

## Phosphorylation of MCM4 at Sites Inactivating DNA Helicase Activity of the MCM4-MCM6-MCM7 Complex during Epstein-Barr Virus Productive Replication

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**Induction of Epstein-Barr virus (EBV) lytic replication blocks chromosomal DNA replication notwithstanding an S-phase-like cellular environment with high cyclin-dependent kinase (CDK) activity. We report here that the phosphorylated form of MCM4, a subunit of the MCM complex essential for chromosomal DNA replication, increases with progression of lytic replication, Thr-19 and Thr-110 being CDK2/CDK1 targets whose phosphorylation inactivates MCM4-MCM6-MCM7 (MCM4-6-7) complex-associated DNA helicase. Expression of EBV-encoded protein kinase (EBV-PK) in HeLa cells caused phosphorylation of these sites on MCM4, leading to cell growth arrest. In vitro, the sites of MCM4 of the MCM4-6-7 hexamer were confirmed to be phosphorylated with EBV-PK, with the same loss of helicase activity as with CDK2/cyclin A. Introducing mutations in the N-terminal six Ser and Thr residues of MCM4 reduced the inhibition by CDK2/cyclin A, while EBV-PK inhibited the helicase activities of both wild-type and mutant MCM4-6-7 hexamers, probably since EBV-PK can phosphorylate MCM6 and another site(s) of MCM4 in addition to the N-terminal residues. Therefore, phosphorylation of the MCM complex by redundant actions of CDK and EBV-PK during lytic replication might provide one mechanism to block chromosomal DNA replication in the infected cells through inactivation of DNA unwinding by the MCM4-6-7 complex.**

A number of replication initiation sites that are present in the genome of eukaryotic cells are utilized in a temporal order during the DNA synthesis (S) phase of the cell cycle. Reinitiation of DNA replication is prevented, and only a single round of DNA replication is performed in a cell cycle. This DNA replication by the so-called replication licensing system is regulated by the loading of the minichromosome maintenance (MCM) complexes on chromatin DNA and their phosphorylation (37, 50, 51).

During the G<sub>1</sub> phase of the cell cycle, replication origins in DNA are licensed by the assembly of prereplicative complexes (pre-RC) comprising the origin recognition complex (ORC), Cdc6, Cdt1, and the MCM complex (47, 51). The ORC binds to origins of DNA replication and remains bound during most of the cell cycle (30, 40, 48). Cdc6 and Cdt1 then bind to the complex and facilitate the loading of the MCM2-MCM7 (MCM2-7) complex. Cdt1 itself is regulated by geminin, which blocks the binding of the MCM complex to the pre-RC (39, 46, 54). Activation of the pre-RC occurs at the G<sub>1</sub>/S boundary after licensing and is mediated by the action of S-phase cyclin-dependent kinases (CDKs), primarily cyclin A/CDK2, cyclin E/CDK2, and Cdc7/Dbf4 (3, 10, 42, 45), which trigger a chain of reactions that lead to the binding of Cdc45 to the

origin and phosphorylation of Cdc6 and the MCM complex. As a result, the DNA duplex unwinds, facilitating loading of the DNA polymerase machinery (24, 41, 52, 59). The phosphorylation of key components of this process by the CDKs leads to initiation of replication and at the same time helps to prevent rereplication during the S and G<sub>2</sub>/M phases of the cell cycle (6, 7, 23, 55).

All of the members of the MCM protein family contain highly conserved DNA-dependent ATPase motifs in the central domain (3, 44) and form several stable subassemblies, including MCM2-3-4-5-6-7, MCM2-4-6-7, MCM4-6-7, and MCM3-5 complexes (35, 49, 56). DNA helicase activity has been identified in the MCM4-6-7 complexes of human, mouse, and fission yeast (*Schizosaccharomyces pombe*) (19, 35, 36, 56), while MCM2 and MCM3-5 are known to inhibit this activity by converting the double-trimer structure into a heterotetramer or a heteropentamer (43, 56). MCM4-6-7 proteins form trimers or hexamers to function as DNA helicases in vitro (37). Such DNA helicase activity is not processive under standard conditions of a DNA helicase assay. During S phase, MCM proteins are released from origins of replication after initiation of DNA replication and move with replication forks, where they are thought to function as DNA helicases. The mechanisms ensuring replication of DNA only once per cycle involve release of MCM proteins from chromatin after firing of the origins of replication and prevention of reloading (2, 11). Moreover, the phosphorylation of MCM4 with CDK2/cyclin A is associated with inactivation of the DNA helicase (unwinding) activity of the

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MCM4-6-7 complex (22) and the sites critical for phosphorylation have been determined previously (20, 22).

Primary infection with Epstein-Barr virus (EBV), a human herpesvirus that infects 90% of individuals, targets resting B lymphocytes and induces their continuous proliferation. In B lymphoblastoid cell lines, there is no production of virus particles (this being termed latent infection), but EBV-infected cell lines sometimes switch from a latent stage of infection into a virus-productive lytic stage, featuring an ordered cascade of viral early and late gene expression. The lytic phase of EBV DNA replication is dependent on seven viral replication proteins and occurs in discrete sites in nuclei, called replication compartments (8). We have demonstrated previously that induction of the EBV lytic program results in inhibition of replication of chromosomal DNA despite replication of viral DNA (32). However, rather high S-phase CDK activity is associated with progression of lytic infection, indicating that the lytic replication occurs under S-phase-like cellular conditions (31–33).

The EBV BGLF4 gene product is the only viral protein kinase which belongs to the group of herpesvirus-encoded Ser/Thr protein kinases, conserved in all herpesviruses (27). Expressed at an early stage after viral reactivation and localized in nuclei, the protein is reported to phosphorylate a number of viral and cellular proteins, including the EBV BMRF1 DNA polymerase processivity factor (EA-D) (5), the EBV nuclear antigen leader protein (EBNA-LP) (26), EBNA2 (58), protein kinase itself, and the cellular translation elongation factor 1 $\delta$  (26, 28). Most interestingly, EBV-encoded protein kinase (EBV-PK) phosphorylates EBNA-LP and elongation factor 1 $\delta$  at the same sites as CDK1 (Cdc2). However, hitherto there has been no evidence that such phosphorylation influences functions of target proteins.

We report here that human MCM4, a subunit of the MCM complex, is phosphorylated in B95-8 and Akata cells when lytic replication is induced. The phosphorylation sites of Thr-19 and Thr-110 in MCM4 were identified as the same amino acid residues targeted by CDK2/CDK1, which inactivates DNA helicase activity of the MCM4-6-7 complex. Furthermore, expression of EBV-PK in HeLa cells phosphorylated the same sites on MCM4. In vitro, the site of the MCM4 protein of the MCM4-6-7 complex was confirmed to be phosphorylated with EBV-PK, with the same loss of enzyme activity as with CDK2/cyclin A. Although introducing mutations in the N-terminal six Ser and Thr residues of MCM4 reduced the inhibition by CDK2/cyclin A, EBV-PK inhibited the helicase activities of both wild-type (WT) and mutant MCM4-6-7 hexamers. It was found that EBV-PK can phosphorylate MCM6 and other sites on MCM4 in addition to the N-terminal residues. Thus, phosphorylation of the MCM4-6-7 complex by redundant actions of CDK and EBV-PK during lytic replication might inhibit chromosomal DNA replication, including rereplication of chromosomal DNA and/or DNA fork progression, through inactivation of DNA unwinding by the MCM4-6-7 complex.

#### MATERIALS AND METHODS

**Cells.** Tet-BZLF1/B95-8 cells, a marmoset B-cell line latently infected with EBV (32), and Tet-BZLF1/Akata cells, human EBV-positive Burkitt's lymphoma cells (31), were maintained in RPMI medium supplemented with 1  $\mu$ g/ml of puromycin, 250  $\mu$ g/ml of hygromycin B, and 10% tetracycline-free fetal calf

serum. To induce lytic EBV replication, a tetracycline derivative, doxycycline, was added to the culture medium at a final concentration of 2  $\mu$ g/ml. HeLa cells were cultured in Dulbecco's modified Eagle's medium (Sigma) containing 10% fetal calf serum.

**Protein preparation.** Cells were harvested, washed with phosphate-buffer saline (PBS), and treated with lysis buffer (0.02% sodium dodecyl sulfate [SDS], 0.5% Triton X-100, 300 mM NaCl, 20 mM Tris-HCl [pH 7.6], 1 mM EDTA, 1 mM dithiothreitol [DTT]) for 20 min on ice. After multiple protease inhibitors (25  $\mu$ l/ml; Sigma), 200  $\mu$ M sodium vanadate, and 20 mM sodium fluoride were added, samples were centrifuged at 18,000  $\times$  g for 10 min at 4°C, and clarified cell extracts were assayed for protein concentration by use of a Bio-Rad kit.

**Cellular fractionation.** Tet-BZLF1/B95-8 cells ( $1.5 \times 10^7$ ) were harvested, washed with cold PBS, and then lysed for 10 min on ice with 1 ml of ice-cold modified cytoskeleton (mCSK) buffer {10 mM PIPES [piperazine-*N,N'*-bis(2-ethanesulfonic acid)], pH 6.8, 100 mM NaCl, 300 mM sucrose, 1 mM MgCl<sub>2</sub>, 1 mM EGTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 10  $\mu$ g/ml aprotinin} containing 0.5% Triton X-100, multiple protease inhibitors (protease inhibitor mixture for mammalian cell extracts, 25  $\mu$ l/ml; Sigma), 0.1 mM ATP, 200 mM sodium orthovanadate, and 20 mM NaF. The samples were then subjected to centrifugation (2,000  $\times$  g, 3 min, 4°C) for separation into a Triton X-100-extractable fraction and a nuclear pellet. The latter was then washed once with mCSK buffer and resuspended in the same buffer containing 0.1% Triton X-100, 1 mM ATP, and 4 mM MgCl<sub>2</sub> at  $1.5 \times 10^7$  nuclei/ml. To separate phosphorylated forms of MCM proteins, samples were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (7.5% polyacrylamide gels [acrylamide, 72; bisacrylamide, 1]).

**Antibodies.** Primary monoclonal antibodies to MCM6, the BMRF1 protein, and Cdk2 were purchased from Santa Cruz (MCM6 H-300), Chemicon (EBV BMRF1-R3), and BD Biosciences (CDK2 clone 55), respectively. Rabbit polyclonal antibodies to the BZLF protein and MCM7 proteins were prepared as described previously (13, 32). Anti-BGLF4 protein antibody was provided by Y. Kawaguchi (Tokyo University) (25). Antisera against phosphothreonine at amino acid (aa) 19 or 110 of human MCM4 were also prepared as detailed earlier (21). MCM4 was detected either with a monoclonal antibody (kindly provided by K. Tamai, MBL Co.) or with a commercially available polyclonal antibody (BD Biosciences). Highly cross-absorbed secondary reagents for dual-color detection (Alexa-488 and -594) were from Molecular Probes.

**Immunoblot analysis.** Equal amounts of proteins were subjected to SDS-10% PAGE (acrylamide, 29.2; bisacrylamide, 0.8). To separate phosphorylated form of MCM proteins, gradient SDS-PAGE (2/15 PAG Mini; Daiichi Pure Chemicals) or SDS-7.5% PAGE (acrylamide, 72; bisacrylamide, 1) was applied. The proteins were then processed as described previously (32). Detection of target proteins was done with an enhanced chemiluminescence detection system (Amersham Biosciences).

**Immunofluorescence analysis.** Cells were treated with 0.5% Triton X-100-mCSK buffer for 10 min on ice, followed by fixation with 70% methanol for 20 min on ice. The fixed cells were washed with PBS and blocked for 20 min in 10% normal goat serum in PBS. Staining with primary antibody (anti-MCM4 or anti-MCM7) was performed overnight at 4°C in PBS-0.5% goat serum. Staining with a mouse monoclonal antibody to BMRF1 protein was carried out for 1 h at room temperature. Species-specific secondary antibodies were then applied for 1 h at room temperature. Slides were mounted with Vectashield (Vector Labs) for analysis under a fluorescence confocal microscope. Images were captured and processed using a Radiance 2000 confocal system (Bio-Rad). All of the primary antibodies were employed at a 1:100 dilution and the secondary antibodies at a 1:500 dilution. All washes after antibody incubations were performed with 0.05% Tween 20 in PBS at room temperature. When cells were stained singly for either antigen with inappropriate combinations of first and second antibodies, no fluorescence was observed. Also, no immunofluorescence was observed with alternate filters.

**Preparation of phosphospecific antibodies.** Antisera against phosphothreonine at amino acids 19 and 110 of human MCM4 were obtained by immunizing rabbits with a synthetic peptide of NH<sub>2</sub>-SRGRG(pT)PAQTPRSEC-COOH (for aa 19) or NH<sub>2</sub>-CGTPRSGVRG(pT)PVRQRPDL-COOH (for aa 110) and purified as described previously (21).

**Purification of human MCM4-6-7 complexes.** Sf21 insect cells were coinfecting with recombinant baculovirus expressing human MCM7 (Bac-MCM7) and virus expressing human histidine-tagged MCM4 and -6 (Bac-MCM4-6) at a multiplicity of infection of 10 and harvested 3 days postinfection. MCM4-6-7 complexes containing histidine-tagged MCM4 were purified by nickel-nitrilotriacetic acid column chromatography and then subjected to glycerol density gradient centrifugation (56) as described previously (21). Fractions containing the 600-kDa

MCM4-6-7 hexamer determined by each specific antibody were collected and pooled.

Site-directed mutagenesis of the human MCM4 gene was conducted using a QuikChange site-directed mutagenesis kit (Stratagene). The oligonucleotide 5'-GATGCCAGGTCAGCTCCCTCTCAGAGACG-3' was used as the primer to introduce a change from Ser to Ala at amino acid 32, and 5'-GGTGTAGGG GCGCACCTGTGAGACAGAGG-3' was used for the change at amino acid 110. The oligonucleotide 5'-CATCACGGATCGGCCCGGCGTCGGCCCCG AGCCGC-3' was used as the primer to change both Ser-3 and Thr-7 to Ala, 5'-CGTGAAGGGCCGCCCGCCAGACG-3' was used for the change at amino acid 19, and 5'-CCGATGCCAACCAGCGCCTGGAGTGGAC-3' was used for the change at residue 54. The mutated MCM4 gene forms were cloned into a pAcUW31 vector (Pharming) in which the MCM6 gene had been cloned (56). The entire MCM4 gene has been sequenced, and all of the mutated sites were confirmed. Sf21 cells were coinfecting with the recombinant baculovirus expressing proteins from mutated MCM4 and MCM6 (Bac-MCM4a-6) and a virus expressing the MCM7 protein (Bac-MCM7). The 600-kDa mutated MCM4a-6-7 hexamer was purified by nickel-nitrilotriacetic acid column chromatography and then by glycerol gradient centrifugation (56).

**Purification of GST-fused BGLF4 protein from baculovirus-infected cells.** Sf21 cells were infected with a baculovirus (either Bac-GST-BGLF4 or Bac-GST-BGLF4<sup>K1021</sup>, where GST is glutathione S-transferase) at a multiplicity of infection of 10 and harvested 3 days thereafter. Cells were suspended in 10 ml of buffer A (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 0.1% Nonidet P-40, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, and a protease inhibitor mixture for mammalian cell extracts [25  $\mu$ l/ml; Sigma]) and then sonicated. After the insoluble materials were removed by centrifugation, the supernatants were reacted with 500  $\mu$ l of a 50% slurry of glutathione-Sepharose beads (Amersham Pharmacia) for 2 h. The beads were then extensively washed with buffer A-2 (50 mM Tris-HCl [pH 7.5], 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, and 1 mM phenylmethylsulfonyl fluoride) and eluted in buffer A-2 containing 10 mM glutathione. The eluted proteins (GST-BGLF4 and GST-BGLF4<sup>K1021</sup>) were finally applied to Mono Q column chromatography, and peak fractions were collected and pooled.

**In vitro kinase assays.** Human MCM4-6-7 or MCM4a-6-7 complexes (500 ng) were incubated with various amounts of EBV BGLF4 protein, kinase-negative BGLF4<sup>K1021</sup> protein, or human CDK2/cyclin A (purchased from Upstate, Inc.) in a 50- $\mu$ l reaction mixture containing 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM dithiothreitol, 10 mM MgCl<sub>2</sub>, 1 mM ATP (or 100  $\mu$ M ATP and 2  $\mu$ Ci [ $\gamma$ -<sup>32</sup>P]ATP), and 0.2 mM sodium orthovanadate. The reaction mixtures were incubated for 1 h at 37°C, and the products were subjected to electrophoresis on 7.5% polyacrylamide gels containing SDS and were detected with an image guider (BAS2500; Fujifilm).

**DNA helicase assays.** For preparation of a substrate for DNA helicase activity, a 17-mer oligonucleotide (5'-GTTTTCCAGTCACGAC-3') complementary to the M13mp18(+) strand was labeled at the 5' end with polynucleotide kinase in the presence of [ $\gamma$ -<sup>32</sup>P]ATP and then annealed to M13 DNA. Approximately 50 fmol of the annealed oligomer was incubated at 37°C for 1 h with MCM4-6-7 proteins in 25 mM HEPES-NaOH, pH 7.5, 1 mM DTT, 4 mM ATP, 10 mM Mg(CH<sub>3</sub>COO)<sub>2</sub>, and 0.1 mg of bovine serum albumin per ml. The reaction was terminated by adding SDS to a final concentration of 0.2%, and an aliquot was electrophoresed on a 12% acrylamide gel in Tris-borate-EDTA. The loaded oligomer in the gel was detected with an image guider (BAS2500; Fujifilm) and quantitated.

To examine the effects of phosphorylation on DNA helicase activity of MCM4-6-7 complexes, EBV BGLF4 protein, kinase-negative BGLF4<sup>K1021</sup> protein, or CDK2/cyclin A was added to the reaction mixture from the start of the DNA helicase assay, and the displaced oligonucleotide was analyzed as described above. Phosphorylation of MCM4 with EBV-PK or with CDK2/cyclin A reached a plateau within 3 min of incubation (data not shown).

**Plasmids.** The BGLF4 expression plasmid pME-BGLF4(F) and the control plasmid pME18S were constructed as described previously (25, 26, 28). In pME-BGLF4(F), the expression of the 3' Flag epitope-tagged BGLF4 is driven by the SR $\alpha$  promoter. HeLa cells were transfected with these plasmid DNAs with Lipofectamine 2000 (Invitrogen).

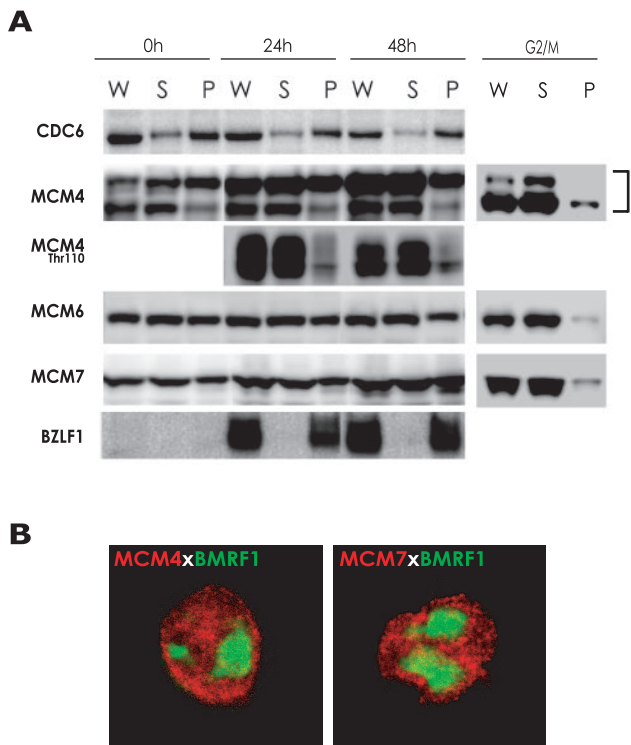
## RESULTS

**Effect of EBV lytic replication on MCM loading onto chromatin.** It has been reported previously that infection of quiescent primary fibroblasts (G<sub>0</sub> phase) with human cytomegalovirus (HCMV) induces expression of all prereplicative factors,

like the ORC, CDC6, Cdt1, and the MCM complex, but that the virus prevents prereplicative complex assembly by inhibiting MCM complex loading onto chromatin (4, 53). Therefore, we examined whether the MCM complexes are detached from chromatin after induction of EBV lytic replication in cycling cells latently infected with EBV. Tet-BZLF1/B95-8 cells were treated with doxycycline to induce lytic replication (32). We have demonstrated previously subcellular dynamics of EBNA1, viral replication proteins, histones, and human chromosomal DNA replication initiation proteins in lytic-program-induced Tet-BZLF1/B95-8 cells by using biochemical fractionation methods with a buffer containing nonionic detergent Triton X-100 at a relatively physiological salt condition (8, 9). The treatment extracts not only cytoplasmic but also nuclear proteins not tightly bound to nuclear structures (8, 9). Proteins remaining in extracted nuclei have been shown previously to indeed bind to chromatin or viral DNA/nuclear matrix material (12, 15). With these procedures, the MCM4 and MCM7 proteins were extracted with the buffer, and most of the remainder were solubilized after DNase I treatment (8). Thus, the detergent-resistant fractions of MCMs represent their chromatin binding. As shown in Fig. 1A, all of the BZLF1 immediate-early protein became bound to chromatin or viral DNA/nuclear matrix material. In contrast, almost all of the MCM4, -6, and -7 proteins in the G<sub>2</sub>/M phase were sensitive to the detergent treatment, indicating release of the MCM complex from chromatin DNA. These results confirmed reproducibility of the fractionation procedure, consistent with our previous observations (8, 9, 16).

In cells latently infected with EBV, about half of the MCM proteins proved resistant to the detergent treatment, indicating MCM loading onto chromatin DNA (Fig. 1A, 0 h). Although two forms of MCM4 were detected, the slower-migrating band represents the hyperphosphorylated form, as determined previously (16), whereas the faster-migrating band is hypophosphorylated. Levels of the slowly migrating forms of MCM4, increased with progression of lytic replication by an unknown mechanism, while levels of CDC6 remained constant. The ratios of the chromatin-bound to unbound forms of MCM6 and MCM7 were essentially unchanged before and after induction. On the other hand, levels of the chromatin-unbound forms of MCM4 increased to some degree after induction. For example, most of the MCM4 protein phosphorylated at Thr-110 was detached from chromatin (Fig. 1A), but about half of MCM4 phosphorylated at Thr-19 became bound to chromatin (data not shown). Thus, the picture of regulation of chromatin binding of the MCM complex is likely to be very complex. Phosphorylation of some sites on MCM4 appears to trigger the dissociation of the MCM complex from chromatin. It was demonstrated previously that EBV lytic replication arrests cell cycle and chromosomal DNA replication (32). Thus, the chromosomal DNA replication arrest during lytic infection might be partly due to dissociation of the MCM complex from chromatin. However, the overall picture of MCM attachment did not change dramatically after induction of EBV lytic replication, suggesting that other mechanisms also operate at stages after loading MCM complex onto chromatin.

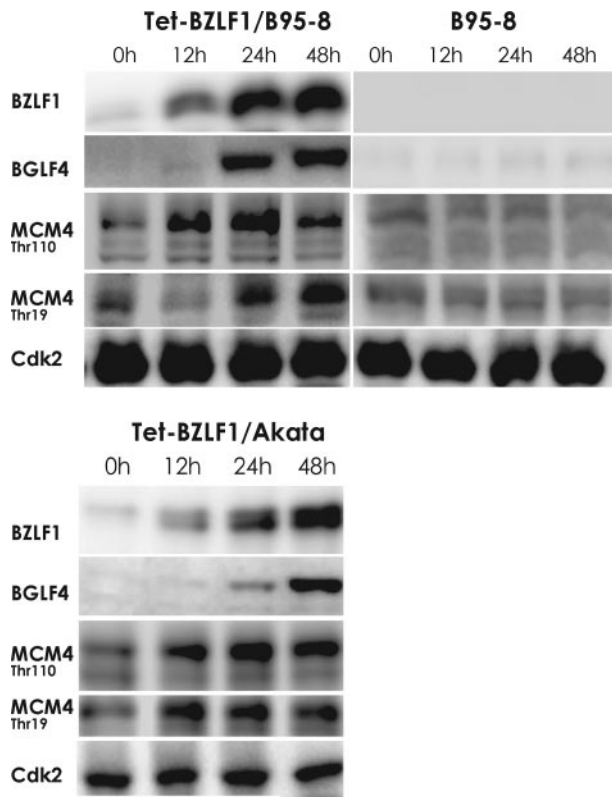
**MCM proteins are localized outside viral replication compartments in nuclei.** Tet-BZLF1/B95-8 cells were treated with



**FIG. 1.** Biochemical analysis of the subcellular distribution of CDC6, MCM4, MCM6, and MCM7 in lytic-program-induced Tet-BZLF1/B95-8 cells. (A) Tet-BZLF1/B95-8 cells were cultured in the presence of 2  $\mu$ g/ml doxycycline, harvested at the indicated times, and subjected to biochemical fractionation as described in Materials and Methods. Tet-BZLF1/B95-8 cells were also treated with paclitaxel (20  $\mu$ M) for 24 h to arrest cell cycle at the G<sub>2</sub>/M phase and processed similarly. The relative abundance of each protein in Triton X-100-extractable supernatants (S) and extracted nuclear pellets (P) was examined by immunoblotting with anti-CDC6, anti-MCM4, anti-phosphorylated Thr-110 of MCM4, anti-MCM6, anti-MCM7, and anti-BZLF1 antibodies. W, whole-cell lysate. (B) Subnuclear localizations of MCM4 and MCM7 in lytic-program-induced Tet-BZLF1/B95-8 cells. Cells were harvested at 24 h.p.i. and treated with 0.5% Triton X-100-mCSK buffer. Nonionic-detergent-extracted cells were fixed with methanol and then immunostained with anti-MCM4 or anti-MCM7 and anti-BMRF1 antibodies. Shown are merged images of MCM4 (red) or MCM7 (red) and BMRF1 (green) proteins.

doxycycline to induce lytic replication (32), harvested, and treated with a buffer containing the nonionic detergent Triton X-100. This treatment is known to disrupt nuclear envelopes, but the nuclear lamina remains intact, thus maintaining nuclear structures (18). By confocal immunofluorescence analyses, the sites stained with anti-BMRF1 replication protein-specific antibodies completely coincided with foci of newly synthesized viral DNA, as judged by 5-bromodeoxyuridine incorporation and fluorescence in situ hybridization analyses (8, 33). Thus, BMRF1 protein-localized sites represent loci of viral DNA synthesis, termed viral replication compartments. As shown in Fig. 1B, the detergent-resistant forms of MCM4 and MCM7 proteins were localized outside these compartments in nuclei, indicating chromatin binding during lytic infection.

**Induction of EBV lytic program elicits phosphorylation of Thr-19 and Thr-110 on MCM4.** Phosphorylation of Thr-19 and



**FIG. 2.** Phosphorylation of Thr-19 and Thr-110 residues of MCM4 upon induction of EBV lytic replication. B95-8, Tet-BZLF1/B95-8, and Tet-BZLF1/Akata cells were cultured in the presence of 2  $\mu$ g/ml doxycycline and harvested at the indicated times. Equal amounts of proteins for each sample (~20 to 50  $\mu$ g) were subjected to immunoblot analysis with the specific antibodies indicated on the left side of each panel. Anti-CDK2 antibody was used to confirm equal protein loading.

Thr-110 on MCM4 in human and mouse MCM4-6-7 complexes is associated with inhibition of the associated DNA helicase activity (20). In order to investigate whether chromosomal replication arrest induced by the EBV lytic replication is partly due to inactivation of the MCM complex, we examined the phosphorylation state of MCM4 on Thr-19 and Thr-110 in Tet-BZLF1/B95-8 cells after induction of lytic replication. Cells were harvested at the indicated times and subjected to Western blot analysis with specific antibodies (Fig. 2). Detailed expression profiles of viral proteins after induction of lytic replication with doxycycline have been described previously for Tet-BZLF1/B95-8 cells (32), with BZLF1 protein becoming detectable 4 hours postinduction (h.p.i.) (32) and reaching a plateau at 24 h.p.i. (Fig. 2, Tet-BZLF1/B95-8). The EBV-PK encoded by the BGLF4 gene also appeared at 6 h.p.i. (data not shown), with a plateau at 24 h.p.i. in lytic replication-induced Tet-BZLF1/B95-8 cells. As shown in Fig. 2, by use of phosphospecific antibodies raised against two sites (Thr-19 and Thr-110) on MCM4, it was established that induction of lytic replication in Tet-BZLF1/B95-8 and Tet-BZLF1/Akata cells caused a time-dependent increase in the phosphorylation of Thr-19 and Thr-110 on MCM4 (Fig. 2), although their phosphorylation levels on MCM4 of B95-8 cells treated with doxy-

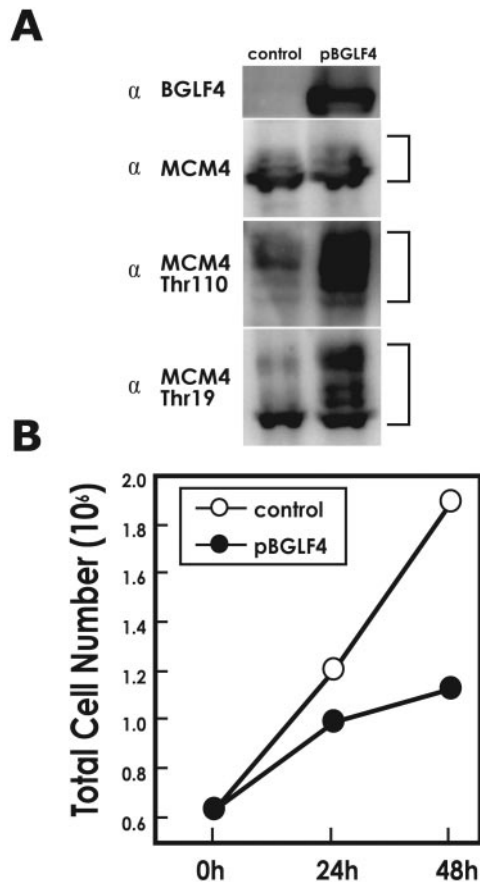


FIG. 3. Expression of the EBV-PK encoded by the BGLF4 gene in HeLa cells results in phosphorylation of MCM4 at Thr-19 and Thr-110. (A) HeLa cells were transiently transfected with the BGLF4 protein expression vector pME-BGLF4(F) or a control vector, pME18S, and harvested after 2 days. Whole-cell extracts were prepared, and equal amounts of proteins for each sample (20  $\mu$ g) were separated by gradient SDS-PAGE and subjected to immunoblot analysis with the specific antibodies indicated on the left side of each panel. (B) Effect of expression of the EBV-PK on the proliferation of HeLa cells. HeLa cells ( $0.6 \times 10^6$  cells/35-mm dish) were transfected with the BGLF4 expression plasmid pME-BGLF4(F) or the control plasmid pME18S and were counted with a hemacytometer at the indicated times.

cyclin as a control were low and essentially constant (Fig. 2). It has been shown that the amino-terminal region of MCM4 in an MCM4-6-7 complex is extensively phosphorylated with CDK2/cyclin A or CDK1/cyclin B (29) and that this phosphorylation inhibits DNA helicase activity of the complex in vitro and in vivo (20, 22). Thus, the finding that MCM4 is phosphorylated at Thr-19 and Thr-110 is suggestive of inactivation of DNA helicase activity of the MCM complexes during EBV lytic replication.

**Expression of the EBV-PK in HeLa cells leads to phosphorylation of Thr-19 and Thr-110 of MCM4.** It has been demonstrated previously that during lytic replication levels of cyclin E and cyclin A are elevated, CDK2 activity eventually rises, and high CDK1/cyclin B activity is maintained (31–33). Thus, the MCM4 phosphorylation is conceivably due to high CDK2 or CDK1 activity. However, EBV also expresses viral protein

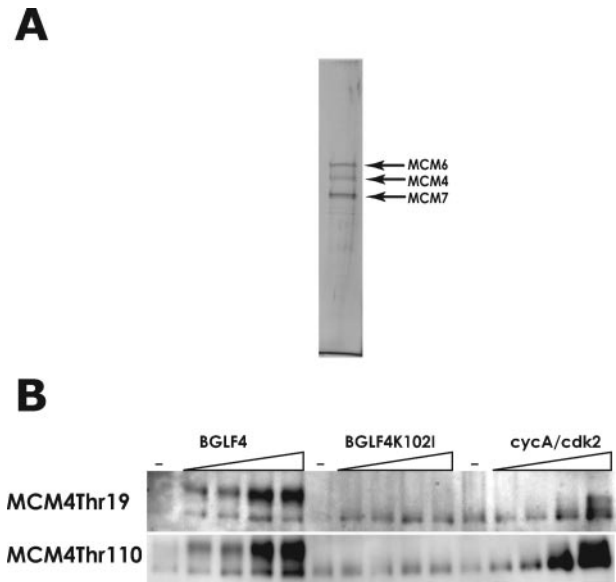


FIG. 4. EBV-PK phosphorylates Thr-19 and Thr-110 residues on MCM4 of the MCM4-6-7 hexamer in vitro. (A) Purification of MCM4-6-7 hexamers. Sf21 cells were coinfecting with recombinant baculoviruses, Bac-Mcm4-6 and Bac-Mcm7, and MCM4-6-7 complexes were purified as described in Materials and Methods, separated by SDS-7.5% PAGE, and stained with silver. The positions of the MCM4, Mcm6, and MCM7 proteins were determined by Western blot analyses (data not shown) and are indicated by arrows. (B) Wild-type or kinase-negative GST-BGLF4 proteins were isolated from Sf21 cells infected with Bac-GST-BGLF4 or Bac-GST-BGLF4<sup>K1021</sup> as described in Materials and Methods. Human MCM4-6-7 hexamer (100 ng) was incubated with increasing amounts of wild-type GST-BGLF4, kinase-negative GST-BGLF4<sup>K1021</sup>, or cyclin A/CDK2 (–, none). The samples were subjected to SDS-7.5% PAGE and analyzed by Western blotting using phosphospecific antibodies against MCM4 at Thr-19 and Thr-110.

kinase encoded by the BGLF4 gene, which is known to phosphorylate the same sites as CDK1 (27). CDK2 also sometimes phosphorylates the target sites of CDK1. Therefore, since there is a possibility that the EBV-PK is involved in MCM4 phosphorylation during lytic infection, we examined phosphorylation of MCM4 at Thr-19 and Thr-110 after BGLF4 expression plasmid pME-BGLF4(F) (25) or the control vector pME18S was transfected into HeLa cells. A marked increase in the level of phosphorylation at Thr-19 and Thr-110 was observed for cells expressing the BGLF4 protein (Fig. 3A), although levels of the slowly migrating forms of MCM4 with expression of EBV-PK were not so distinct. These observations clearly indicate that expression of EBV-PK results in phosphorylation of MCM4, including at least Thr-19 and Thr-110 sites in vivo.

**Expression of the EBV-PK inhibits cell proliferation.** To determine whether expression of the EBV-PK inhibits cell proliferation, HeLa cells were seeded at  $6 \times 10^5$  per ml, and the cells were counted 0, 24, and 48 h after BGLF4 expression plasmid pME-BGLF4(F) or the control vector pME18S was transfected into HeLa cells (Fig. 3B). Growth of HeLa cells slowed down following transfection of pME-BGLF4(F). In contrast, when cells were transfected with the control vector,

the cells continued to proliferate normally. Thus, it was demonstrated clearly that the expression of the BGLF4 protein inhibits the proliferation of Tet-BZLF1/B95-8 cells.

**Both the EBV-PK and CDK2/cyclin A phosphorylate Thr-19 and Thr-110 sites on MCM4 of the MCM4-6-7 complex in vitro.** Next, we examined whether the EBV-PK can directly phosphorylate Thr-19 and Thr-110 sites on MCM4 of the MCM4-6-7 complex in vitro (Fig. 4). As shown in Fig. 4A, the recombinant His-tagged MCM4-6-7 hexamer was purified through Ni-nitrilotriacetic acid column chromatography and glycerol density gradient centrifugation from extracts of recombinant-baculovirus-infected cells and used as a substrate in in vitro kinase assays (Fig. 4A). Also, WT and kinase-inactive BGLF4 proteins were purified from the recombinant-baculovirus-infected cells. Both the WT BGLF4 protein and the CDK2/cyclin A complex could phosphorylate MCM4 of the MCM4-6-7 hexamer at Thr-19 and Thr-110 sites in a dose-dependent manner, whereas the kinase-inactive BGLF4<sup>K102I</sup> protein did not (Fig. 4B). The mobilities of MCM4 phosphorylated by the BGLF4 protein and by CDK2/cyclin A differed, suggesting that EBV-PK might phosphorylate many sites on MCM4 in addition to those phosphorylated by CDK2. Whatever the case, the data indicate that EBV-PK is able to phosphorylate at least Thr-19 and Thr-110 of MCM4 in MCM complexes directly.

**DNA helicase activity of MCM4-6-7 hexamers is inhibited by EBV-PK as well as by CDK2/cyclin A.** To date, DNA helicase activity has been identified for the MCM4-6-7 complex exclusively. The effect of EBV-PK on MCM4-6-7 complex-associated DNA helicase activity was examined using a <sup>32</sup>P-labeled 17-mer annealed to M13 single-stranded DNA as the substrate (Fig. 5A). The purified MCM4-6-7 hexamer was able to effectively unwind the 17-mer primers from annealed M13 DNA substrates (Fig. 5B), and this was inhibited by addition of CDK2/cyclin A, as reported previously (20). Similarly, EBV-PK inhibited the DNA helicase activity of the complex in a dose-dependent manner, whereas kinase-negative GST-BGLF4<sup>K102I</sup> (up to 200 ng) did not (Fig. 5B).

**EBV-PK, unlike CDK2/cyclin A, inhibits helicase activity of MCM4-6-7 hexamers containing mutations in amino-terminal phosphorylation sites of MCM4.** To determine whether the suppression of MCM4-6-7 hexamer-associated helicase activity by EBV-PK is actually due to phosphorylation of specific sites on MCM4, mutated MCM4-6-7 hexamers containing six amino acid mutations (to alanine) at amino-terminal phosphorylation sites on MCM4 (aa 3, 7, 19, 32, 54, and 110), which are therefore resistant to phosphorylation by CDK2/cyclin A, were examined for DNA helicase activity in the presence of EBV-PK. As shown in Fig. 6A, the mutated MCM was as sensitive to the inhibition of DNA helicase activity by EBV-PK as the WT MCM complex. In contrast, the mutated MCM was resistant to the inhibition by CDK2/cyclin A (compare Fig. 5B and 6A). Thus, EBV-PK inhibited the MCM complex-associated helicase activity more powerfully than CDK2/cyclin A. Although phosphorylation of the N-terminal six amino acids in MCM4 is mainly responsible for inhibition of MCM4-6-7-associated DNA helicase, EBV-PK might further phosphorylate other sites of MCM4 or other subunits of the MCM complex, causing inhibition of the helicase activity.

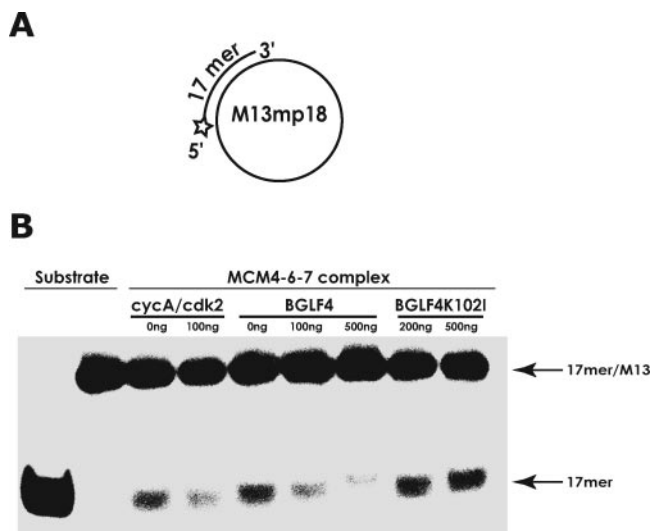


FIG. 5. EBV-PK, like CDK2/cyclin A, inhibits DNA helicase (unwinding) activity associated with the MCM4-6-7 complex. (A) Substrate for DNA helicase assays. A 5' <sup>32</sup>P-labeled oligonucleotide (17-mer) annealed to M13 single-stranded DNA is depicted. (B) DNA helicase assays were performed with 100 fmol of the helicase substrate and human MCM4-6-7 hexamers (100 ng) in the presence of cyclin A/CDK2 (100 ng), BGLF4 protein (100 and 500 ng), or kinase-negative BGLF4<sup>K102I</sup> protein (200 and 500 ng) as described in Materials and Methods. Positions of the DNA substrate (17mer/M13) and displaced oligonucleotide (17mer) are indicated by arrows. The left two lanes show results for heat-denatured and native DNA substrates, respectively.

**EBV-PK phosphorylates MCM6 and another site(s) of MCM4 in addition to the amino-terminal six Ser/Thr residues.**

We examined whether the EBV-PK can directly phosphorylate another site(s) besides the amino-terminal six Ser/Thr residues on MCM4 and other subunits of the MCM4-6-7 complex in vitro (Fig. 6B). Human intact MCM4-6-7 and mutated MCM4a-6-7 hexamers containing six amino acid mutations (to alanine) at amino-terminal phosphorylation sites on MCM4 (aa 3, 7, 19, 32, 54, and 110) were used as substrates for in vitro kinase assays. CDK2/cyclin A phosphorylated mainly MCM4 of the MCM4-6-7 hexamer but hardly the N-terminal-mutated MCM4. Thus, CDK2/cyclin A phosphorylates the N-terminal Ser/Thr residues mainly on MCM4. In contrast, EBV-PK could clearly phosphorylate the mutated MCM4 of the MCM4a-6-7 hexamers, indicating that EBV-PK phosphorylates another site(s) in addition to amino-terminal phosphorylation sites on MCM4. Phosphorylation of MCM6 and -7 by CDK2/cyclin A was scarcely observed (Fig. 6B). When intact MCM4-6-7 complex was reacted with EBV-PK, a broad band of MCM4 was observed by SDS-PAGE, probably due to phosphorylation (Fig. 6C). However, when the mutated complex was reacted with EBV-PK, the mobility of the mutated MCM4 by SDS-PAGE was virtually unchanged, distinguishing the mutated MCM4 from MCM6 (Fig. 6C). As shown in Fig. 6B, the EBV-PK phosphorylated both MCM6 and the mutated MCM4. Collectively, EBV-PK inhibits the MCM complex-associated DNA helicase activity through phosphorylation of many sites, including six amino-terminal phosphorylation sites on MCM4 and also MCM6 of the MCM4-6-7 hexamer.

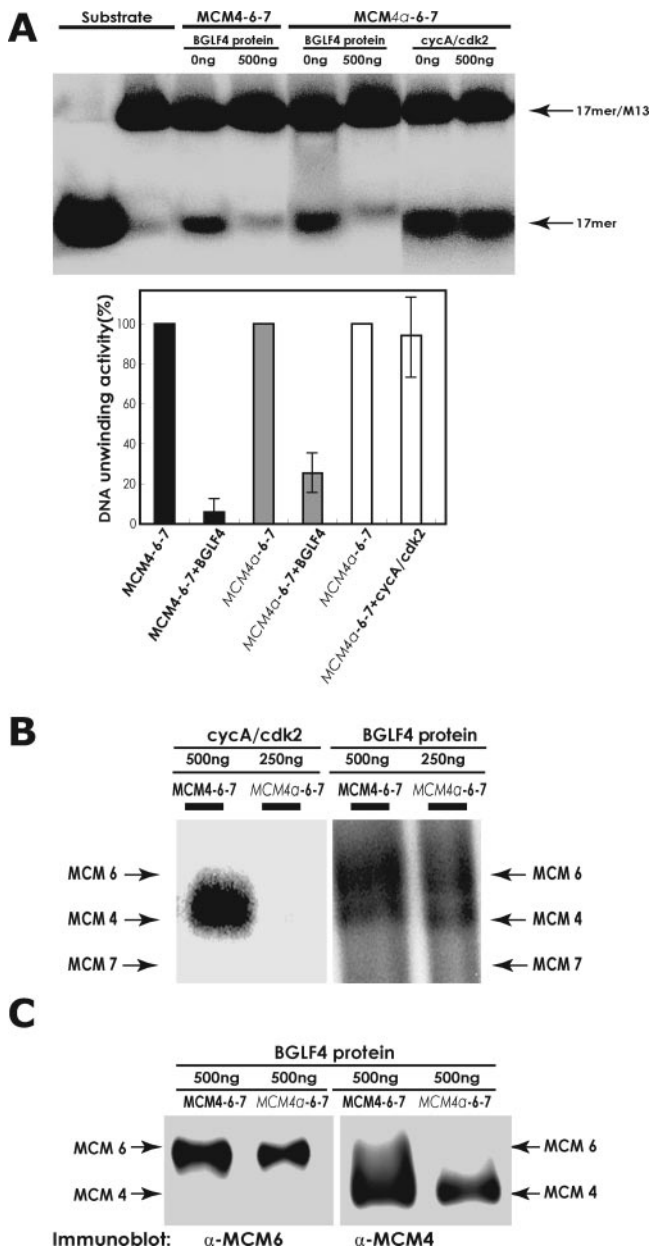


FIG. 6. EBV-PK, unlike CDK2/cyclin A, still inhibits helicase activity of MCM4-6-7 hexamers containing mutations in amino-terminal phosphorylation sites of MCM4. (A) MCM4-6-7 hexamers containing mutant MCM4 (MCM4a/6/7) (200 ng) or the wild type (MCM4-6-7) (100 ng) were examined for DNA helicase activity in the presence or absence of BGLF4 protein (500 ng) or cyclin A/CDK2 (500 ng) as described in Materials and Methods. The left two lanes show results for heat-denatured and native DNA substrates, respectively. Quantitative analysis of the DNA unwinding activity is shown in the graph. The percentage of 17-mer oligonucleotide displaced with each MCM complex in the presence of the BGLF4 EBV-PK or CDK2/cyclin A was calculated from the signal intensity, with that in the absence of the kinase taken as 100%. Data are means  $\pm$  standard deviations of three independent experiments. (B) Human MCM4-6-7 or MCM4a-6-7 complexes (1  $\mu$ g) were incubated with indicated amounts of CDK2/cyclin A (left) or the BGLF4 protein (right) in a 50- $\mu$ l reaction mixture containing 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM dithiothreitol, 10 mM MgCl<sub>2</sub>, 100  $\mu$ M ATP, 2  $\mu$ Ci [ $\gamma$ -<sup>32</sup>P]ATP, and 0.2 mM sodium orthovanadate. <sup>32</sup>P-labeled proteins were separated by SDS-7.5% PAGE followed by autoradiography. (C) Human MCM4-6-7 or MCM4a-6-7 complexes (1  $\mu$ g) were phosphorylated by 500 ng of the

## DISCUSSION

EBV lytic infection induces hyperphosphorylation of retinoblastoma protein, with elevated levels of E2F-1, cyclin E, and cyclin A and their associated CDK2 activities, while cyclin B/CDK1 activity remains constant (32, 33). The present study provided evidence with phosphospecific antibodies that virus-mediated changes occur in the phosphorylation of MCM4 of the MCM complex, with involvement not only of CDK2/CDK1 but also of EBV-encoded protein kinase. These molecules are likely to act redundantly.

MCM4 exhibits different phosphorylation states that depend on the cell cycle phases and their association with chromatin (16). In the normal cell cycle, MCM4 is phosphorylated by CDK2/cyclin A and CDK1/cyclin B, primarily during S phase and mitosis, respectively (17, 22). The phosphorylation of a mutant complex containing mouse MCM4 mutated at six sites (aa 3, 7, 19, 32, 53, and 109) showed almost no phosphorylation in this region of MCM4 (20). Thus, these three Ser-Pro (aa 3, 32, and 53) and three Thr-Pro (aa 7, 19, and 109) sites are required for phosphorylation of MCM4 in the MCM4-6-7 complex by CDK2/cyclin A. The same sites in the amino-terminal region of MCM4 appeared to be required for the phosphorylation by CDK1/cyclin B in vitro (17), suggesting that both CDK2/cyclin A and CDK1/cyclin B have similar specificities of substrate recognition. Furthermore, Komamura-Kohno et al. have reported recently that phosphorylation of MCM4 at Thr-7, Thr-19, Ser-32, Ser-88, and Thr-110 in the M phase requires CDK1 (Cdc2), although they do not deny the possibility of the phosphorylation of these sites on MCM4 by CDK2 (29). Therefore, it must be noted that there is also a possibility of the involvement of CDK1/cyclin B in MCM4 phosphorylation, since the CDK1/cyclin B activity remains essentially constant and high throughout lytic replication (33).

MCM proteins are present in cells in chromatin-bound as well as unbound forms, but at G<sub>2</sub>/M phase, almost all are heavily phosphorylated and present as the unbound form (16). Hyperphosphorylation of the MCM complex prohibits inappropriate reloading of MCMs onto chromatin and thereby contributes suppression of rereplication. In heterohexameric Mcm2-7 complexes, either the chromatin-bound or the unbound form may be formed (16). Although phosphorylation of MCM4 partly resulted in the dissociation of the MCM complex from chromatin, as shown in Fig. 1A, the preparation of the chromatin-bound form of MCM proteins was found to be almost unchanged. It is suggested here that inhibition of the DNA unwinding function of the hexameric MCM complex on chromatin occurs during EBV lytic replication as another role for the phosphorylation of MCM4.

In the case of HCMV, it has been reported that the virus prevents prereplicative complex assembly by inhibiting MCM complex loading onto chromatin (4, 53), but quiescent primary

BGLF4 protein in a 50- $\mu$ l reaction mixture containing 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM dithiothreitol, 10 mM MgCl<sub>2</sub>, 1 mM ATP, and 0.2 mM sodium orthovanadate, and products were separated by SDS-7.5% PAGE. Proteins were analyzed by Western blotting using MCM6 and MCM4 antibodies.

fibroblasts ( $G_0$  phase) were used so that there was no expression of prereplicative factors like the ORC, CDC6, Cdt1, and the MCM complex. However, both reactivation of EBV from latently infected B cells and infection of HCMV into cycling cells result in cell cycle arrest and blockage of chromosomal DNA replication. Since levels of the chromatin-bound forms of MCM4, MCM6, and MCM7 were essentially unchanged before and after induction in our investigation, the chromosomal DNA replication arrest during EBV lytic infection might not be explained only by dissociation of MCM complexes from chromatin. Overlap of multiple factors might result in the chromosomal DNA replication arrest.

Components of the replication fork include the trimeric, single-stranded DNA binding RPA complex and the DNA helicase. The identity of the replicative helicase which functions in the elongation step remains equivocal. Genetic and biochemical evidence supports a role for the MCM2-7 complex providing helicase activity in unwinding DNA at replication origins during initiation (37). The MCM2-7 proteins form a stable complex in vivo (14), although detectable helicase activity is observed only with the MCM4-6-7 subcomplex (19). Current models suggest that this subcomplex may represent the active helicase, while the remaining subunits may have an essential role in regulating its activity (38, 57). Moreover, a role for the MCM complex has also been suggested to occur during the elongation step, and in budding yeast (*Saccharomyces cerevisiae*), MCM4 appears to move away from replication origins after initiation of DNA synthesis (1, 34, 47). Although the MCM2-7-related protein MCM8 recently has been reported to possess ATP-dependent helicase activity (38), the MCM4-6-7 complex is still thought to be a strong candidate for replicative helicase in the elongation step of DNA replication. Taking this into consideration, MCM4 phosphorylation during EBV productive replication might suppress refring of chromosomal DNA replication by blocking DNA unwinding activity and also prevent fork movement by blocking replicative DNA helicase activity. EBV might have adopted multiple redundant pathways to halt the cell cycle. Whatever the underlying mechanism, phosphorylation of MCM4 provides one means to block chromosomal DNA replication in lytic infected cells.

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