Postreplicative Mismatch Repair Factors Are Recruited to Epstein-Barr Virus Replication Compartments*

The mismatch repair (MMR) system, highly conserved throughout evolution, corrects nucleotide mispairing that arise during cellular DNA replication. We report here that proliferating cell nuclear antigen (PCNA), the clamp loader complex (RF-C), and a series of MMR proteins like MSH-2, MSH-6, MLH1, and hPMS2 can be assembled to Epstein-Barr virus replication compartments, the sites of viral DNA synthesis. Levels of the DNA-bound form of PCNA increased with progression of viral productive replication. Bromodeoxyuridine-labeled chromatin immunodepletion analyses confirmed that PCNA is loaded onto newly synthesized viral DNA as well as BALF2 and BMRF1 viral proteins during lytic replication. Furthermore, the anti-PCNA, -MSH2, -MSH3, or -MSH6 antibodies could immunoprecipitate BMRF1 replication protein probably via the viral DNA genome. PCNA loading might trigger transfer of a series of host MMR proteins to the sites of viral DNA synthesis. The MMR factors might function for the repair of mismatches that arise during viral replication or act to inhibit recombination between moderately divergent (homologous) sequences.

Mismatch repair (MMR) systems play a primary role in mutation avoidance by removing base-base and small insertion-deletion mismatches that arise during DNA replication (1). Prokaryotes and eukaryotes have evolved similar systems for repair (2) and in *Escherichia coli* MMR is initiated when MutS binds to mismatched DNA, possibly through its interaction with the β-clamp accessory protein that is required for processive DNA replication (3–6). MutL binds to MutS to form MutS-MutL-DNA complexes that stimulate MutH binding and cleavage of unmethylated DNA strands at GATC sequences, either 5’ or 3’ of recognized mismatches. Exonucleases then chew away at the DNA beyond the mismatch site so that highly accurate DNA polymerase III can correctly re-synthesize the strand.

In eukaryotes, mismatch recognition is accomplished by MSH2 (MutS homolog 2) forming a heterodimer with either MSH3 or MSH6 to bind to distinct but overlapping spectra of mismatches (7). In both the yeast *Saccharomyces cerevisiae* and humans, the repair of base-base mismatches appears to be solely dependent on MSH2-MSH6, whereas both MSH2-MSH6 and MSH2-MSH3 can participate in the repair of small (1 to 12-nucleotide) loop insertions. Currently, it is thought that MSH heterodimer binding to a mismatch triggers ATP-dependent steps that allow interactions with MLH (MutL homolog) heterodimers composed of MLH1-Pms1 or MLH1-MLH3 (7, 8). No MutH homolog, however, has been identified in eukaryotes, and the exact details of strand discrimination and error removal are not known, although in both yeast and humans a number of other proteins have been implicated in MMR, including proliferating cell nuclear antigen (PCNA), replication factor C (RF-C), and DNA polymerases δ and ε (9–16).

PCNA was originally characterized as a DNA sliding clamp for replicative DNA polymerases, but subsequent studies have revealed its striking ability to interact with multiple partners involved in several metabolic pathways (17). One role of PCNA in MMR is mediated by interactions with MSH2-MSH6 or MSH2-MSH3 complexes through PCNA interaction motifs present in MSH6 and MSH3 (18–20). PCNA increases the *in vitro* mispair binding specificity of the MSH2-MSH6 complex, and this is eliminated by amino acid substitutions in PCNA that cause MMR defects (19). It has been suggested that MMR may be coupled to DNA replication through the physical interaction between MutS family proteins and PCNA and through co-localization of PCNA with MSH6 and MSH3 in replication foci (18–20). The PCNA-MSH6-MSH2 complex can form a stable ternary complex with fully base-paired DNA, but when a mispair is present, PCNA is excluded from the complex in an ATP-dependent fashion (21), suggesting that MSH2-MSH6 binds to PCNA to search of mispairs on newly replicated DNA and is then transferred from PCNA to the mispaired bases.

PCNA also interacts with RF-C, which contains five subunits ranging between 36 and 140 kDa and possesses an DNA-dependent ATPase that is stimulated by PCNA. At present, its chief known function is to load PCNA onto DNA (22–24). Following its association with DNA at a primer end, RF-C recruits PCNA (the clamp) and loads it onto DNA in the presence of ATP (clamp loading), this being required for MMR (25). This RF-C-dependent PCNA loading mechanism is conserved among human, *E. coli*, and the T4 bacteriophage.

Viral DNA genome replication is closely linked with host cellular DNA replication, repair, and recombination. The Epstein-Barr virus (EBV) is a human lymphotropic herpesvirus with a linear double-stranded DNA, 172 kb in length (26). Primary EBV infection targets resting B lymphocytes, inducing their continuous proliferation. In resultant B lymphoblastoid cell lines only limited numbers of viral genes are usually expressed and there is no production of virus particles, this being termed latent infection. Reactivation from latency, which can occur spontaneously or be induced artificially, is characterized by the expression of lytic genes, which leads to the production of virions and eventually to host cell lysis. The EBV genome is amplified 100–1,000-fold by viral DNA replication machinery composed of seven viral replication proteins: BZLF1, an oriLyt-bounding protein; BALF5, a DNA polymerase; BMRF1, a polymerase processivity factor; BALF2, a single-stranded DNA-binding protein; and BBLF4, BSLF1, and BBLF2/3, predicted to be helicase, primase, and helicase-primase-associated pro...
Association of MMR Factors with EBV DNA Replication Sites

Cell Culture—Tet-BZLF1/B95-8 cells (30) were maintained in RPMI 1640 medium supplemented with 1 μg/ml puromycin, 250 μg/ml hygromycin B, and 10% tetracycline-free fetal calf serum (Clontech) at 37 °C in a humidified 5% CO₂ atmosphere. To induce lytic EBV replication, the tetracycline derivative, doxycycline, was added to the culture medium at a final concentration of 2 μg/ml.

Antibodies—Anti-BALF2 and anti-BMRF1 rabbit polyclonal antibodies were prepared as follows. Recombinant BALF2 or BMRF1 proteins were individually expressed in Sf21 cells infected with AcBALF2 or AcBMRF1 baculoviruses. PCNA and RF-C are precisely co-localized within viral replication compartments. RF-C might load PCNA onto newly synthesized viral DNA. Because MSH-3 and MSH-6 interact with MMR proteins to sites of viral DNA synthesis, such loading might trigger transfer of a series of host PCNA (19, 20), such loading might trigger transfer of a series of host MMR proteins to sites of viral DNA synthesis.

EXPERIMENTAL PROCEDURES

Cell Culture—Tet-BZLF1/B95-8 cells (30) were maintained in RPMI 1640 medium supplemented with 1 μg/ml puromycin, 250 μg/ml hygromycin B, and 10% tetracycline-free fetal calf serum (Clontech) at 37 °C in a humidified 5% CO₂ atmosphere. To induce lytic EBV replication, the tetracycline derivative, doxycycline, was added to the culture medium at a final concentration of 2 μg/ml.

Antibodies—Anti-BALF2 and anti-BMRF1 rabbit polyclonal antibodies were prepared as follows. Recombinant BALF2 or BMRF1 proteins were individually expressed in Sf21 cells infected with AcBALF2 or AcBMRF1 recombinant baculovirus and purified as described previously (31, 32). The anti-BALF2 or anti-BMRF1 protein-specific rabbit polyclonal antibodies were raised against ~200 μg of each purified protein mixed with RIBI adjuvant system R-730 (RIBI Immunochem Research) and affinity purified (33). The antibodies recognized proteins migrating at 130 and 48–52 kDa in whole cell lysates from lytic-induced B95-8 cells, demonstrating specific immunoprecipitation. Anti-BALF5 protein-specific rabbit antibody (34) was affinity-purified with the BALF5 protein coupled-Sepharose 4B as described (33).

An anti-EBV EA-D-p52/50 (BMRF1 gene product) protein-specific mouse monoclonal antibody, clone name R3, was purchased from Chemicon Inc. and anti-PCNA mouse monoclonal and rabbit polyclonal antibodies from Transduction Laboratories and Oncogene, respectively. Anti-MSH2, -MSH3, and -MSH6 monoclonal antibodies were obtained from Transduction Laboratories, BD Biosciences, and anti-hpMS2 and anti-MLH1 antibodies from Calbiochem and BD Pharmingen International, respectively. Alexa Fluor 594-conjugated anti-BrdUrd mouse monoclonal antibody was purchased from Molecular Probes, Inc. Anti-human RF-C p140 monoclonal antibody was kindly provided by Dr. Waga (Osaka University) (35) and anti-DNA polymerase δ rabbit polyclonal antibody by Dr. Tamai (Medical & Biological Laboratories Co., Ltd.). The secondary goat anti-rabbit or -mouse IgG antibodies conjugated with Alexa 488 or 594 were purchased from Molecular Probes.

Biochemical Cellular Fractionation—Tet-BZLF1/B95-8 cells (1.5 × 10⁶) treated with or without doxycycline at a concentration of 2 μg/ml were cultured and harvested at the indicated times. The cells were washed twice with phosphate-buffered saline at room temperature and were lysed for 10 min on ice with 1 ml of ice-cold 0.5% Triton X-100/mCSK buffer (10 mM Pipes, pH 6.8, 100 mM NaCl, 300 mM sucrose, 1 mM MgCl₂, 1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 0.5% Triton X-100) containing multiple protease inhibitors (protease inhibitor mixture for mammalian cell extracts, Sigma; 25 μl/ml), 200 μM Na₃VO₄, and 20 mM NaF. The samples were then subjected to centrifugation (2000 × g, 3 min, 4°C) to obtain Triton X-100-extractable nuclear fractions that were digested with 250 units/ml DNase I (10 units/ml, Roche Molecular Biochemicals) in 0.1% Triton X-100/mCSK containing 2 mM MgCl₂ and 1 mM MnCl₂ at 25 °C for 20 min. The samples were then centrifuged (2000 × g, 3 min, 4°C) to obtain the solubilized chromatin fraction and the remaining non-chromatin nuclear structures. Each sample was adjusted to the same volume by adding 2× SDS sample buffer, boiled, and aliquots corresponding to 1.8 × 10⁶ cells per lane were applied for SDS-PAGE.

Immunofluorescence Analysis—All staining procedures were carried out at room temperature except for extraction and incubation with primary antibodies. For immunofluorescence experiments, cells were washed with ice-cold PBS and extracted with 0.5% Triton X-100/mCSK buffer on ice for 2 min. Multiple protease inhibitors (Sigma; 25 μl/ml), 200 μM Na₃VO₄, and 20 mM NaF, were also added to the buffer. Cells were fixed with 70% methanol for 30 min on ice, washed with PBS, permeabilized with 0.5% Triton X-100 in PBS for 15 min, blocked for 1 h in 10% fetal calf serum in PBS, and then incubated overnight with the primary antibodies diluted in PBS containing 10% fetal calf serum. The rabbit anti-PCNA mouse monoclonal antibodies and control mouse IgG were used at 2.5 μg/ml, the anti-BALF2 rabbit polyclonal antibody at 2 μg/ml, and the control rabbit IgG and anti-BMRF1 mouse monoclonal antibodies at 5 μg/ml. The samples were then incubated for 1 h with secondary goat anti-rabbit or mouse IgG antibodies conjugated with Alexa Fluor 488 or 594. Neither of the control antibodies yielded specific signals in either single-staining or double-staining experiments.

For BrdUrd staining, newly synthesized DNA was labeled by incubating Tet-BZLF1/B95-8 cells with 10 μM BrdUrd added directly to the incubation medium for 1 h prior to harvesting. For the pulse-chase experiment, the cells were pulse-labeled with 10 μM BrdUrd for 1 h at 24 h post-induction and then chased for 24 h. Cells were washed with ice-cold PBS and extracted with 0.5% Triton X-100/mCSK buffer on ice for 2 min. Multiple protease inhibitors (Sigma; 25 μl/ml), 200 μM Na₃VO₄, and 20 mM NaF, were also added to the buffer. Cells were then fixed with 70% methanol for 30 min on ice and treated for 10 min with 2 N HCl containing 0.5% Triton X-100 to expose the incorporated BrdUrd residues before blocking. The cells were washed twice with PBS and neutralized with 0.1 M sodium tetraborate, pH 9.0, for 5 min.

For fluorescence in situ hybridization, an EBV BamHI-W fragment was labeled with Chroma Tide Alexa Fluor 594-5-dUTP (Molecular Probes, Inc.) and used for the detection of amplified EBV genomes. First, immunostaining was performed as described above and then cells were refixed in 4% paraformaldehyde to cross-link bound antibodies. After permeabilizing in 0.2% Triton X-100 (20 min on ice), cells were digested with RNase A, dehydrated in ethanol, air dried, and immediately covered with a probe mixture containing 50% formamide in 2× SSC containing probe DNA (10 ng/μl), 10% dextran sulfate, salmon sperm DNA (0.1 μg/μl), and yeast tRNA (1 μg/μl). Probes and cells were simultaneously heated at 94 °C for 4 min and incubated overnight at 37 °C. After hybridization, specimens were washed at 37 °C with 50% formamide in 2× SSC (two times for 15 min each) and then 2× SSC. Finally,
cells were equilibrated in PBS containing 0.1% Tween 20 and mounted in Vectashield (Vector Laboratories, Inc). Image acquisition was performed using a Bio-Rad Radiance 2000 confocal laser-scanning microscope equipped with a PL Apo 100 × 1.4 NA oil-immersion objective lens. Images were processed and assembled in an Apple G4 computer using Adobe Photoshop 5.0.

**Immunoblot Analysis**—Cells were harvested at the indicated times post-treatment with doxycycline, washed with PBS, and treated with lysis buffer (0.02% SDS, 0.5% Triton X-100, 300 mM NaCl, 20 mM Tris-HCl, pH 7.6, 1 mM EDTA, 1 mM dithiothreitol) for 20 min on ice. Multiple protease inhibitors, 200 μM Na3VO4, and 20 mM NaF were added to the buffer. Samples were centrifuged at 18,000 × g for 10 min at 4 °C, and clarified cell extracts were assayed for their protein concentrations using a Bio-Rad protein assay kit. Images were processed and assembled in an Apple G4 computer using Adobe Photoshop 5.0.

**Determination of Target Proteins**—Detection of target proteins was with an enhanced chemiluminescence detection system (Amersham Biosciences).

**BrdUrd-labeled Chromatin Immunodepletion Assays**—Chromatin immunodepletion assays were performed as follows. 5 × 10^7 Tet-BZLF1/B95-8 cells per sample were cultured in 100-mm plates in the presence of 2 μg/ml doxycycline for 27 h. Newly synthesized DNA was labeled by incubation with 50 μM BrdUrd added directly to the incubation medium for 3 h prior to harvesting. After washing twice in PBS, cells were lysed in 0.5% Triton X-100/mCSK buffer for 10 min on ice and centrifuged (2000 × g, 5 min) to obtain Triton X-100-extractable fractions. The Triton X-100-extracted nuclei were cross-linked in situ with 5 ml of 1% formaldehyde in 0.5% Triton X-100/mCSK buffer for 20 min at room temperature and then centrifuged at 2000 × g for 5 min. Cell pellets were suspended in Solution 1 (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.5 mM EGTA, 150 mM NaCl) at 4 °C and sonicated to reduce the average DNA fragment length to 500 – 1000 bp (verifed on agarose gels; data not shown). After centrifugation, the supernatants were reacted for 1 h with 1 μg each of anti-BALF2, anti-BMRF1, anti-MCM7-specific rabbit IgGs, anti-PCNA mouse IgG, or 1 μg of non-immune mouse or rabbit IgGs as controls. Immune complexes of proteins cross-linked to BrdUrd-incorporated DNA were precipitated with protein G-Sepharose CL-4B beads and immunodepleted samples were treated with 0.5% SDS and 0.7 mg/ml proteinase K for 5 h at 45 °C. Protein-DNA cross-links were reversed by incubation at 65 °C for 6 h and then DNA was purified using a PCR purification kit from Qiagen and recovered by precipitation with 2 volumes of ethanol and 20 μg/ml proteinase K for 3 h at 37 °C. DNA was quantified by fluorescence (PCR product) and purity was assessed by gel electrophoresis. DNA was treated with 0.5% Triton X-100/mCSK buffer. After centrifugation, Triton X-100-extracted nuclei were sonicated. The lysates were diluted with NET buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 1 mM EDTA). The aliquots were mixed with 1.5 μg each of control IgG, anti-BMRF1, -PCNA, -MSH2, -MSH3, and -MSH6 mouse monoclonal antibodies, respectively, for 90 min at 4 °C. After the addition of 20 μl of Protein G-Sepharose beads, the mixtures were further incubated for 1 h at 4 °C, rocking gently. The beads were then washed with NET buffer, and the immunoprecipitates were boiled in SDS sample buffer without β-mercaptoethanol. The supernatant was collected, added to β-mercaptoethanol, reboiled, and subjected to immunoblot analysis.

**Asdy of PAA, a herpesvirus DNA polymerase inhibitor, viral prereplicative sites represented by staining of the BALF2 protein were formed (Fig. 1A, panels a and c). The sites stained with anti-BMRF1 protein-specific antibodies completely coincided with the foci of newly synthesized viral DNA as judged by BrdUrd incorporation and fluorescence in situ hybridization analyses on confocal immunofluorescence analyses. Furthermore, we assumed that viral DNA would move out of the nucleus as a component of maturing virus but cellular DNA would remain in the nucleus and so assessed the fate of incorporated BrdUrd label by chasing newly synthesized DNA. When Tet-BZLF1/B95-8 cells were pulse-labeled with BrdUrd for 1 h at 24 h post-induction and then chased for 24 h, fluorescence intensity of BrdUrd-labeled DNA in nucleus was greatly reduced during the chase period (compare Fig. 1A, panels a and b). Thus, the demonstration that a majority of the BrdUrd pulse-labeled DNA moved out of the nucleus with time clarified that BrdUrd-labeled DNAs at 24 h post-induction are mostly viral and not cellular DNAs. Collectively, because BMRF1 protein-localized sites represent loci of viral DNA synthesis, BMRF1 protein staining was used as a marker for viral replication compartments.

**To investigate whether the MMR system is involved in EBV lytic DNA replication, we first examined subcellular localization of PCNA, a protein involved in the early step of MMR, in lytic replication-induced Tet-BZLF1/B95-8 cells (Fig. 1B). PCNA is an abundant protein that is generally distributed throughout the nucleus. However, in S phase, it appears to be found in foci of aggregate that have been shown to colocalize with newly replicated DNA labeled with BrdUrd in nuclei (38). Because both hMSH6 and hMSH3 are known to colocalize with PCNA to these foci (20), it has been suggested that the MMR system is coupled with chromosomal DNA replication. As shown in Fig. 1B, PCNA was found to become resistant to mild detergent extraction and was localized as distinct spots within the BMRF1 protein-localized replication compartments. In contrast, PCNA was sensitive to detergent treatment in most of the latently infected cells (data not shown), although in a portion of the cells PCNA was resistant to detergent extraction and was observed as punctate spots throughout nuclei (Fig. 1B, panel c), probably representing in S phase cells. As shown in Fig. 1C, BALF2 single-stranded DNA-binding proteins were also co-distributed as distinct spots within viral replication compartments. PCNA staining loci coincided with and/or were nearby those for the BALF2 protein (Fig. 1C, panels a and b). When viral DNA replication was blocked by the addition of PAA, a herpesvirus DNA polymerase inhibitor, viral prereplicative sites represented by staining of the BALF2 protein were formed (Fig. 1C, panel c), as reported previously (39). It remains unclear how the BALF2 protein is recruited to the sites. However, PCNA was solubilized by detergent treatment and not evident at the BALF2 protein-localized prereplicative sites in nuclei. Thus, PCNA appears to localize at viral replication compartments when viral DNA genomes are synthesized.
Association of MMR Factors with EBV DNA Replication Sites

PCNA clamp by itself does not possess DNA binding activity and needs to interact with the clamp loader, RF-C, for loading onto DNA in an ATP-dependent manner (40). As shown in Fig. 1D, RF-C was found to completely colocalize with PCNA within the replication compartments.

Although Total Levels of PCNA Remain Constant, the DNA-Bound Form Increases as Lytic Infection Proceeds—To investigate whether the levels of PCNA change during lytic replication, we prepared whole cell lysates from lytic program-induced Tet-BZLF1/B95-8 cells at the indicated times and analyzed them by immunoblotting. As shown in Fig. 2A, the levels of PCNA proved constant throughout the productive infection.

We have previously demonstrated subcellular dynamics of EBNA1, viral replication proteins, core histones, and human chromosomal DNA replication initiation proteins such as CDC6, MCM4, and MCM7 in lytic program-induced Tet-BZLF1/B95-8 cells using biochemical fractionation methods (28, 41). In this method, cells were first treated with mCSK buffer containing the nonionic detergent Triton X-100 in relatively physiological conditions, extracting not only cytoplasmic proteins but also nuclear proteins that are not tightly bound to nuclear structures. Detergent-extracted nuclei were digested with DNase I to remove the bulk of chromosomal and EBV DNA, releasing DNA-binding proteins into DNAse I-solubilized supernatants. The proteins remaining in the pellets have been shown to bind to nuclear matrix (33, 37). As shown in Fig. 2B, about two-thirds of MCM7 protein was extracted with the buffer and most of the remainder was solubilized after DNase I treatment, showing their chromatin binding. Core histones were almost resistant to detergent treatment but were liberated by DNase I digestion. On the other hand, almost all of the CDC6 protein was detected in the non-detergent-extractable fraction and was resistant to DNase I treatment. These results were consistent with our previous reports (28, 41). As shown in Fig. 2B, about half of the BALF2 single-stranded DNA-binding protein was extracted with mild detergent buffer at 24 h post-induction, whereas almost all of the BMRF1 protein remained bound. Most of remainder was solubilized after DNase I treatment. Levels of the DNA-bound form of the BALF2 protein increased with progression of the lytic replication. Broad bands of the BMRF1 protein in SDS-PAGE were due to different phosphorylation states as judged by phosphatase treatment (data not shown), indicating that most PCNA exists as a DNA-unbound form in the latent phase of EBV replication. After induction of lytic replication the levels of the DNA-bound form increased with time to attain about 40% of the total after 48 h post-induction (Fig. 2, B and D).

FIGURE 1. Accumulation of PCNA in viral replication compartments after induction of lytic replication. Lytic replication-induced Tet-BZLF1/B95-8 cells were harvested at the indicated times, extracted with 0.5% Triton X-100/mCSK buffer, and processed as described under “Experimental Procedures.” A, shown are profiles of the BMRF1 protein (green) and BrdUrd-incorporated DNA (red) (panels a and b) or viral DNA hybridized with the EBV BamHI-W fragment labeled with Chroma Tide Alexa 594-dUTP (red) (panel c). Cells were pulse-labeled with 10 μM BrdUrd for 1 h at 23 h post-induction (h.p.i.) and harvested at 24 h post-induction (panel a). Cells were pulse-labeled with 10 μM BrdUrd for 1 h at 23 h post-induction and then chased for 24 h (panel b). B, shown are profiles of BMRF1 (green) and PCNA (red) proteins. Panels a and b are for early (16 h post-induction) and late (36 h post-induction) stages of EBV lytic replication, respectively. Panel c presents profiles in S-phase cells before induction of lytic replication. C, profiles of BALF2 (green) and PCNA (red) proteins. Panels a and b are for early (16 h post-induction) and late (36 h post-induction) stages of EBV lytic replication, respectively. Panel c presents profiles in the presence of PAA. D, profiles of RF-C (green) and PCNA (red) proteins at an early (16 h post-induction) stage of EBV lytic replication.
PAA inhibits lytic viral DNA replication but not chromosomal DNA replication although EBV immediate-early and early proteins are expressed (28, 30). In the presence of PAA, levels of the DNA bound form of the BMRF1 protein were found to be low while almost half of the BALF2 protein was bound (Fig. 2, C and D), well consistent with the observations of the confocal microscopy study (Fig. 1, B and C).
More than half of the BALF2 protein was retained in the pellet fraction even after DNase I treatment (Fig. 2C, lower panel). The BALF2 protein might interact with the cellular structure rather than to DNA in the presence of PAA. Alternatively, the protein might behave like the HSV ICP8 single-stranded DNA-binding protein, which can assemble into long filamentous structures in the absence of single-stranded DNA (43). The levels of the DNA-bound form of PCNA were also very low as well as those of the BMRF1 protein and essentially constant before and after addition of doxycycline (Fig. 2, C and D), consistent with the results that PCNA staining was not observed in the confocal immunofluorescence study when viral DNA synthesis was blocked by PAA (Fig. 1C, panel c). These observations strongly suggest that PCNA is actively loaded onto EBV genomes as well as BMRF1 protein when viral DNA genomes are synthesized during lytic replication.

PCNA Is Loaded onto Newly Synthesized Viral DNA during the EBV Lytic Replication—To determine further whether PCNA is loaded onto newly synthesized viral DNA, BrdUrd-labeled chromatin immunodepletion analyses were performed. During EBV lytic infection chromosomal DNA replication is arrested while viral DNA synthesis occurs, although the molecular mechanisms remain unknown (28, 30). Furthermore, as was demonstrated in the pulse-chase experiment and fluorescence in situ hybridization analysis of Fig. 1A, almost all BrdUrd-incorporating DNA is thus mostly viral newly synthesized DNA. Lytic infection-induced Tet-BZLF1/B95-8 cells were pulse-labeled with BrdUrd for 2 h prior to harvesting and then treated with 0.5% Triton X-100/mCSK buffer, cross-linked in situ with formaldehyde, and sonicated to reduce the average DNA fragment size. After samples were reacted with anti-BALF2-, anti-BMRF1-, anti-PCNA-, or anti-MCM7-specific IgGs or non-immune mouse or rabbit IgGs as control, immune complexes of proteins cross-linked to BrdUrd-labeled DNA were depleted from the samples using protein G-Sepharose CL-4B beads. DNA was purified from the immunodepleted samples, blotted to membrane, and stained with anti-BrdUrd monoclonal antibody, and secondary horseradish peroxidase-labeled anti-mouse IgG (upper panel). Signal intensities were obtained and quantified with a LuminoVision Image Analyzer. The proportion of signal intensity immunodepleted by each indicated antibody was calculated with that by the control IgG taken as 100%. Data are mean of two independent experiments (lower panel).

To confirm whether each antibody can immunodeplete the corresponding protein, samples untreated with formaldehyde fixation were prepared in parallel and immunodepleted with each primary antibody and protein G-Sepharose CL-4B beads. As shown in Fig. 3A, each specific antibody could immunodeplete the corresponding protein from the samples except non-immune mouse or rabbit IgGs. As shown in Fig. 3B, the anti-BALF2- and -BMRF1-specific IgGs immunodepleted ~80% of BrdUrd-labeled DNA from the samples, demonstrating that these viral replication proteins resistant to the detergent treatments bind to newly synthesized viral DNAs. Similarly, anti-PCNA-specific IgG also depleted BrdUrd-labeled DNA, whereas non-immune control IgG did not. The detergent-resistant form of MCM7 protein was localized outside of the viral replication compartments in nuclei, suggesting chromosomal DNA binding of the MCM complex. The anti-MCM7-specific IgG could not deplete BrdUrd-labeled DNA as much. These observations further support the idea that PCNA is loaded onto viral DNA genomes synthesized after induction of lytic infection.

MSH6, MSH2, MLH1, and hPMS2 Co-localize with PCNA to Viral Replication Compartments—PCNA interacts with multiple partners including MMR proteins (17). To determine whether MMR is also involved in viral genome replication, we examined localization of MMR

\[ A \quad \text{IP supernatant} \]

- control IgG
- anti-BALF2
- anti-BMRF1
- anti-PCNA
- anti-MCM7

**FIGURE 3.** PCNA is loaded onto newly synthesized DNA during EBV lytic replication as assessed by the BrdUrd-labeled DNA immunodepletion assay. Lytic replication was induced in Tet-BZLF1/B95-8 cells and newly synthesized DNAs were labeled by incubation with 50 μM BrdUrd 3 h prior to harvesting. The cells were subjected to BrdUrd-labeled chromatin immunodepletion assays as described under “Experimental Procedures.” Cross-linked DNA was immunoprecipitated (IP) using anti-BALF2, -BMRF1, -MCM7 rabbit polyclonal antibodies, and anti-PCNA mouse monoclonal antibody, respectively, whereas non-immune mouse or rabbit IgGs were applied as negative controls. A, samples untreated with formaldehyde fixation were prepared and processed as described under “Experimental Procedures.” The indicated protein was immunodepleted from the samples with each corresponding antibody and protein G-Sepharose beads. The immunodepleted supernatants were analyzed by immunoblotting with the indicated antibodies. B, quantification of BrdUrd-incorporated DNAs by immunoblotting with an anti-BrdUrd antibody. Samples treated with formaldehyde fixation were prepared and processed in a similar way. BrdUrd-incorporated DNA was blotted to Hybond N+ membrane and detected with 5 μg/ml anti-BrdUrd monoclonal antibody, and secondary horseradish peroxidase-labeled anti-mouse IgG (upper panel). Signal intensities were obtained and quantified with a LuminoVision Image Analyzer. The proportion of signal intensity immunodepleted by each indicated antibody was calculated with that by the control IgG taken as 100%. Data are mean of two independent experiments (lower panel).

\[ \text{Percentage of control} \]

- cont IgG
- BALF2
- BMRF1
- PCNA
- MCM7

3 A. Kudoh, T. Daikoku, and T. Tsurumi, unpublished result.
proteins in the lytic replication-induced nucleus using specific antibodies to MSH6, MSH2, MLH1, and hPMS2. Lytic replication-induced cells were treated with a buffer containing detergent and then applied for confocal microscopy. Confocal immunofluorescence studies showed that in cells undergoing viral productive DNA replication, MSH6, MSH2, MLH1, and hPMS2 co-localized with PCNA in the viral replication compartments. Fig. 4A shows that the overlap of PCNA and the MutS homologue (MSH6 and MSH2) signals is essentially complete, consistent with the reported direct interaction between PCNA and MutS to form a complex, searching for mismatched sites during DNA replication (19, 21). On the other hand, although MutL homologues (MLH1 and hPMS2) were localized within PCNA staining sites, free excess PCNA signals were visible in the merged images (Fig. 4A, panels c and d). This might reflect assembly of the eukaryotic MLH1-containing heterodimeric complexes with either MSH2-MSH6 or MSH2-MSH3 at mispaired bases in the DNA (44–46). Thus, our results clearly showed that proteins involved in MMR are redistributed to viral replication compartments, this possibly being mediated by PCNA.

DNA polymerase δ- and ε-associated exonucleases and DNA polymerase δ, respectively, function in excision of single-stranded DNA and accurate repair DNA synthesis in late steps of the MMR (2, 10, 47). These polymerases, however, were not detected in viral replication compartments (Fig. 4B, panels a and b), although signals for polymerase δ and ε were detected in S-phase nuclei of uninduced cells (data not shown). On the other hand, the BALF5 polymerase catalytic protein was overlapped with PCNA and MSH2 in viral replication compartments (Fig. 4B, panels c and d).

The anti-PCNA, -MSH2, -MSH3, or -MSH6 Antibody Immunoprecipitated BMRF1 Replication Protein—To further determine whether PCNA and the MMR proteins were loaded onto viral genome DNA during lytic replication, immunoprecipitation analyses were performed. It has been predicted that the BMRF1 protein not only acts as a viral polymerase processivity factor but also binds to the entire viral genome synthesized during lytic replication (28). Lytic infection-induced Tet-BZLF1/B95-8 cells were harvested at 48 h post-induction, treated with 0.5% Triton X-100/mCSK buffer to remove detergent-soluble proteins, and then sonicated. After samples were reacted with anti-BMRF1-, -PCNA, -MSH2, -MSH3, or -MSH6 mouse monoclonal antibody for 90 min at 4 °C, Samples were subjected to immunoblot analysis with anti-BMRF1 or -PCNA mouse monoclonal antibody.

FIGURE 4. Co-localization of mismatch repair proteins, MSH6, MSH2, MLH1, or hPMS2 with PCNA involved in the early steps of MMR within viral replication compartments. Tet-BZLF1/B95-8 cells were cultured in the presence of 2 μg of doxycycline/ml, harvested at 24 h post-induction, and treated with 0.5% Triton X-100/mCSK buffer. The nonionic detergent-extracted cells were fixed with methanol and double immunostained with the indicated antibodies. A, shown are profiles of MSH6, MSH2, MLH1, and hPMS2 (green) with PCNA (red), immunostained with anti-MSH6, -MSH2, -MLH1, and -hPMS2 mouse monoclonal antibodies and anti-PCNA rabbit polyclonal antibody, respectively. B, shown are profiles of DNA polymerase δ and ε and BALF5 protein (green) with PCNA, MSH2, or BMRF1 protein (red), immunostained with anti-polymerase δ, -BMRF1, -PCNA, -MSH2 mouse monoclonal antibodies and anti-PCNA, -polymerase δ, -BALF5 rabbit polyclonal antibodies. C, co-immunoprecipitation of BMRF1 replication protein by anti-PCNA, -MSH2, -MSH3, or -MSH6 antibody. Tet-BZLF1/B95-8 cells were harvested at 72 h post-induction, and treated with 0.5% Triton X-100/mCSK buffer. After centrifugation, Triton X-100-extracted nuclei fraction were sonicated and the resultant lysates were diluted with NET gel buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 1 mM EDTA) and the aliquots were mixed with 1.5 μg each of control IgG, anti-BMRF1, -PCNA, -MSH2, -MSH3, or -MSH6 mouse monoclonal antibody for 90 min at 4 °C. Samples were subjected to immunoblot analysis with anti-BMRF1 or -PCNA mouse monoclonal antibody.
probably due to an excess amount of the viral genome-bound form of BMRF1 protein compared with those for PCNA and the MMR proteins. The anti-MSH2 antibody immunoprecipitated PCNA from the sample and vice versa, suggesting a complex formation between PCNA and MutSα. These observations further support the idea that PCNA and a series of MMR proteins are associated with viral DNA genomes during the lytic infection.

**DISCUSSION**

The present study demonstrated that PCNA and a series of MMR factors involved in the early steps of MMR are recruited to the sites of viral genome synthesis during EBV lytic infection, suggesting coupling of MMR and EBV genome DNA synthesis as is known for the eukaryotic chromosomal DNA replication system. The BrdU pulse-chase experiment demonstrated that BrdUrd-labeled DNA moved out of the nucleus during the chase period (Fig. 1A, *panels a and b*). From this observation and the BrdUrd-labeled DNA immunodepletion experiments it is strongly suggested that the material associated with PCNA and a series of MMR proteins should be viral DNA. It might be necessary for PCNA to interact with a mismatch binding factor(s) during the early stages of the process (12, 13) to be loaded and to remain bound at strand interruptions (48).

Mismatch excision in eukaryotic cells might be conducted by 3′ to 5′ single-stranded DNA exonucleases intrinsic to replicative polymerases. Consistent with this idea is evidence implicating the 3′ to 5′ exonucleases of polymerase δ and polymerase ε in mismatch excision (9, 47). Also, the requirement for PCNA as a processivity factor with DNA polymerase δ during the late, gap-filling step of MMR is clear. However, neither polymerase δ nor polymerase ε could be detected in viral replication compartments in the present study, whereas the BALE5 EBV DNA polymerase was distributed within the replication compartments (28). It might be possible that a EBV DNA polymerase possessing intrinsic 3′ to 5′ exonuclease could substitute for mismatch excision and accurate repair resynthesis at late stages of MMR during lytic replication, although we cannot deny the possibility of involvement of host DNA repair polymerases in the steps. This issue remains to be clarified.

To test for physical interactions between viral replication proteins and the sets of MMR proteins, co-immunoprecipitation experiments were performed using cellular lysates from Tett-BZLF1/B95-8 cells after induction of lytic replication. Although the anti-BMRF1, -BALF2, -BALF5, or -BBLF2/3 protein-specific antibody could not immunoprecipitate any PCNA or MMR proteins (data not shown), the anti-PCNA, -MSH2, -MSH3, and -MSH6 antibodies could immunoprecipitate the BMRF1 protein (Fig. 4C). DNase I treatment of the samples resulted in failure of precipitation of the BMRF1 protein with these antibodies (data not shown), suggesting no specific protein-protein interactions between viral and MMR proteins. Thus, it is likely that PCNA and the MMR factors interact with the BMRF1 protein via viral genome DNAs. The coupling of MMR and EBV lytic DNA replication could occur either by targeting MMR proteins to synthesized DNA or by incorporating MMR proteins as part of the viral replication machinery. Overall, the former hypothesis may be more likely.

With herpes simplex virus type 1, it has been reported that PCNA similarly accumulates in viral replication compartments (49, 50). Furthermore, Taylor and Knipe (51) have recently found using immunoprecipitation and mass spectrometry over 50 cellular and viral proteins including PCNA, MSH2, MSH3, and MSH6 to be associated with the herpes simplex virus type 1 ICP8 single-stranded DNA-binding protein. Our observations with MMR in the EBV system thus strongly suggests similar roles also in herpes simplex virus type 1 DNA replication.

Although mismatch repair systems play a primary role in mutation avoidance by removing base-base and small insertion-deletion mismatches that arise during DNA replication, MMR factors are also required for the repair of mismatches in heteroduplex DNA (52, 53). Recombination between two similar but nonidentical sequences will form a hetero-duplex intermediate containing mispaired DNA and such mismatches might be corrected by MMR (54, 55). In addition, MMR acts to inhibit recombination between moderately divergent (homologous) sequences (56, 57). During EBV lytic infection, homologous recombination also occurs frequently between identical DNA sequences (58) and the interaction of MMR factors with mismatches that arise in hetero-duplex DNA or possibly with other structures, such as Holliday junctions during recombination in lytic replication as an alternative possibility of involvement of the MMR system in EBV genome replication.

It is known that MMR proteins are required for both cell cycle arrest and induction of apoptosis by DNA damage (59, 60). Although the specific mechanisms have yet to be identified, one hypothesis is that MMR proteins recognize misincorporated nucleotides or damaged base sites as mismatches and during attempts to repair them DNA breaks and gaps might be continuously produced and consequently cell cycle arrest and apoptosis are activated. However, the EBV DNA polymerase possesses an intrinsic proofreading 3′ to 5′ exonuclease activity (34, 61), fidelity of viral genome replication is thought to be high. Furthermore, the HSV DNA polymerase is more accurate than purified eukaryotic DNA polymerases (62). Thus, coupling of the MMR system with viral genome synthesis might function to increase viral genome integrity, rather than to activate cytotoxic responses.

**Acknowledgments**—We thank Dr. S. Waga (Osaka University) for providing RF-C p140 monoclonal antibody and Dr. Hiroshi Kumimoto (Aichi Cancer Center) for technical suggestions and providing anti-MLH1 and -hPMS2 mouse monoclonal antibodies.

**REFERENCES**

1. Modrich, P., and Lahue, R. (1996) *Annu. Rev. Biochem.* 65, 101–133
2. Kunkel, T. A., and Erie, D. A. (2005) *Annu. Rev. Biochem.* 74, 681–710
3. Lopez de Saro, F. J., and O’Donnell, M. (2001) *Proc. Natl. Acad. Sci. U. S. A.* 98, 8376–8380
4. Su, S. S., Lahue, R. S., Au, K. G., and Modrich, P. (1988) *J. Biol. Chem.* 263, 6829–6835
5. Jiricny, J., Su, S. S., Wood, S. G., and Modrich, P. (1988) *Nucleic Acids Res.* 16, 7843–7853
6. Parker, B. O., and Marinus, M. G. (1992) *Proc. Natl. Acad. Sci. U. S. A.* 89, 1730–1734
7. Kolodner, R. D., and Marsischky, G. T. (1999) *Curr. Opin. Genet. Dev.* 9, 89–96
8. Jiricny, J. (2000) *Nat. Genet.* 24, 6–8
9. Tran, H. T., Gordonin, D. A., and Resnick, M. A. (1999) *Mol. Cell. Biol.* 19, 2000–2007
10. Longley, M. J., Pierce, A. J., and Modrich, P. (1997) *J. Biol. Chem.* 272, 10917–10921
11. Johnson, R. E., Kovvali, G. K., Guzder, S. N., Amin, N. S., Holm, C., Habraken, Y., Sung, P., Prakash, L., and Prakash, S. (1996) *J. Biol. Chem.* 271, 27987–27990
12. Uma, A., Buermeyer, A. B., Simon, J. A., Thomas, D. C., Clark, A. B., Liskay, R. M., and Kunkel, T. A. (1996) *Cell* 87, 65–73
13. Gu, L., Hong, Y., McCulloch, S., Watanabe, H., and Li, G. M. (1998) *Nucleic Acids Res.* 26, 1173–1178
14. Lin, Y. L., Shiyii, M. K., Chen, C., Kolodner, R., Wood, R. D., and Dutta, A. (1998) *J. Biol. Chem.* 273, 1453–1461
15. Szankasi, P., and Smith, G. R. (1995) *Science* 267, 1166–1169
16. Tishkoff, D. X., Boerger, A. L., Bertrand, P., Filosi, N., Gaida, G. M., Kane, M. F., and Kolodner, R. D. (1997) *Proc. Natl. Acad. Sci. U. S. A.* 94, 7487–7492
17. Maga, G., and Hubscher, U. (2003) *J. Cell Biol.* 161, 3051–3060
18. Clark, A. B., Valle, F., Drotschmann, K., Gary, R. K., and Kunkel, T. A. (2000) *J. Biol. Chem.* 275, 36498–36501
19. Flores-Rozas, H., Clark, D., and Kolodner, R. D. (2003) *Nat. Genet.* 36, 375–378
20. Kleczykowska, H. E., Marra, G., Lettieri, T., and Jiricny, J. (2001) *Genes Dev.* 15, 724–736
21. Lau, P. J., and Kolodner, R. D. (2003) *J. Biol. Chem.* 278, 14–17
22. Cai, J., Uhlmann, F., Gibbs, E., Flores-Rozas, H., Lee, C. G., Phillips, B., Finkelstein, J.
Association of MMR Factors with EBV DNA Replication Sites

Yao, N., O’Donnell, M., and Hurwitz, J. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 12896–12901

23. Yao, N., Turner, J., Kelman, Z., Stukenberg, P. T., Dean, F., Shechter, D., Pan, Z. Q., Hurwitz, J., and O’Donnell, M. (1996) Genes Cells 1, 101–113

24. Zhang, G., Gibbs, E., Kelman, Z., O’Donnell, M., and Hurwitz, J. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 1869–1874

25. Xie, Y., Counter, C., and Alani, E. (1999) Genetics 151, 499–509

26. Baer, R., Bankier, A. T., Biggin, M. D., Deininger, P. L., Farrell, P. J., Gibson, T. J., Hatfull, G., Hudson, G. S., Satchwell, S. C., Seguin, C., Tuffnell, P. S., and Barrell, B. G. (1984) Nature 310, 207–211

27. Fixman, E. D., Hayward, G. S., and Hayward, S. D. (1995) J. Virol. 69, 2998–3006

28. Daikoku, T., Kudoh, A., Fujita, M., Sugaya, Y., Isomura, H., Shirata, N., and Tsurumi, T. (2004) J. Biol. Chem. 279, 54817–54825

29. Chen, M. R., Chang, S. J., Huang, H., and Chen, J. Y. (2000) J. Virol. 74, 3093–3104

30. D’Andrea, A. K., and Hickman, J. W. (2000) J. Biol. Chem. 275, 19473–19476

31. Tsurumi, T. (1991) Virology 182, 376–381

32. Abbotts, J., Nishiyama, Y., Yoshida, S., and Loeb, L. A. (1987) Nucleic Acids Res. 15, 1185–1198