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Identification and Functional Characterization of Epstein-Barr Virus DNA Polymerase by In Vitro Transcription-Translation of a Cloned Gene

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In order to identify the gene encoding the Epstein-Barr virus (EBV) DNA polymerase, a portion of the *Bam*HI-A fragment containing the fifth leftward open reading frame (BALF5) of the EBV genome was cloned into SP6 and T7 promoter-containing vectors for in vitro transcription-translation. The RNA synthesized in vitro was used to program rabbit reticulocyte lysates, which were analyzed for the synthesis of the putative polymerase polypeptide (110 kDa) and assayed directly for EBV DNA polymerase activity. The polypeptide synthesized by the full-length BALF5 genomic fragment had a molecular mass of 110 kDa. 5′-truncated BALF5 with the first and second ATGs deleted produced 95- and 83-kDa polypeptides, respectively. All three translation products were enzymatically active and displayed resistance to high salt concentrations. The identity of the largest polypeptide as the viral polymerase was established by (i) immunoprecipitation with EBV-positive sera from patients with nasopharyngeal carcinoma and by a rabbit polyclonal antiserum prepared with a synthetic peptide derived from the DNA sequence of BALF5; (ii) identification of a polypeptide of identical size (110 kDa) immunoprecipitated from superinfected Raji cell extracts by these antibodies; and (iii) salt-resistant enzymatic activity which was neutralized by the rabbit EBV antiserum. Thus, BALF5 encodes a functional polymerase identical to that induced in superinfected Raji cells.

We have had a long-standing interest in Epstein-Barr virus (EBV) DNA polymerase because of the interaction between this enzyme and antiviral drugs (2, 5, 10–14). Since EBV DNA polymerase is essential for viral replication, the study of this enzyme should provide information useful for the rational design of drugs for antiviral chemotherapy and a deeper understanding of the control mechanism of EBV replication.

The EBV DNA polymerase has been tentatively assigned to the fifth leftward open reading frame (ORF) of the *Bam*HI-A fragment (BALF5) of the EBV genome. This determination is based on the sequence homology between BALF5 and the coding sequences for the herpes simplex virus type 1 (HSV-1) and human cytomegalovirus DNA polymerase genes (8, 16) as well as the close match between the predicted amino acid composition of the BALF5-encoded protein (1) and the amino acid composition of partially purified EBV DNA polymerase (7). In this article, we report the identification of the EBV DNA polymerase gene and functional characterization of its encoded enzyme by in vitro transcription and translation of the cloned gene with the use of an antiserum specific for EBV DNA polymerase.

BALF5 lying within the EBV *Eco*RI-C fragment was isolated by sequential digestion with *Bgl*II and *Eco*RI, followed by digestion with *Sph*I to generate a 3.9-kbp fragment containing the entire BALF5 ORF. The *Sph*I site is 380 bp upstream of BALF5. The *Sph*I fragment containing BALF5 and a series of 5′-truncated BALF5 fragments were cloned into pGEM3Z or pGEM2 with the *pol* gene downstream from the SP6 or T7 promoter (Fig. 1). Transcription of linearized pGEM3Z or pGEM2 yielded uncapped polymerase (*pol*) runoff transcripts which were used for in vitro translation.

The major translation product of the full-length *pol* gene from pGEM3Z-POL1 and pGEM3Z-POL2 constructs, which contain three ATGs, migrated as a 110-kDa polypeptide (Fig. 2B, lane 2 and 5). This size corresponds to that of the predicted product of the *pol* ORF (1) and the partially purified EBV *pol* from an EBV-producing cell line (7; unpublished data). RNA from pGEM3Z-POL2 was translated consistently about threefold more efficiently than RNA from pGEM3Z-POL1. The products of the 5′-truncated constructs pGEM2-POL3 (Fig. 2B, lane 3) and pGEM3Z-POL4 (lane 4) could be distinguished from the full-length *pol* gene product. Deletion of the first ATG in the pGEM2-POL3 construct resulted in a translation product of 95 kDa (lane 3); with the pGEM3Z-POL4 construct, which has the first two ATGs deleted, the translation product was 83 kDa (lane 4). These translation products (110, 95, and 83 kDa) were detected only with the sense transcripts and not with either the antisense or the no-RNA control.

To determine whether the EBV DNA polymerase translated in vitro could be recognized by EBV-positive serum, serum from a patient with nasopharyngeal carcinoma (NPC) with a high titer of early antigen/viral capsid antigen antibodies was used to immunoprecipitate the translation products. Figure 2A clearly shows that this antiserum specifically recognized polymerase polypeptides of the expected sizes (110 kDa for both pGEM3Z-POL1 and pGEM3Z-POL2 and 95 and 83 kDa for pGEM2-POL3 and pGEM3Z-POL4, respectively). EBV-negative serum was unable to precipitate these polymerase polypeptides (data not shown).

To ascertain whether the in vitro-translated product represents the authentic polymerase polypeptide, we analyzed immunoprecipitates of radiolabeled polymerase from super-

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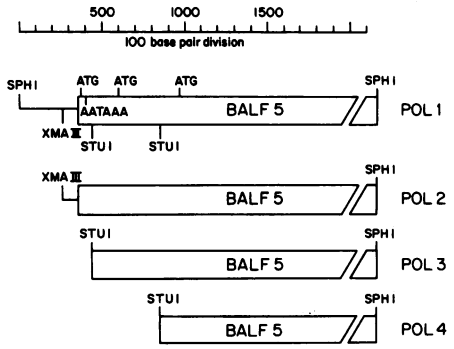

FIG. 1. Construction of four EBV polymerase templates for in vitro transcription-translation. The *Sph*I fragment (3.9 kbp) containing the entire BALF5 ORF was isolated from the EBV *Eco*RI-C fragment by sequential digestion with appropriate restriction enzymes. The 5'-truncated BALF5 fragments were isolated and cloned into pGEM2 or pGEM3Z. The clones were designated pGEM3Z-POL1, pGEM3Z-POL2, pGEM2-POL3, and pGEM3Z-POL4.

infected Raji cells in parallel with the in vitro-translated polymerase with serum from an NPC patient. Figure 3 shows that the human serum precipitated a spectrum of EBV-specific polypeptides from superinfected Raji cells (panel II, lane 2). Among these, a 110-kDa polypeptide comigrated with the in vitro-translated polymerase (lane 3); this polypeptide was not detected in mock-infected Raji cells (lane 1). To further confirm the identity of the 110-kDa polypeptide, a rabbit antibody prepared by immunization with a synthetic peptide derived from the DNA sequence of BALF5 was used

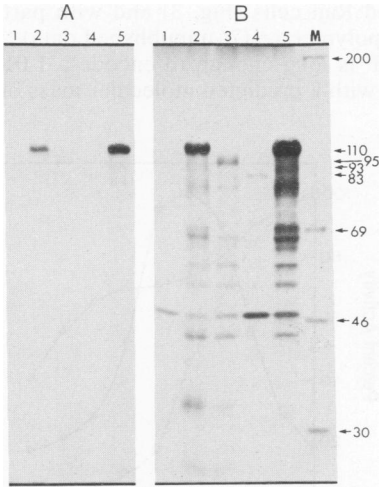

FIG. 2. Expression of EBV DNA polymerase by in vitro transcription-translation. The templates were linearized at the *Hin*dIII or *Eco*RI site, depending on the construct. Transcripts were made by in vitro transcription of the templates with either SP6 or T7 RNA polymerase. The *pol* RNA was translated in rabbit reticulocyte lysates containing [^{35}S]methionine, and the translated products were analyzed by electrophoresis and fluorography. (A) Immunoprecipitation of polymerase polypeptides by EBV-positive serum from an NPC patient. Lane 1, Reticulocyte lysate with no RNA; lane 2, pGEM3Z-POL1; lane 3, pGEM2-POL3; lane 4, pGEM3Z-POL4; lane 5, pGEM3Z-POL2. (B) In vitro-translated products. Lane 1, reticulocyte lysate with no RNA; lane 2, pGEM3Z-POL1; lane 3, pGEM2-POL3; lane 4, pGEM3Z-POL4; lane 5, pGEM3Z-POL2. Lane M, Molecular size markers (in kilodaltons).

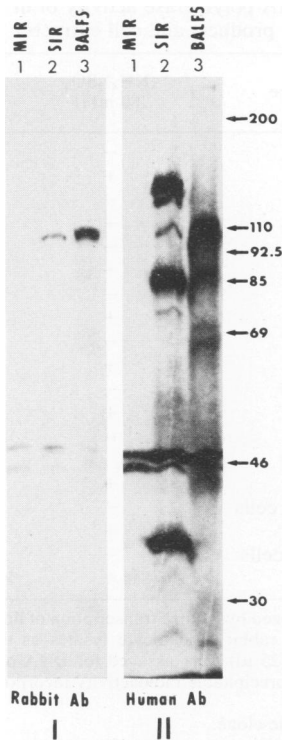

FIG. 3. Determination of authenticity of polymerase polypeptide made in vitro by immunoprecipitation. (I) Immunoprecipitation with a rabbit antibody (Ab) against synthetic peptide derived from the DNA sequence of BALF5. Lane 1, mock-infected Raji (MIR) cells; lane 2, superinfected Raji (SIR) cells; lane 3, in vitro-translated product of pGEM3Z-POL2. (II) Same samples as in panel I were immunoprecipitated with serum from an NPC patient. Sizes are marked in kilodaltons.

for immunoprecipitation. This rabbit antibody specifically precipitated a 110-kDa polypeptide from both the in vitro-translated product (Fig. 3, panel I, lane 3) and superinfected Raji cells (lane 1). These results clearly indicate that the EBV polymerase from the Raji cells contains the first ATG of the ORF and the sequence of amino acids downstream of it.

To ascertain whether the polymerase protein translated in vitro possessed enzymatic activity, translation mixtures from transcripts made from these constructs were assayed directly for EBV DNA polymerase activity (3). The EBV DNA polymerase from superinfected Raji cells was most active in the presence of 80 mM ammonium sulfate; therefore, the translation products were assayed in 80 mM salt. All the *pol* constructs produced functional polymerase with enzymatic activity detectable above the background activity found in the reticulocyte lysate (Table 1), although the activity was not stimulated by salt. However, the salt-resistant polymerase activity was highest in pGEM3Z-POL2, approximately 13-fold above that in the reticulocyte lysate control. The enzymatic activities in pGEM3Z-POL1, pGEM2-POL3, and pGEM3Z-POL4 were about 6-, 2.2-, and 1.8-fold above the control level, respectively. A pT7-7.2 construct, containing the HSV-1 *pol* gene (3), used as a positive control, exhibited a property similar to EBV *pol* in terms of salt-resistant but not salt-stimulated polymerase activity.

To compare the authentic EBV DNA polymerase made in vivo, superinfected Raji cell extracts were assayed under the

TABLE 1. DNA polymerase activity of in vitro-translated products and cell extracts[a]

Polypeptide source	$(NH_4)_2SO_4$ (80 mM)	Polymerase activity (cpm of ^{32}P/100 μg of protein) ± SD
Reticulocyte lysate	−	1,400 ± 250
	+	1,250 ± 230
pGEM2-POL1	−	9,440 ± 480
	+	7,950 ± 370
pGEM3Z-POL2	−	20,450 ± 1,130
	+	16,890 ± 890
pGEM2-POL3	−	2,950 ± 390
	+	2,760 ± 410
pGEM2-POL4	−	2,680 ± 310
	+	2,220 ± 280
pT7-7.2[b] (HSV *pol*)	−	29,270 ± 880
	+	23,660 ± 760
pGEM3Z-POL2 (antisense)	−	1,680 ± 340
	+	1,450 ± 290
Mock-infected Raji cells	−	394,800 ± 60,120
	+	212,750 ± 28,930
Superinfected Raji cells	−	55,520 ± 7,930
	+	301,750 ± 12,450

[a] RNA was synthesized by in vitro transcription of linearized templates and translated in vitro in rabbit reticulocyte lysates as described in the text. Translation mixtures (25 μl) were assayed for DNA polymerase activity by measurement of acid-precipitable radioactivity incorporated by using [α-^{32}P] dCTP.

[b] An HSV-1 *pol* gene clone.

same conditions. The polymerase activity, when assayed in 80 mM ammonium sulfate in superinfected Raji cells, was approximately 18-fold higher than that of the in vitro-translated product of pGEM3Z-POL2. The EBV polymerase activity in superinfected Raji cells was stimulated approximately sixfold by salt, in contrast to the cellular polymerase in mock-infected Raji cells as well as in the reticulocyte lysate, both of which were inhibited by salt.

The effect of ammonium sulfate on the activity of in vitro-translated EBV *pol* was determined and compared with the effect on the enzymes from superinfected and mock-infected Raji cells. With increasing salt concentration, the *pol* activity from the in vitro-translated product decreased gradually, with a lag between 80 and 100 mM (Fig. 4). With further increases in salt concentration up to 180 mM, approximately 40% of *pol* activity was retained. In contrast, the *pol* activity from superinfected Raji cells showed optimal salt stimulation at 80 mM salt; at this concentration the *pol* activity from mock-infected Raji cells was inhibited approximately 55%. Thus, it appeared that 80 mM salt was optimal for measuring the salt-resistant *pol* activity from the in vitro-translated product and the salt-stimulated *pol* activity from superinfected Raji cells.

The inability to demonstrate salt-stimulated polymerase activity of in vitro-translation product may indicate that BALF5 encodes only a core polymerase which requires an additional factor for salt stimulation. EBV early antigen diffuse component (EA-D) has been implicated as a putative cofactor (9). Since EA-D is encoded by the BMRF1 ORF of the EBV genome, BMRF1 was cloned into the pGEM3Z vector for in vitro transcription and translation. When the cold translation product of BMRF1 was included in the DNA polymerase assay, we were repeatedly unable to demonstrate salt-stimulated polymerase activity in the presence of EA-D; in several cases, the polymerase activity was decreased by the addition of EA-D (data not shown).

TABLE 2. Effect of antiserum on EBV DNA polymerase activity[a]

Polypeptide source	Polymerase activity (cpm of ^{32}P/100 μg of protein) ± SD		% Inhibition
	Without antiserum	With antiserum	
Reticulocyte lysate	1,740 ± 130	1,590 ± 120	9
pGEM3Z-POL2	19,940 ± 960	2,990 ± 340	85
Mock-infected Raji cells	167,450 ± 14,250	150,940 ± 17,620	10
Superinfected Raji cells	275,600 ± 30,310	50,632 ± 5,450	82

[a] Polymerase activities were assayed in the presence of 80 mM $(NH_4)_2SO_4$. Prior to assay, the enzyme was incubated with 5 μl of rabbit antiserum to polymerase peptide at room temperature for 30 min. The polymerase activity was then determined as described in the text.

To test whether the rabbit antibody against the synthetic peptide can neutralize the polymerase activity, active protein extracts were first incubated with antiserum and then assayed for polymerization of DNA. The polymerase activity from superinfected Raji cells and from the cloned gene product (pGEM3Z-POL2) was inhibited by more than 80%, in contrast to approximately 10% inhibition observed in mock-infected Raji cells and reticulocyte lysate (Table 2).

In this study, transcripts made from the BALF5 fragment (pGEM3Z-POL1) containing the three in-frame ATG initiation codons were translated efficiently into a full-length polymerase polypeptide (110 kDa). Transcripts made from 5′-truncated templates with deletion of either the first or the first two ATGs yielded smaller polypeptides (95 and 83 kDa) (Fig. 2). It appears that the translation of EBV polymerase in vivo initiates at the first in-frame ATG, located 12 bp from the start of BALF5, since a polypeptide (110 kDa) of identical size and activity was found both in vitro and in superinfected Raji cells (Fig. 3) and with partially purified EBV DNA polymerase (7; unpublished data). The 3,045-bp BALF5 ORF is long enough to encode a 1,015-amino-acid polypeptide with a predicted molecular mass of 111,447 Da.

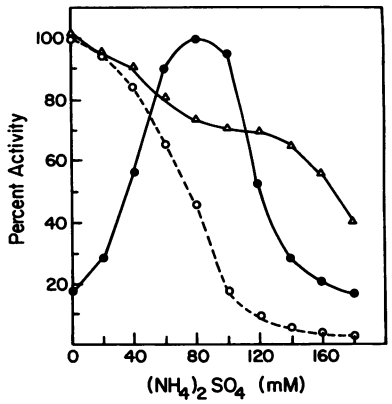

FIG. 4. Effect of ammonium sulfate on DNA polymerase activity. Cell extracts prepared from superinfected (●) and mock-infected (○) Raji cells and the in vitro translation product of pGEM3Z-POL2 (△) were assayed for DNA polymerase activity at increasing concentrations of $(NH_4)_2SO_4$. The 100% enzymatic activity for mock-infected Raji cells and the in vitro translation product was determined based on the activity obtained in the absence of $(NH_4)_2SO_4$, after substraction of the background, whereas the 100% activity for superinfected Raji cell extract was determined based on the concentration of ammonium sulfate (80 mM) at which enzymatic activity was the highest.

Other direct evidence that the full-length BALF5 ORF is the coding sequence for the EBV DNA polymerase is provided by our preliminary RNA analyses with S1 nuclease mapping with primers covering different regions of the genomic sequence. The results indicated that the protected fragments all extended beyond the first ATG (6).

While the in vitro-translated polymerase activity was not stimulated by salt in contrast to that of superinfected Raji cell extracts, the in vitro-translated product exhibited considerably higher salt-resistant polymerase activity than that of the endogenous reticulocyte activity. The presence of the 110-kDa polypeptide and the demonstration of polymerase activity in the in vitro-translated product strongly suggest that the *pol* enzymatic activity resides in the 110-kDa polypeptide and no additional viral gene products, such as BMRF1 (EA-D), are necessary to obtain core polymerase activity in vitro. However, we have not excluded the possibility that BMRF1 or other gene products act as cofactors that might enhance the polymerase activity in vivo. The lack of salt-stimulated polymerase activity could be due to the inherent nature of the in vitro-translated product, similar to what was found with the cloned gene of HSV-1 *pol* (3). Alternatively, the stimulatory effect of salt on the core polymerase activity may require a modified cofactor such as the BMRF1 gene product, the effect of which we were not able to demonstrate in vitro.

Evidence for the authenticity of the polymerase polypeptide synthesized in vitro was provided by immunoprecipitation assays and comparison with activity from EBV-infected cells. Antisera from patients with NPC or prepared against a synthetic peptide derived from the DNA sequence of BALF5 precipitated a 110-kDa polypeptide only from superinfected Raji cells, which comigrated with the polypeptide precipitated from the in vitro-translated product. Furthermore, the polymerase enzymatic activities from both sources were inhibited to the same extent by antiserum. Thus, BALF5 encodes a functional polymerase identical to that induced in superinfected Raji cells.

The development of a system in which a functional EBV DNA polymerase can be characterized will help provide a detailed understanding of the mechanism of viral DNA replication and its inhibition by antiviral drugs. With this system, the effect of gene deletions on enzyme activity can be studied (4, 15, 17), and the functional domains within the polymerase can be identified and mapped. The present study is the first step towards these goals.

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