

# The Epstein-Barr Virus Polymerase Accessory Factor BMRF1 Adopts a Ring-shaped Structure as Visualized by Electron Microscopy\*

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Epstein-Barr virus (EBV) encodes a set of core replication factors used during lytic infection in human cells that parallels the factors used in many other systems. These include a DNA polymerase and its accessory factor, a helicase/primase, and a single strand binding protein. The EBV polymerase accessory factor has been identified as the product of the *BMRF1* gene and has been shown by functional assays to increase the activity and processivity of the polymerase. Unlike other members of this class of factors, BMRF1 is also a transcription factor regulating certain EBV genes. Although several polymerase accessory factors, including eukaryotic proliferating cell nuclear antigen, *Escherichia coli*  $\beta$  protein, and *T4* gene 45 protein have been shown to form oligomeric rings termed sliding clamps, nothing is known about the oligomeric state of BMRF1 or whether it forms a ring. In this work, BMRF1 was purified directly from human cells infected with an adenovirus vector expressing the *BMRF1* gene product. The protein was purified to near homogeneity, and examination by negative staining electron microscopy revealed large, flat, ring-shaped molecules with a diameter of  $15.5 \pm 0.8$  nm and a distinct 5.3-nm diameter hole in the center. The size of these rings is consistent with an oligomer of 6 monomers, nearly twice as large as the trimeric proliferating cell nuclear antigen ring. Unlike the herpes simplex virus UL42 homologue, BMRF1 was found to self-associate in solution. These findings extend the theme of polymerase accessory factors adopting ring-shaped structures and provide an example in which the ring is significantly larger than any previously described sliding clamp.

The human  $\gamma$ -herpesvirus Epstein-Barr virus (EBV)<sup>1</sup> has both a latent and a lytic replicative cycle. In the latent phase, the virus is maintained as an episome and replicates using the OriP origin of replication and cellular machinery (1–4). Lytic infection uses a separate origin, OriLyt and several virally encoded proteins (5–7), and is currently believed to occur via a

rolling circle mechanism producing head to tail concatemers (6). Rolling circle replication would utilize the EBV gene products BALF1 (catalytic component of viral DNA polymerase), BMRF1 (the polymerase accessory protein), BALF2 (single strand binding protein), BSLF1 (primase), BBLF4 (helicase), and BBLF2/3 (helicase/primase-associated protein) (8, 9). Ligase and RNase H functions would also be required.

The BMRF1 polymerase accessory factor exists in two phosphorylated forms of 50 and 52 kDa, while a third unphosphorylated 48-kDa form is seen in baculovirus-infected cells (10). BMRF1 co-purifies with the 110-kDa EBV DNA polymerase, and they appear to function together as a holoenzyme (11–13). Polymerase activity is stimulated by more than 10-fold when BMRF1 is included in reactions containing the catalytic subunit and an activated DNA template (14). Additionally, BMRF1 appears to increase the processivity of the viral polymerase, suggesting a model in which the accessory protein acts as a sliding clamp that prevents dissociation of the polymerase from the active template. The herpes simplex virus type 1 UL42 polymerase accessory protein does not share extensive amino acid homology with BMRF1, but in both proteins the amino-terminal two-thirds of the molecule contains a DNA-binding domain and a region of interaction with the catalytic subunit (15). Both BMRF1 and UL42 bind to double strand DNA with high affinity in contrast to the well characterized sliding clamps *Escherichia coli*  $\beta$ , *T4* gene 45 protein, and PCNA, which lack an intrinsic DNA-binding activity (16–18). A unique feature of BMRF1 is that it also serves as a transcriptional activator, a function that to date has not been found with other polymerase accessory proteins (19). Transcriptional activation may function to induce viral proteins that are required for lytic replication.

*E. coli*  $\beta$ , *T4* gene 45 protein, and PCNA have been shown to form oligomeric rings that can translocate along DNA without direct contacts that could inhibit polymerase movement (16–18). Despite the high affinity for DNA, BMRF1 does not appear to retard the holoenzyme, and this has also been observed with UL42 (20). PCNA and *T4* gene 45 proteins form homotrimers, whereas *E. coli*  $\beta$  is a homodimer (21), and the checkpoint sliding clamp Rad9-Rad1-Hus1 is a heterotrimeric ring (22). Each of these sliding clamps has a homologous ATP-dependent clamp loading factor that actively places the clamp around DNA. Clamp loaders have not been identified for BMRF1 or UL42. In this study, we used electron microscopy (EM) to examine the structural organization of BMRF1 in solution. We find it exists as a ring-shaped structure with a distinct hole in the center. The size of the rings is suggestive of an oligomer of 6 monomers.

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<sup>1</sup> The abbreviations used are: EBV, Epstein-Barr virus; PCNA, proliferating cell nuclear antigen; EM, electron microscopy; GST, glutathione *S*-transferase.

## MATERIALS AND METHODS

**Cells and Adenovirus Vectors**—A replication-deficient adenovirus vector (E1/E3 deleted) expressing the BMRF1 protein (AdBMRF1) controlled by the human cytomegalovirus immediate-early promoter was made using the Cre-lox-mediated recombination systems as described previously (23). The control vector contained the bacterial  $\beta$ -galactosidase gene (*AdLacZ*) instead of the *BMRF1* gene. HeLa cells were infected with the adenovirus vectors at a multiplicity of infection of 10.

**Plasmid Vectors**—A vector expressing full-length BMRF1 protein linked at its amino terminus in-frame with glutathione *S*-transferase (GST) was a gift from David Dorsky (University of Connecticut Health Center). A plasmid containing the *BMRF1* gene inserted into the SG5 vector (Stratagene) was used for *in vitro* translation studies and was a gift from David Dorsky.

**Protein Purification of BMRF1 Expressed in Cells**—Adenovirus-infected HeLa cells ( $10 \times 150$ -mm plates) were harvested 48 h after infection. Cells were washed twice with  $1 \times$  phosphate-buffered saline (5 ml/plate) and then scraped into 2 ml of phosphate-buffered saline/plate and pelleted by low speed centrifugation ( $1000 \times g$  for 10 min at  $4^\circ\text{C}$ ). Cells were resuspended in 10 ml of lysis buffer (50 mM Tris, pH 7.5, 100 mM NaCl, 0.5 mM  $\text{MgCl}_2$ , 1 mM EDTA, 2 mM dithiothreitol, 0.2% Nonidet P-40, and 1 mM phenylmethylsulfonyl fluoride) followed by one freeze-thaw cycle and sonication (three 15-s pulses, 50% duty cycle). Lysates were clarified by high speed centrifugation at  $10,000 \times g$  for 40 min at  $4^\circ\text{C}$ . Using a fast protein liquid chromatography system, samples were applied to a 10-ml heparin-Sepharose (Amersham Biosciences) column pre-equilibrated with Buffer H (20 mM Hepes, pH 7.9, 0.2 mM EDTA, 20% glycerol, 2 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride) containing 100 mM KCl at a flow rate of 0.1 ml/min. The column was washed with 10 column volumes of Buffer H plus 100 mM KCl and eluted with a 30-ml gradient of 100–800 mM KCl in Buffer H. Peak fractions were dialyzed against 4 liters of Buffer H plus 100 mM KCl and then loaded on a 10-ml DNA-cellulose column (Amersham Biosciences) pre-equilibrated with Buffer H plus 100 mM KCl at a flow rate of 0.1 ml/min. The column was washed with Buffer H plus 100 mM KCl, and proteins were eluted with a 30-ml gradient of 100–800 mM KCl in Buffer H. Peak fractions were dialyzed against Buffer H containing 100 mM KCl and 20% glycerol prior to storage. Protein concentrations were determined by Bradford assays.

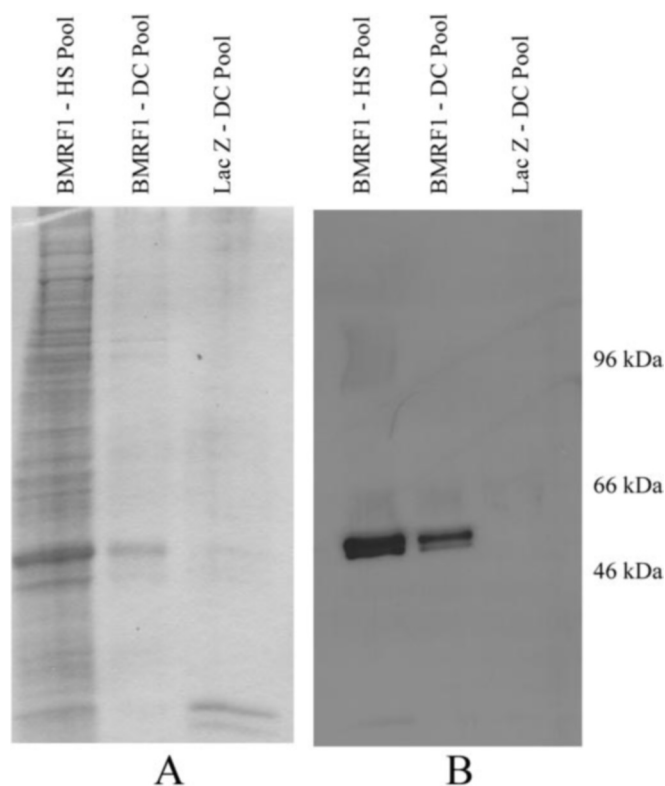
**Western Blot Analysis**—Proteins were separated on 10% discontinuous SDS-PAGE followed by immobilization on nitrocellulose membranes using an electrotransfer system (Bio-Rad). Membranes were blocked with 5% nonfat dried milk in  $1 \times$  TBS-T (20 mM Tris, pH 7.6, 137 mM NaCl, 0.1% Tween 20) for 1 h at room temperature and incubated with a 1:500 dilution of a mouse monoclonal antibody to BMRF1 (Capricorn, Inc.) in blocking buffer at  $4^\circ\text{C}$  overnight. Blots were washed three times (10 min/wash) in  $1 \times$  TBS-T, incubated with a 1:5000 dilution of the secondary antibody, goat anti-mouse conjugated to horseradish peroxidase, for 1 h at room temperature, washed three times with  $1 \times$  TBS-T, and detected by chemiluminescence (ECL kit, Amersham Biosciences).

**BMRF1 Dimerization Assays**— $^{35}\text{S}$ -Labeled *in vitro* translated BMRF1 protein was produced from the SG5-BMRF1 vector using the TNT-T7 quick-coupled *in vitro* translation system (Promega). GST or GST-BMRF1 proteins coupled to 50  $\mu\text{l}$  of glutathione beads (50% slurry) (Sigma) were incubated with 3  $\mu\text{l}$  of the  $^{35}\text{S}$ -labeled *in vitro* translated BMRF1 protein in 500- $\mu\text{l}$  reactions containing binding buffer (20 mM Tris pH 7.9, 75 mM KCl, 25 mM  $\text{MgCl}_2$ , 0.1 mM EDTA, 10 mM dithiothreitol, 0.15% Nonidet P-40) for 1 h at  $4^\circ\text{C}$ . The beads were washed five times in binding buffer, and bound proteins were analyzed by 10% SDS-PAGE and autoradiography.

**Electron Microscopy**—For negative staining, the protein was diluted to a concentration of 30–50  $\mu\text{g}/\text{ml}$  in 20 mM Tris-HCl, pH 7.5, 50 mM NaCl, and 5 mM  $\text{MgCl}_2$ , adsorbed to a thin carbon foil according to the method of Valentine and Green (24), and negatively stained with 2% uranyl acetate. Transmission electron microscopy was performed using a Philips CM12 electron microscope. The micrographs were recorded at a  $\times 60,000$  magnification and scanned using a Nikon LS4500 film scanner. Morphometric measurements were made using Gatan Digital Image software. Images were arranged into panels using Adobe PhotoShop software.

## RESULTS AND DISCUSSION

**Purification of BMRF1 from Cells**—Previous studies with purified BMRF1 have utilized either baculovirus vectors or *in vitro* transcription and translation systems to express the protein. One of the concerns with these systems is whether all of



**FIG. 1. Purification of BMRF1.** HeLa cells were infected with Ad-BMRF1 or AdLacZ and purified by chromatography using heparin-Sepharose (HS) and DNA-cellulose (DC) columns as described under "Materials and Methods." Pooled fractions from both columns were visualized by Coomassie Blue staining (A) or Western blot analysis (B) using the anti-BMRF1 antibody.

the post-translational modifications placed on the protein in infected human cells have been added. For example, BMRF1 purified from baculovirus-infected insect cells is a mixture of two phosphorylated and one unphosphorylated form (10). To ensure that the BMRF1 protein used for these studies was as close as possible to its cellular state, we used an adenovirus-based vector system to express BMRF1 in HeLa cells. These have been used to demonstrate that, *in vivo*, BMRF1 can enhance the gastrin gene expression.<sup>2</sup> As a control, a vector containing the bacterial *LacZ* gene was used. Lysates from HeLa cells infected with the AdBMRF1 or AdLacZ were prepared 48 h after infection. The expressed proteins were first passed over a heparin-Sepharose column, and bound proteins were eluted using a salt gradient. A single peak was observed by  $A_{320\text{ nm}}$  measurement, and fractions within this peak were found to contain BMRF1 by Western blot analysis. Appropriate fractions were pooled, dialyzed to decrease the salt concentration, and then applied to a DNA-cellulose column. Proteins were eluted using a salt gradient, and peak fractions were tested by Western blot analysis for the presence of BMRF1 (see under "Materials and Methods"). SDS-PAGE and Western blot analysis from pooled fractions of both columns are shown in Fig. 1. As seen in Fig. 1A, pooled fractions from the heparin-Sepharose and DNA-cellulose columns showed a strong band around 50 kDa in Coomassie Blue-stained gels. Western blots analysis revealed two bands in both column pools that presumably represent the 50- and 52-kDa proteins (Fig. 1B). The BMRF1 band in the heparin-Sepharose fractions was by far the most dominant species, representing  $\sim 60\%$  of the total protein,

<sup>2</sup> Holley-Guthrie, E. A., Seaman, W. T., Bhende, P., Merchant, J. L., and Kenney, S. C. (2004) *J. Virol.*, in press.

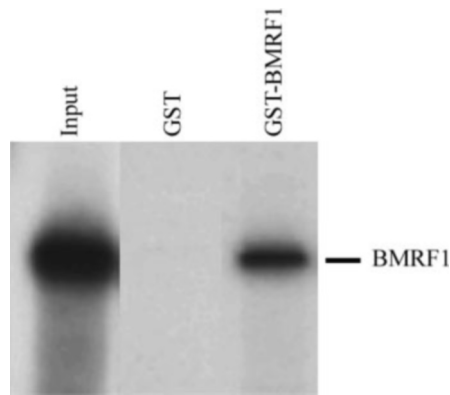


FIG. 2. **BMRF1 associates with itself in solution.** Glutathione beads conjugated with GST or GST-BMRF1 were incubated with  $^{35}\text{S}$ -labeled *in vitro* translated BMRF1, and proteins immobilized to the beads were analyzed by SDS-PAGE and autoradiography. Input from the *in vitro* translated material was included as control.

with the remaining 40% spread over a wide range of molecular weights. The DNA-cellulose pool was near homogeneity as determined by Coomassie Blue staining. Parallel purification using lysates from AdLacZ-infected cells showed negligible amounts of contaminating protein after the DNA-cellulose column (Fig. 1).

**BMRF1 Interacts with Itself**—Although the herpes simplex virus type 1 DNA polymerase processivity factor UL42 does not form a dimer (20), the Kaposi sarcoma herpesvirus DNA polymerase processivity factor was recently shown to dimerize (25), particularly when bound to DNA. To determine whether BMRF1 can interact with itself,  $^{35}\text{S}$ -labeled *in vitro* translated BMRF1 protein was incubated with either GST protein or a GST-BMRF1 conjugated to glutathione-linked beads, and proteins bound to the beads were analyzed by SDS-PAGE and autoradiography. As shown in Fig. 2, *in vitro* translated BMRF1 did not interact with the GST protein alone but clearly interacted with the GST-BMRF1 fusion protein. Thus, BMRF1 can dimerize and/or form higher order structures with itself in solution.

**Visualization of BMRF1 Rings**—To directly examine the oligomeric state of BMRF1, unfixed protein in a buffer containing 50 mM NaCl and 5 mM  $\text{MgCl}_2$  was prepared for EM by negative staining with 2% uranyl acetate (see under “Materials and Methods”). Examination of BMRF1 purified through the DNA-cellulose column step to homogeneity revealed molecules with a distinct ring-shaped structure, but the relatively low protein concentration resulted in the rings being scattered at a distance in the fields. Efforts to concentrate the sample led to a loss of the protein. However, when BMRF1 purified only through the heparin-Sepharose step (which was at a higher protein concentration) was examined, fields with the same rings much more closely spaced were observed (Fig. 3A). Although the heparin-Sepharose pool was contaminated with other proteins, BMRF1 was the predominant protein detected by Coomassie Blue staining, and the protein concentrations were high enough for negative staining. The BMRF1 particles seen in the heparin-Sepharose pool matched the size and shapes observed in the highly pure DNA-cellulose peak, and thus we concluded that these rings are indeed formed by BMRF1. There was no evidence as seen by EM from either protein pool for a significant concentration of protein particles of sizes that would correspond to BMRF1 dimers, compact trimers, or tetramers. Closer examination of the rings showed that the majority had a very distinct appearance (Fig. 3B). Analysis of their dimensions revealed a diameter of  $15.5 \pm 0.8$  nm ( $n = 12$ ), with a  $5.3 \pm 0.8$  nm ( $n = 10$ ) hole in the center.

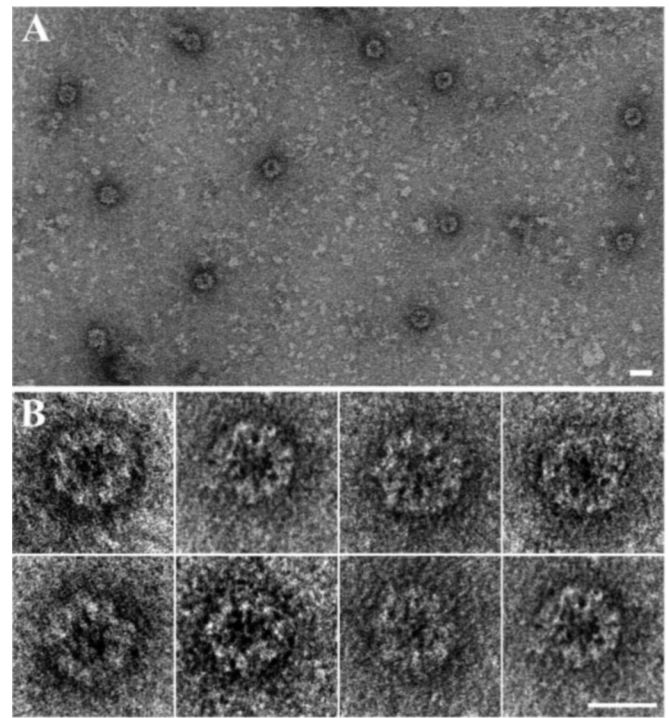


FIG. 3. **Visualization of BMRF1 by electron microscopy.** BMRF1 was negatively stained with uranyl acetate and adsorbed to a thin carbon foil as described under “Materials and Methods.” *A*, a field of dispersed BMRF1 molecules is shown. Scale bar = 20 nm. *B*, a gallery of BMRF1 particles at a higher magnification showing ring-like particles with a diameter of 15.5 nm and a central hole diameter of 5.5 nm. Scale bar = 10 nm.

Some particles appeared deformed possibly because of the staining procedure or interaction with the carbon foil. The only projection of the BMRF1 ring observed was that of a flat disk with a large hole, suggesting that the rings are too thin to be stable lying edgewise on the EM support.

Simple modeling of these structures suggests that the most probable oligomeric state is that of a ring with six subunits. The BMRF1 ring appears as a flat toroid constructed of a 5-nm-wide protein tube that forms a smooth contoured ring  $15.5 \pm 0.8$  nm wide with a  $5.3 \pm 0.8$ -nm hole in the center. The molecular weight of streptavidin is close to that of a BMRF1 monomer, and its diameter is  $5.6 \pm 1.3$  nm. Modeling indicates that six such protein balls could easily be arranged side by side to generate the rings observed here. Other arrangements and oligomeric states, however, are possible depending on the shape of the BMRF1 monomer. Unfortunately, efforts at simple rotational analysis were not successful, and more complex single particle reconstruction will be required in the future to resolve individual monomers in the rings and obtain a higher resolution structure.

**The Classic Sliding Clamps**—*E. coli*  $\beta$ , *T4* gene 45 protein, and eukaryotic PCNA all share a nearly exact “Christmas wreath”-shaped ring structure (16–18) in spite of being formed from two (*E. coli*  $\beta$ ) or three (PCNA and *T4* gene 45 protein) subunits. Furthermore, the checkpoint clamp Rad9-Rad1-Hus1 is a heterotrimer with a shape similar to the latter proteins consisting of an 8.5-nm-diameter ring with a 3.5-nm hole (22). All of these rings have molecular masses of approximately 100 kDa. It is not clear what the significance of a larger ring would be and how this may be related to its association with the EBV DNA polymerase and other proteins. Additionally, it is not known how BMRF1 or UL42 is assembled around DNA in the apparent absence of ATP-dependent clamp loading factors such as those utilized by the classic sliding clamps. Possibly the nonspecific affinity of

BMRF1 and UL42 for duplex DNA allows them to self-assemble around DNA. Future studies of both proteins assembled onto DNA may aid in answering such questions.

The multisubunit structures observed by EM are consistent with the data demonstrating that BMRF1 can associate with itself in solution. In contrast, UL42 does not form dimers or other higher order oligomers in solution (20). The polymerase processivity factor of another  $\gamma$ -herpesvirus, Kaposi sarcoma herpesvirus, has been shown to self-associate, and oligomerization appears to be required for enhanced polymerase processivity and DNA binding (25). In this latter study, however, cross-linking reagents were required to observe oligomerization.

BMRF1 is unique among the polymerase processivity factors in performing a second function as a transcriptional activator. BMRF1 activates one of the two EBV OriLyt promoters (the early viral BHLF1 promoter) as well as the cellular gastrin promoter<sup>2</sup> (19, 26). The BMRF1-responsive regions of the BHLF1 and gastrin promoters contain binding sites for the cellular transcription factors SP1 and ZBP-89, although the precise mechanisms for the BMRF1 transcriptional effect have not yet been defined. Whether the ring-like structures observed in this study also mediate the transcriptional effects of BMRF1 remains unknown. It is possible that alternative conformations or structures of BMRF1 mediate its replication *versus* transcriptional effects. For example, BMRF1 is hyperphosphorylated by the virally encoded kinase BGLF4 (27, 28) in infected cells. Clearly, this hyperphosphorylation is not required for ring formation, but it may provide a mechanism that could regulate these structures presumably involved in its polymerase processivity function, as contrasted with possible alternative conformations that may be needed for the regulation of transcription.

## REFERENCES

1. Kieff, E. and Liebowitz, D. (1990) in *Fields Virology* (Fields, B., and Knipe, D., eds) 2nd Ed., pp. 1889–1920, Raven Press, New York
2. Miller, G. (1990) in *Fields Virology* (Fields, B. K. D., ed) 2nd Ed., pp. 1921–1957, Raven Press, New York
3. Reisman, D., Yates, J., and Sugden, B. (1985) *Mol. Cell. Biol.* **5**, 1822–1832
4. Yates, J. L., Warren, N., and Sugden, B. (1985) *Nature* **313**, 812–815
5. Gruffat, H., Renner, O., Pich, D., and Hammerschmidt, W. (1995) *J. Virol.* **69**, 1878–1886
6. Hammerschmidt, W., and Sugden, B. (1988) *Cell* **55**, 427–433
7. Schepers, A., Pich, D., Mankertz, J., and Hammerschmidt, W. (1993) *J. Virol.* **67**, 4237–4245
8. Fixman, E. D., Hayward, G. S., and Hayward, S. D. (1992) *J. Virol.* **66**, 5030–5039
9. Fixman, E. D., Hayward, G. S., and Hayward, S. D. (1995) *J. Virol.* **69**, 2998–3006
10. Tsurumi, T. (1993) *J. Virol.* **67**, 1681–1687
11. Kallin, B., Sternas, L., Saemundssen, A. K., Luka, J., Jornvall, H., Eriksson, B., Tao, P. Z., Nilsson, M. T., and Klein, G. (1985) *J. Virol.* **54**, 561–568
12. Li, J. S., Zhou, B. S., Dutschman, G. E., Grill, S. P., Tan, R. S., and Cheng, Y. C. (1987) *J. Virol.* **61**, 2947–2949
13. Tsurumi, T., Daikoku, T., and Nishiyama, Y. (1994) *J. Virol.* **68**, 3354–3363
14. Tsurumi, T., Daikoku, T., Kurachi, R., and Nishiyama, Y. (1993) *J. Virol.* **67**, 7648–7653
15. Kiehl, A., and Dorsky, D. I. (1995) *J. Virol.* **69**, 1669–1677
16. Kong, X. P., Onrust, R., O'Donnell, M., and Kuriyan, J. (1992) *Cell* **69**, 425–437
17. Krishna, T. S., Fenyo, D., Kong, X. P., Gary, S., Chait, B. T., Burgers, P., and Kuriyan, J. (1994) *J. Mol. Biol.* **241**, 265–268
18. Moarefi, I., Jeruzalmi, D., Turner, J., O'Donnell, M., and Kuriyan, J. (2000) *J. Mol. Biol.* **296**, 1215–1223
19. Zhang, Q., Holley-Guthrie, E., Ge, J. Q., Dorsky, D., and Kenney, S. (1997) *Virology* **230**, 22–34
20. Randell, J. C., and Coen, D. M. (2004) *J. Mol. Biol.* **335**, 409–413
21. Jeruzalmi, D., O'Donnell, M., and Kuriyan, J. (2002) *Curr. Opin. Struct. Biol.* **12**, 217–224
22. Griffith, J. D., Lindsey-Boltz, L. A., and Sancar, A. (2002) *J. Biol. Chem.* **277**, 15233–15236
23. Westphal, E. M., Mauser, A., Swenson, J., Davis, M. G., Talarico, C. L., and Kenney, S. C. (1999) *Cancer Res.* **59**, 1485–1491
24. Valentine, R. C., and Green, N. M. (1967) *J. Mol. Biol.* **27**, 615–617
25. Chen, X., Lin, K., and Ricciardi, R. P. (2004) *J. Biol. Chem.* **279**, 28375–28386
26. Zhang, Q., Hong, Y., Dorsky, D., Holley-Guthrie, E., Zalani, S., Elshiekh, N. A., Kiehl, A., Le, T., and Kenney, S. (1996) *J. Virol.* **70**, 5131–5142
27. Chen, M. R., Chang, S. J., Huang, H., and Chen, J. Y. (2000) *J. Virol.* **74**, 3093–3104
28. Gershburg, E., and Pagano, J. S. (2002) *J. Virol.* **76**, 998–1003