situation for the T4 DNA polymerase accessory protein gene 44/62 and gene 45 (14) or DNA polymerase δ accessory protein RF-C and proliferating cell nuclear antigen (27).

The BMRF1 gene products exhibit no DNA-dependent ATPase activity. It is known that T4 gene 44/62 proteins (T4 DNA polymerase accessory proteins) or RF-C (eukaryotic DNA polymerase δ accessory proteins) exhibit DNA-dependent ATPase activities for their specific primer terminus binding (14, 27). To test whether the EBV DNA polymerase accessory proteins, the BMRF1 gene products, also exhibit the activity, the peak fractions of the final chromatography of the heparin-agarose column were assayed for DNA-dependent ATPase activity. It was found that the BMRF1 gene products did not exhibit any detectable ATP hydrolysis (data not shown).

Discussion and conclusions. To induce EA-D proteins (BMRF1 proteins) in EBV-transformed B cells, the cells are usually treated with chemical agents such as tetradecanoyl phorbol acetate and sodium *n*-butyrate or superinfected with cell-free EBV. However, the induction procedure is subject to irreproducibility, and induction efficiency is usually low. The expression of the BMRF1 gene products in the baculovirus system can be controlled by adjusting for the multiplicity of infection and is more reproducible. Alternatively, in the chemically induced EBV-transformed B-cell lines, high levels of alkaline exonuclease activity are induced. To avoid contamination of the nuclease activity, more purification steps are needed for the BMRF1 protein purification from the EBV-transformed B-cell lines (32). In contrast, in the AcBMRF1-infected SF9 cells there was little nuclease activity and the purified BMRF1 protein fraction was free from any detectable nuclease activity. Thus, the expression and purification systems described here will be very useful in attempts to understand the molecular basis of protein-protein interactions between the EBV DNA polymerase catalytic subunit and polymerase accessory protein during DNA replication and structure-function relationships with its substrates.

Although the estimated size of the BMRF1 gene product from the open reading frame of the EBV genome is 43 kDa (1), the molecular mass of the BMRF1 gene product expressed in the baculovirus expression system is 48 to 52 kDa. The 48-kDa unphosphorylated form of the BMRF1 gene product expressed in the baculovirus system is in close agreement with 46 to 47 kDa, the reported size of the BMRF1 protein in the reticulocyte lysate in vitro translation system (16, 22). Therefore, the 48-kDa protein appears to be a primary form of the BMRF1 gene product. The EBV BMRF1 gene products of 52 and 50 kDa were phosphorylated in the recombinant baculovirus-infected insect cells. This observation suggests that the BMRF1 gene product may be phosphorylated by host cell-derived protein kinases and not by the EBV-encoded protein kinase, the BGLF4 protein (25).

So far, several authors have described the EBV BMRF1 gene products in EBV-transformed B-cell lines. Pearson et al. (22) reported that MAb R3, which is identical to the anti-BMRF1 protein-specific MAb 9240 used in this study, recognized 50- and 52-kDa polypeptides, which were phosphorylated in vivo. Roeckel and Müller-Lantzsch (24) also reported that MAb R3 recognized two phosphoproteins of 58 and 50 kDa and that the 58-kDa protein contained phosphoserine and phosphothreonine whereas the 50-kDa protein contained only phosphoserine. In contrast, Epstein (6) identified the 55-kDa phosphoprotein and the 50-kDa unphosphorylated protein as EBV EA-D proteins. Moreover, Cho et al. (4) observed that 48- and 50-kDa proteins were dominant in the BMRF1 gene products expressed in B95-8 and Raji cells, corresponding to my results obtained in the baculovirus expression system. In light of these studies and the results obtained here, it seems reasonable to assume that the BMRF1 gene products exist in vivo as at least two phosphorylated forms and one unphosphorylated form and that the relative amount of each protein may depend on the degree of phosphorylation, which varies with the cell types and induction conditions. It will be very interesting to find whether the function of the BMRF1 protein is regulated by phosphorylation. Further studies are needed.

Previously I have reported that the purified EBV DNA polymerase fraction contained 50- and 48-kDa polypeptides in addition to the 110-kDa polymerase catalytic subunit. Anti-BMRF1 protein-specific MAb 9240 recognized these 50- and 48-kDa polypeptides (32). Also, Li et al. have reported that the BMRF1 proteins are associated with the EBV DNA polymerase through the course of the purification (18). Furthermore, the purified EBV DNA polymerase from P3HR-1 cell extracts has an apparent molecular mass of 185 kDa by gel filtration (15), and the BMRF1 proteins can stimulate the EBV DNA polymerase activity (3, 16). These observations suggest that the BMRF1 gene product acts as the EBV DNA polymerase accessory protein, although more detailed biophysical and biochemical studies are needed to clarify the relationship of the EBV DNA polymerase catalytic subunit and the BMRF1 gene products.

Most replicative polymerases consist of a polymerase catalytic subunit and polymerase accessory proteins. Bacteriophage T4 polymerase accessory proteins, gene 44/62 proteins, function as a DNA-dependent ATPase and primer terminus recognition protein complex (14). The primer terminus-binding activity is stimulated by gene 45 protein, and together the gene 44/62 and gene 45 proteins cooperate in a complex to stimulate the processivity of the gene 43 protein,

the polymerase catalytic subunit, to forming a polymerase holoenzyme. Also, the eukaryotic replication factors RF-C and PCNA (accessory proteins to DNA polymerase δ) have been demonstrated to be strikingly similar to proteins encoded by T4 gene 44/62 and gene 45, respectively (13, 27). RF-C exhibits DNA-dependent ATPase activity and functions as a primer terminus recognition protein. It cooperates with PCNA to stimulate processive DNA synthesis by DNA polymerase δ. The DNA-binding activity of RF-C is stimulated by the addition of ATP. It binds specifically to the primer terminus junction (28) but not to ssDNA or dsDNA (27). On the other hand, bacteriophage T7 DNA polymerase complex differs from these systems in its simplicity. T7 DNA polymerase has only one accessory protein, *E. coli* thioredoxin, which can stimulate the polymerase activity but neither binds to DNA by itself nor possesses DNA-dependent ATPase activity (12). Herpes simplex virus type 1 (HSV-1) DNA polymerase complex consists of a polymerase catalytic subunit (UL30 protein) and polymerase accessory protein (UL42 protein) with a stoichiometry of 1:1 (5, 9) and exhibits highly processive DNA replication (21). Challberg and coworkers have recently reported on the primer terminus-binding site of the HSV-1 DNA polymerase complex (2, 8, 9). The polymerase catalytic subunit is centered on the 3′ terminus of the primer (15 bp of the primer template duplex region and an adjacent 18 bases of single-stranded template). The UL42 protein binds a short contiguous region of the primer terminus duplex. Free UL42 protein binds nonspecifically to the duplex region of the substrate but does not bind to the single-stranded region (2). It does not exhibit any DNA-dependent ATPase activity (7) but functions to increase the processivity of the UL30 polymerase catalytic polypeptide (9, 11). Although EBV BMRF1 protein possesses no significant amino acid sequence homology to HSV UL42 protein (7), the DNA-binding property of the EBV BMRF1 protein obtained in this study was in good agreement with that of HSV UL42 protein. EBV DNA polymerase accessory protein makes a tight complex with DNA polymerase catalytic subunit and binds nonspecifically to dsDNA without ATP hydrolysis. Although the relationship between the dsDNA-binding activity and the function of the BMRF1 protein on EBV DNA replication is not known with certainty, it is reasonable to speculate that the BMRF1 protein may increase the affinity of the polymerase for the primer terminus by its dsDNA-binding activity and that it may act as a "clamp" to decrease the dissociation of the polymerase from the template DNA.

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