Primer terminus recognition and highly processive replication by Epstein–Barr virus DNA polymerase

Tatsuya TSURUMI

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

The Epstein–Barr virus (EBV) DNA polymerase is essential for viral DNA replication in the lytic phase of the EBV life cycle. It efficiently extends RNA primers on the template DNA, suggesting the possible involvement of the EBV DNA polymerase in synthesizing Okazaki fragments from RNA primers on the lagging strand template. Competition experiments revealed that the EBV DNA polymerase had significantly higher affinity for primer termini hybridized to the template DNA than for the single-stranded DNA template or the single-stranded primer itself. ATP was not required either for primer terminus recognition or for sustainment of polymerization. The stimulation of the enzyme by $(\text{NH}_4)_2\text{SO}_4$ was dependent on the template/primers utilized. These observations suggest that the primary and secondary structure of the template/primers are important factors for primer terminus recognition by the EBV DNA polymerase. The enzyme elongated synthetic RNA primer annealed to circular single-stranded M13 DNA coated with *Escherichia coli* single-stranded DNA-binding protein without dissociation. The processivity of the EBV DNA polymerase was strikingly high (> 7200 nucleotides) and the rate of polymerization was 12 nucleotides/s per polymerase molecule. The high processing capacity is a desirable feature in the synthesis of multiple copies of the EBV genome in rolling-circle DNA replication.

INTRODUCTION

The genome of Epstein–Barr virus (EBV), a human lymphotropic herpes virus, consists of a linear duplex DNA which is 172 kb in length and encodes approx. 84 proteins [1]. EBV DNA polymerase is coded by the BALF5 open reading frame of the EBV genome, and the predicted molecular mass from the open reading frame is 113 kDa. During the latent phase of the viral life cycle the EBV genome is maintained as a circular plasmid molecule identical to the template DNA by host cell DNA polymerases [2]. However, after induction of the lytic phase of viral replication, the EBV genome is amplified 100–1000 times. The replication product is a head-to-tail concatemer, which is synthesized by the EBV DNA polymerase, presumably via a rolling-circle mechanism initiated from *oriLyt* [3].

EBV DNA polymerase has been partially purified and characterized in several laboratories [4–11]. The enzyme activity is stimulated by salt and is sensitive to aphidicolin, phosphonoacetic acid and *N*-ethylmaleimide. More recently, EBV DNA polymerase was shown to have a putative proofreading 3'-to-5' exonuclease activity [11]. These enzymic properties are common to DNA polymerases in the herpes virus family. However, further enzymic characterization of the EBV DNA polymerase, especially primer terminus recognition and processivity, is necessary for understanding the polymerization process of rolling-circle EBV DNA replication. First, the present study was undertaken to reveal whether EBV DNA polymerase has the capacity to recognize specific template primers as initiation signals for DNA replication. My approach was to test the EBV DNA polymerase for synthesis with various combinations of homopolymer templates and primers. Secondly, the processivity (i.e. the average number of nucleotides incorporated with each polymerase–template binding event) of the EBV DNA polymerase was determined by using singly primed M13 single-stranded (ss)DNA as a natural template primer. Grossberger & Clough [6] have previously reported, in contrast to the results reported here, that the processivity of EBV DNA polymerase is

about 10 nucleotides, which was lower than that of DNA polymerase α or *Escherichia coli* Pol I. Such quasi-processive DNA replication requires recycling of DNA polymerase molecules to the primer termini for elongation, making it inherently less rapid and efficient than highly processive DNA synthesis. This seems unlikely to explain the behaviour in a processive manner of rolling-circle EBV DNA replication, and therefore the dynamics of this process on ssDNA templates were examined.

MATERIALS AND METHODS

Materials

$[\alpha\text{-}^{32}\text{P}]\text{dCTP}$ (3000 Ci/mmol), $[\text{H}]\text{TTP}$ (50 Ci/mmol), $[1',2'\text{-}^3\text{H}]\text{dGTP}$ (50 Ci/mmol), $[1',2',2,8\text{-}^3\text{H}]\text{dATP}$ (100 Ci/mmol), and unlabelled deoxynucleoside 5'-triphosphates were from Amersham Corp. Unlabelled 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)₆₀₀₀ was from the Midland Certified Reagent Co. Activated calf thymus DNA [12] and single-stranded M13 mp18 DNA were prepared as described [13]. *E. coli* single-stranded DNA-binding protein (SSB) was from Stratagene Inc.

Template/primer preparation

The oligoribonucleotide CACAATTCACACAAC, complementary to nucleotides 6170–1685 of M13 mp18 single-stranded DNA [14], was synthesized on an Applied Biosystems instrument. The primer sequence was selected to minimize non-specific annealing to other regions of M13 ssDNA. The oligodeoxynucleotide CACAATTCACACAAC, corresponding to the RNA primer sequence, was purchased from New England Biolabs, Inc. To form singly RNA-primed DNA, a synthetic 16-mer RNA was annealed at a molar ratio of 20:1 to M13 mp18 ssDNA in buffer A (20 mM-Tris/HCl, pH 8.0, 5 mM-MgCl₂ and 0.3 M-NaCl). The hybridization mixture was incubated at 90 °C for 5 min, allowed to cool to room temperature over 1 h, and incubated for a further 1 h at 30 °C. Excess primer was removed

Abbreviations used: DTT, dithiothreitol; EBV, Epstein–Barr virus; SSB, *Escherichia coli* single-stranded DNA-binding protein; PMA, phorbol 12-myristate 13-acetate; RF II, circular duplex DNA with a small gap or nick in one strand.

Table 1. Template/primer preference of EBV DNA polymerase

All DNA polymerase assays contained 50 mM-Tris/HCl, pH 8.0, 6 mM-MgCl₂, 1 mM-DTT, 100 µg of BSA/ml, 10 % glycerol, 40 µM-[³H]dNTP complementary to the template, and the indicated template/primer. Reactions were performed for 20 min at 35 °C in the presence or absence of 80 mM-(NH₄)₂SO₄. All templates and primers were present at 80 µg/ml and 40 µg/ml respectively. The template/primer concentration in reactions with activated DNA was 80 µg/ml. ATP (0.5 mM) was added to the reaction or not, as indicated. Results are the means ± S.E.M. (*n* = 3).

Template/primer	(NH ₄) ₂ SO ₄	ATP	[³ H]dNMP incorporated	
			(pmol)	(%)
Activated DNA	—	—	7.3 ± 0.8	100
	—	+	7.5 ± 0.6	102
	+	—	16.9 ± 2.2	232
	+	+	16.6 ± 2.8	227
Poly(dT)·oligo(dA)	—	—	2.7 ± 0.03	37
	+	—	0.7 ± 0.06	9
Poly(dT)·oligo(rA)	—	—	48.7 ± 4.2	667
	—	+	46.5 ± 5.2	637
	+	—	78.7 ± 5.3	1078
	+	+	80.4 ± 5.9	1101
Poly(dC)·oligo(dG)	—	—	213.8 ± 10.7	2918
	—	+	205.4 ± 11.5	2814
	+	—	7.9 ± 1.0	108
	+	+	6.5 ± 0.8	89
Poly(dA)·oligo(dT)	—	—	54.2 ± 4.8	742
	—	+	56.7 ± 4.7	776
	+	—	0.5 ± 0.08	7
	+	+	0.6 ± 0.1	8
Poly(rA)·oligo(dT)	—	—	0.2 ± 0.05	2
	+	—	0.1 ± 0.04	1
Poly(rU)·oligo(dA)	—	—	0.1 ± 0.05	1
	+	—	0.1 ± 0.06	1
Poly(rU)·oligo(rA)	—	—	0.25 ± 0.04	3
	+	—	0.1 ± 0.02	1
Poly(dT)	—	—	0.1 ± 0.02	1
	+	—	0.1 ± 0.04	1
Poly(dC)	—	—	0.1 ± 0.01	1
	+	—	0.1 ± 0.01	1
Oligo(dT)	—	—	0.1 ± 0.02	1
	+	—	0.1 ± 0.01	1

by gel filtration through a Bio-Gel A1.5m column equilibrated with a solution containing 10 mM-Tris/HCl, pH 7.5, 1 mM-EDTA and 50 mM-NaCl.

Template/primers used in the template/primer preference and competition experiments were prepared as follows. Oligonucleotide was hybridized at a ratio of 1:2 (w/w) to the complementary polynucleotide in buffer A 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.

Enzyme preparation

EBV DNA polymerase was purified from B95-8 cells, which is a virus-producing marmoset lymphoblastoid cell line immortalized with human EBV from a mononucleosis patient, and treated with phorbol 12-myristate 13-acetate (PMA) and sodium *n*-butyrate as described in a previous report [11]. The purified DNA polymerase was sensitive to phosphonoacetic acid, *N*-ethylmaleimide and aphidicolin, and had template primer preference for poly(dC)·oligo(dG). The activity was stimulated by salt. These enzymic properties were specific for the EBV DNA polymerase, as reported previously [7,10,15]. The polymerase fraction also contained 3'-to-5' exonuclease activity, but lacked 5'-to-3' exonuclease, endonuclease, primase and ATPase activities. The specific activity of the purified EBV DNA polymerase was 3028846 units/mg [11].

Measurement of DNA synthesis

EBV DNA polymerase was routinely assayed in a reaction mixture (25 µl) that contained 50 mM-Tris/HCl, pH 8.0, 10 % glycerol, 80 mM-(NH₄)₂SO₄, 6 mM-MgCl₂, 100 µg of BSA/ml, 80 µg of activated calf thymus DNA/ml, 1 mM-dithiothreitol (DTT), 25 µM each of dATP, dGTP and dCTP, and 10 µM-[³H]TTP (1800 c.p.m./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 dNMP into acid-insoluble material in 60 min at 35 °C. Specific details of DNA synthesis on the singly primed M13 ssDNA and competition experiments are given in the Figure and Table legends.

RESULTS

Template/primer preference of EBV DNA polymerase in the presence and the absence of (NH₄)₂SO₄

EBV DNA polymerase utilized poly(dC)·oligo(dG) and poly(dA)·oligo(dT) 29-fold and 7.4-fold more efficiently than activated DNA when (NH₄)₂SO₄ was removed from the reaction mixture (Table 1). The DNA template/RNA primer poly(dT)·oligo(rA) was also a good template for EBV DNA polymerase, whereas poly(dT)·oligo(dA) was poorly utilized.

This may be due to the physical structure of the template/primer. Poly(dT)·oligo(rA) can form a duplex structure, whereas poly(dT)·oligo(dA) is reported to form a three-stranded structure, which inhibits DNA synthesis [16]. The observation that EBV DNA polymerase could utilize RNA primers on the DNA template suggests the possible involvement of the EBV DNA polymerase in the synthesis of Okazaki fragments from RNA primers on the lagging strand template. Poly(rA)·oligo(dT), poly(rU)·oligo(dA), poly(rU)·oligo(rA), poly(dT), poly(dC) and oligo(dT) were not utilized at all. When 80 mM- NH_4SO_4 was added to the reaction mixture, the DNA polymerase activity changed dramatically depending on the template/primers used. Activated DNA and poly(dT)·oligo(rA) were utilized 2.3-fold and 1.6-fold more efficiently than in the absence of the salt. In contrast, 80 mM- $(\text{NH}_4)_2\text{SO}_4$ inhibited polymerase activity by 96% and 99% when poly(dC)·oligo(dG) and poly(dA)·oligo(dT) respectively were used. In the presence of 80 mM- $(\text{NH}_4)_2\text{SO}_4$, poly(dT)·oligo(rA) was the best template/primer for EBV DNA polymerase. The above results indicate that the stimulation of EBV DNA polymerase by salt is dependent on the template/primer utilized.

ATP is not required in primer terminus recognition

No requirement for ATP was observed in DNA synthesis using various template/primers by EBV DNA polymerase (Table 1) and with RNA- or DNA-primed M13 ssDNA (results not shown). Unlike *E. coli* DNA polymerase III holoenzyme [17], T4 DNA polymerase [18] or DNA polymerase δ [19], EBV DNA polymerase did not require ATP hydrolysis in its binding to primer termini or in its polymerization process.

Competition experiment

The ability of various template/primer combinations to compete for EBV DNA polymerase was compared when two

template/primer systems were present in the same reaction mixture (Table 2). Reactions contained the same amounts (80 μg of template/ml and 40 μg of primer/ml) of the two template/primer systems in the absence of $(\text{NH}_4)_2\text{SO}_4$. $[\text{H}]$ poly(dG) synthesis directed by poly(dC)·oligo(dG) was inhibited by 44% and 33% by the addition of poly(dT)·oligo(rA) and poly(dA)·oligo(dT) respectively. However, activated DNA was less inhibitory in the poly(dC)·oligo(dG) system. This observation may reflect a 29-fold preference for poly(dC)·oligo(dG) compared with activated DNA (Table 1). It is interesting to note that the EBV genome has a high G + C content (60%). Unexpectedly, poly(dT) or poly(dA) alone did not have any effect in competing for the enzyme under these assay conditions. Also, oligo(rA) or oligo(dT) alone did not have any effect at all. With $[\text{H}]$ poly(dA) synthesis directed by poly(dT)·oligo(rA), the poly(dC)·oligo(dG) and activated DNA systems inhibited DNA synthesis by 34% and 33% respectively. However, poly(dC) alone did not affect the DNA synthesis at all, and neither did oligo(dG) alone. These observations indicate that EBV DNA polymerases strongly recognize primers hybridized to template DNA rather than long single-stranded templates or short single-stranded primers with 3'-OH termini.

Differential inhibitory effects of primed and unprimed homopolymers on EBV DNA polymerase activity with poly(dC)·oligo(dG)

The above observation that the unprimed homopolymers did not compete for EBV DNA polymerase with the primed homopolymers was in sharp contrast with that reported for DNA polymerase α , which exhibits a high affinity for single-stranded homopolymers, especially poly(dT) [20,21]. To analyse the interaction between EBV DNA polymerase and the template/primer in detail, kinetic studies were performed. Rate determinations were based on 15 min incubations under reaction conditions in which polymerase activity was linear with time for at least 40 min. Apparent K_m values for poly(dC)·oligo(dG) and

Table 2. Competition by various template/primer combinations for EBV DNA polymerase

All templates and primers were present at 80 $\mu\text{g}/\text{ml}$ and 40 $\mu\text{g}/\text{ml}$ respectively. The template/primer concentration in reactions with activated DNA was 80 $\mu\text{g}/\text{ml}$. Reactions contained the same amounts of the two template/primer systems and were performed in the absence of $(\text{NH}_4)_2\text{SO}_4$ in a range where the DNA polymerase activity was proportional to the amount of enzyme. Reactions were carried out as described in Table 1. Results are the means $\pm$ S.E.M. ($n = 3$).

Competitive template/primer	Amount of $[\text{H}]$ dNMP incorporated	
	(pmol)	(%)
(a) $[\text{H}]$ Poly(dG) synthesis with poly(dC)·oligo(dG)		
None	135 $\pm$ 7	100
Poly(dT)·oligo(rA)	75 $\pm$ 4	56
Poly(dT)	134 $\pm$ 8	99
Oligo(rA)	132 $\pm$ 8	98
Poly(dA)·oligo(dT)	91 $\pm$ 6	67
Poly(dA)	134 $\pm$ 7	99
Oligo(dT)	130 $\pm$ 7	96
Activated DNA	129 $\pm$ 11	95
(b) $[\text{H}]$ Poly(dA) synthesis with poly(dT)·oligo(rA)		
None	37 $\pm$ 4	100
Poly(dC)·oligo(dG)	24.5 $\pm$ 4	66
Poly(dC)	37 $\pm$ 3	100
Oligo(dG)	36 $\pm$ 3	97
Activated DNA	25 $\pm$ 2	67

Fig. 1. Lineweaver-Burk analysis of the inhibition of $[\text{H}]$ poly(dG) synthesis with poly(dC)·oligo(dG) by poly(dT)·oligo(rA), poly(dT) and oligo(rA)

EBV DNA polymerase (8 units) was incubated for 15 min at 35 $^{\circ}\text{C}$ in a reaction (25 μl) containing 50 mM-Tris/HCl, pH 8.0, 6 mM- MgCl_2 , 1 mM-DTT, 100 μg of BSA/ml, 10% glycerol, 40 μM - $[\text{H}]$ dGTP at 454 c.p.m./pmol, and poly(dC)·oligo(dG) at the concentrations indicated. Poly(dT)·oligo(rA), poly(dT) and oligo(rA) were added to each series of incubations at final concentrations of 10 $\mu\text{g}/\text{ml}$, 40 $\mu\text{g}/\text{ml}$ and 40 $\mu\text{g}/\text{ml}$ respectively. $\circ$, control reaction without competitors; $\bullet$, poly(dT)·oligo(rA); $\blacktriangle$, poly(dT); $\blacksquare$, oligo(rA).

Fig. 2. Dixon plot analysis of the inhibition of $[^3\text{H}]$ poly(dG) synthesis with poly(dC)·oligo(dG) by poly(dT)·oligo(rA) (a), poly(dT) (b) and oligo(rA) (c)

Reactions were formulated and run essentially as described in Fig. 1, with specific modifications as indicated below. Poly(dC)·oligo(dG) was at 5 $\mu\text{g}/\text{ml}$ (●), 10 $\mu\text{g}/\text{ml}$ (▲) or 20 $\mu\text{g}/\text{ml}$ (■). Apparent K_i values: (a) 3.4 $\mu\text{g}/\text{ml}$, (b) 76 $\mu\text{g}/\text{ml}$, (c) 280 $\mu\text{g}/\text{ml}$.

poly(dT)·oligo(rA) were 6.6 $\mu\text{g}/\text{ml}$ (Fig. 1) and 3.0 $\mu\text{g}/\text{ml}$ (results not shown) respectively. Lineweaver–Burk analysis of the inhibition of $[^3\text{H}]$ poly(dG) synthesis by poly(dT)·oligo(rA), poly(dT) and oligo(rA) indicated that they are kinetically competitive with the poly(dC)·oligo(dG) substrate (Fig. 1). Thus the unprimed homopolymer poly(dT), which is completely unreactive as a polymerization substrate (Table 1), was inhibitory to EBV DNA polymerase at low concentrations of the poly(dC)·oligo(dG) substrate. Fig. 2 shows the kinetics of EBV DNA polymerase inhibition by each of the competitors, as assessed by Dixon plot analysis. Values of apparent K_i for the competitors were determined: poly(dT)·oligo(rA), 3.4 $\mu\text{g}/\text{ml}$; poly(dT), 76 $\mu\text{g}/\text{ml}$; oligo(rA), ~280 $\mu\text{g}/\text{ml}$. The K_i value for the primed homopolymer poly(dT)·oligo(rA) was about 22-fold and 82-fold lower than that for the unprimed homopolymer [poly(dT)] and that for the primer with 3'-OH terminus [oligo(rA)] respectively. Thus EBV DNA polymerase has significantly higher affinity for the primed template DNA than for the ssDNA template or the single-stranded primer itself.

Processivity and rate of polymerization of EBV DNA polymerase

Assays that determine the processivity and the rate of polymerization of EBV DNA polymerase were performed using

Fig. 3. Time course of DNA replication of singly RNA-primed M13 ssDNA

The reaction mixture (200 μl) contained 50 mM-Tris/HCl, pH 8.0, 3 mM-MgCl₂, 1 mM-DTT, 100 μg of BSA/ml, 10% glycerol, 1.2 μg (500 fmol as circle) of RNA-primed M13 ssDNA, 0.5 mM-ATP, 50 μM each of dATP, dGTP and dTTP, 2 μM - $[\alpha\text{-}^{32}\text{P}]\text{dCTP}$ (3000 Ci/mmol), 12 μg of *E. coli* SSB and 120 units of EBV DNA polymerase. The replication reaction was incubated at 32 °C. Samples (25 μl) were removed at the times indicated and quenched by adding them to an equal volume of 1% SDS/50 mM-EDTA. After quenching, the samples were divided into two parts. One half was analysed for DNA synthesis by measuring the radioactivity incorporated into acid-insoluble material (a). The other half was used for the analysis of the replication products by electrophoresis in neutral 0.7% agarose gels (b). For autoradiography, the dried gel was exposed to Kodak X-AR X-ray film. The arrows mark the positions of RF II DNA (open circular double-stranded DNA) and singly primed ssDNA standards. Removal of ATP from the reactions did not have any effect on DNA synthesis.

singly RNA-primed M13 mp18 ssDNA template coated with SSB as a natural template/primer. EBV DNA polymerase recognized the ribo-oligomer as primer 5-fold more efficiently than the corresponding deoxyoligomer (results not shown). Singly primed ssDNA circles were more than 5-fold in molar excess over EBV DNA polymerase in the reaction, so that each polymerase molecule was bound to a primer terminus. EBV DNA polymerase activity was stimulated about 2-fold on the primed ssDNA in the presence of SSB, and a series of discontinuous products smaller than RF II (circular duplex DNA with a small gap or nick in one strand) were synthesized in the absence of SSB (results not shown). DNA synthesis by EBV DNA polymerase on SSB-coated RNA-primed M13 DNA reached a plateau after 20 min (Fig. 3a). Analysis of replication products by neutral agarose gel electrophoresis (Fig. 3b) showed that full-length products (RF II) were formed within 10 min. Most of the primed ssDNAs remained unused, as detected by u.v.-induced

ethidium bromide fluorescence (results not shown). The 10 min required for the complete replication of an M13 mp18 ssDNA molecule (7200 bases) yielded a rate of polymerization of 12 nucleotides/s per enzyme molecule. When one-fifth of the amount of enzyme was used, the same gel pattern was observed (results not shown). The limited DNA synthesis from 20 min to 50 min and disappearance of radioactivity between the primed ssDNA and RF II after 10 min indicate highly processive replication, where each polymerase molecule extends an RNA primer around the ssDNA circle, without dissociation and resulting slowness in recycling of the polymerase molecule to other primed ssDNA.

DISCUSSION

The DNA template/RNA primer poly(dT)-oligo(rA) was utilized very well by EBV DNA polymerase as template/primer in the presence or the absence of $(\text{NH}_4)_2\text{SO}_4$. Furthermore, when M13 ssDNA was used as a natural template DNA, EBV DNA polymerase utilized ribo-oligomer primers more efficiently than deoxyoligomers (T. Tsurumi, unpublished work). Thus EBV DNA polymerase, in contrast to β -, γ - and δ -DNA polymerases [22,23], is able to elongate *in vitro* synthesized and natural RNA primers such as DNA polymerase α [22]. The ability of EBV DNA polymerase to extend such primers suggests the possible involvement of EBV DNA polymerase in RNA-primed discontinuous-lagging-strand DNA synthesis.

Competition and kinetic studies have revealed that EBV DNA polymerase had significantly higher affinity for primed template DNA than for the unprimed template or the single-stranded primer itself. However, EBV DNA polymerase exhibited weak affinity for the single-stranded template DNA (Figs. 1 and 2): In the case of DNA polymerase α , binding of the enzyme to DNA is absolutely dependent on single-stranded template DNA. It is postulated that the α -polymerase molecule possesses two highly interactive DNA-binding sites [24]: one is a template-binding site and the other is a primer-binding site. Therefore it may be reasonable to speculate about the interaction of EBV DNA polymerase with the template/primer as follows. EBV DNA polymerase first recognizes the single-stranded template. However, this interaction of the enzyme with DNA may be weak, so the polymerase can easily move on the ssDNA or translocate on to other templates. Once the polymerase recognizes the primer on the template DNA, it binds very tightly to the primer terminus and then initiates DNA synthesis without dissociation. This primer terminus recognition by EBV DNA polymerase does not require ATP hydrolysis.

EBV DNA polymerase extends an RNA primer on M13 ssDNA (7200 nucleotides) without dissociation. The polymerization rate on SSB-coated singly primed M13 ssDNA of 12 nucleotides/s per enzyme molecule at 32 °C is more than twice that found by O'Donnell *et al.* [25] for the HSV-1 DNA polymerase on singly primed ϕ X174 DNA coated with SSB at 30 °C. The rate of fork movement of pseudorabies virus, which is a member of the herpes virus family, is approx. 1 $\mu\text{m}/\text{min}$, or 50 nucleotides/s at 37 °C [26]. Although *E. coli* SSB was used in the present study to melt out hairpins or other secondary structures within M13 ssDNA [27], EBV itself encodes an ssDNA-binding protein, the BALF2 protein, which is a functional analogue of *E. coli* SSB. If the BALF2 protein was used in the replication assay in place of *E. coli* SSB, the polymerization rate might have approached the rate *in vivo* due to a specific protein-protein interaction between EBV DNA polymerase and the BALF2 protein.

EBV DNA polymerase exhibited highly processive polymerization (> 7200 nucleotides). In contrast, the processivity of EBV DNA polymerase reported by Grossberger & Clough [6] was

very low (~ 10 nucleotides). The reason for this discrepancy is not known with certainty, although it is most likely due to the template DNAs utilized and the presence of SSB. The assay system of Grossberger & Clough [6] contained only ϕ X174 ssDNA as template, which easily forms secondary structures, such as loops or hairpins, within the circular single-stranded DNA molecule [28]. To remove these sequence-specific barriers, M13 ssDNA coated with SSB was utilized for my processivity assay. It has been reported that HSV-1 DNA polymerase also has high processivity (> 5400 nucleotides) when using SSB-coated singly primed ϕ X174 ssDNA [25].

The eukaryotic chromosomal replication apparatus uses two different DNA polymerases [19,29,30]. Leading-strand synthesis is carried out by DNA polymerase δ , which has high processivity [19]. On the other hand, lagging-strand synthesis is accomplished by DNA polymerase α , which can efficiently extend RNA primers on template DNA [20]. However, in other systems such as T4 phage [31], T7 phage [32] or *E. coli* DNA replication [28], the same DNA polymerase is used in the synthesis of both strands. The high processivity of EBV DNA polymerase and its ability to extend RNA primers suggest the possible involvement of this polymerase in leading- and lagging-strand DNA synthesis in the lytic phase of EBV DNA replication.

HSV-1 DNA polymerase purified from HSV-infected Vero cells is a heterodimer complex which consists of the products of the UL 30 (Pol) and UL 42 genes [33–35]. The UL 42 protein interacts with the UL 30 (Pol) protein to enhance its processivity of deoxynucleotide polymerization, as does the β subunit of the *E. coli* DNA polymerase III holoenzyme [34,36]. Similarly, there is a possibility that EBV DNA polymerase purified from the cultured cells is a complex of the BALF5 (Pol) and BMRF1 gene products, as EA-D polypeptide (the BMRF1 gene product) [8,37], which can stimulate EBV DNA polymerase activity [38], is reported to be associated with EBV DNA polymerase [8]. Further studies will be needed to clarify the relationship between the highly processive replication and these proteins.

I thank Dr. K. Maeno, Dr. Y. Nishiyama, and Dr. I. R. Lehman for encouragement, and T. Tsuruguchi and E. Iwata for technical assistance. This work was supported by a Grant-in-Aid for Cancer Research from the Ministry of Education, Science and Culture of Japan, by the research fund of the Ishida Foundation, and by the Hibino Memorial Medical Research Fund.

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Received 15 May 1991/18 July 1991; accepted 24 July 1991