

## Cell Cycle-dependent Proteolysis and Phosphorylation of Human Mcm10\*

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**Mcm10 (Dna43) is an essential protein for chromosomal DNA replication in *Saccharomyces cerevisiae*. Recently, we identified a human Mcm10 homolog that interacts with the mammalian Orc2 and Mcm2–7 complex. We additionally demonstrated that human Mcm10 binds nuclease-resistant nuclear structures during S phase and dissociates from them in G<sub>2</sub> phase. In this study, we have further characterized the subcellular localization, modification, and expression levels of human Mcm10 protein throughout the cell cycle. Human Mcm10 protein decreased in late M phase, remained low during G<sub>1</sub> phase, started to accumulate, and bound chromatin at the onset of S phase. Proteasome inhibitors stabilized Mcm10 levels, suggesting that proteolysis is involved in the down-regulation of the protein in late M/G<sub>1</sub> phase. Dissociation of Mcm10 from chromatin in G<sub>2</sub>/M phase was concomitant with alterations in the electrophoretic mobility of the protein. Treatment with λ phosphatase revealed that mobility shifts were due to hyperphosphorylation. These results indicate that human Mcm10 is regulated by proteolysis and phosphorylation in a cell cycle-dependent manner. It is further suggested that mammalian Mcm10 is involved in S phase progression, and not the formation of a prereplicative complex, as previously proposed from data on the *S. cerevisiae* protein.**

In eukaryotes, initiation of DNA replication is a strictly controlled process, so that chromosomal DNA is precisely duplicated once per cell cycle. Recent studies using different systems show that a number of proteins are involved in the initiation of DNA replication, a process that is largely conserved from yeast to human (1, 2). Current models indicate that initiation consists of two steps. In the first step, the origin recognition complex (ORC),<sup>1</sup> Cdc6, Cdt1, and Mcm2–7 proteins sequentially assemble on the replication origins to form the prereplicative complex, from late mitosis to early G<sub>1</sub> phase. The ORC, a complex of six proteins (Orc1–6), binds replication

origins (3, 4) and recruits Cdc6. Cdc6 in turn coordinates with Cdt1 to load the Mcm2–7 complex (5–10), a presumed replicative helicase (11, 12), on the chromatin template. In the second step, Cdc7/Dbf4 kinase and S phase cyclin-dependent kinases (S-Cdks) activate the prereplicative complex and trigger DNA replication by loading Cdc45 onto each origin with programmed timing (13–16). Cdc45 facilitates assembly of the replication machinery by recruiting replication protein A and DNA polymerases (17, 18).

Eukaryotes contain multiple parallel pathways to ensure that the prereplicative complex is not re-assembled until the segregation of chromosomes in mitosis. Cdc6 is either degraded via the ubiquitin-dependent pathway (19) or exported out of the nucleus (20). Phosphorylation of Mcm2–7 complex by cdc2 kinase initiates dissociation from chromatin during S phase (21). Cdt1 is regulated by protein expression as well as interactions with geminin to ensure that it is active only in the G<sub>1</sub> phase (22, 23).

Mcm10 (Dna43) was originally discovered in *Saccharomyces cerevisiae* while screening to identify other *mcm* mutants (24). Previous studies performed in *S. cerevisiae* suggest that Mcm10 has multiple roles in DNA replication. The *mcm10* mutant is defective in initiation of DNA replication at the non-permissive temperature (24) and causes stalling of replication forks when the replication machinery passes through origins that do not fire (24). In addition, Mcm10 mediates the loading of the Mcm2–7 complex onto replication origins (25), and interacts genetically with Cdc45, DNA polymerase δ and ε, which are required for the elongation steps of DNA replication (26). Therefore, it appears that Mcm10 is involved in both origin activation and elongation, although the mechanisms by which the protein interacts with multiple replication factors at each step remain to be elucidated.

Mcm10 homologs have additionally been discovered in *Schizosaccharomyces pombe* and *Caenorhabditis elegans* (25, 27). Recently, we identified *Drosophila* and human homologs of Mcm10 and demonstrated that human Mcm10 interacts with the mammalian Orc2 and Mcm2–7 complex (28). We also confirmed that human Mcm10 binds chromatin during S phase and dissociates in G<sub>2</sub> phase (28), whereas yeast Mcm10 remains bound to chromatin throughout the cell cycle (25, 26). To clarify the mechanism of regulation of Mcm10 function in higher eukaryotes, we further characterized the subcellular localization, modification, and protein levels of human Mcm10 throughout the cell cycle in this study. In contrast to yeast Mcm10, expression of the human homolog fluctuated during the cell cycle. Human Mcm10 was phosphorylated in a cell cycle-dependent manner. Our data further established that human Mcm10 bound chromatin only in S phase, suggesting that mammalian Mcm10 is required for S phase progression.

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<sup>1</sup> The abbreviations used are: ORC, origin recognition complex; Mcm, minichromosome maintenance; PCNA, proliferating cell nuclear antigen; BrdUrd, bromodeoxyuridine; CSK, cytoskeleton; PIPES, 1,4-piperazinediethanesulfonic acid; APC, anaphase-promoting complex.

## EXPERIMENTAL PROCEDURES

**Cell Culture and Synchronization**—HeLa S3 cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% calf serum. For synchronization at mitotic phase, exponentially growing HeLa S3 cells were cultured in the presence of 50 ng/ml nocodazole (Aldrich) for 4 h and collected by mitotic shake-off. Mitotic cells were washed twice with nocodazole-free medium, replated with fresh medium, and harvested at indicated timepoints. For synchronization at G<sub>1</sub>/S boundary, mitotic cells were collected as described above and replated, and arrested with 15  $\mu$ M aphidicolin (Sigma) for 12–14 h. Cells were released in aphidicolin-free medium and harvested at indicated timepoints.

To monitor DNA replication, cells were pulse-labeled with 20  $\mu$ g/ml bromodeoxyuridine (BrdUrd) for 10 min, and the incorporated label was detected by immunofluorescent staining, using a rat anti-BrdUrd antibody (Harlan Sera-Lab) and Texas red-conjugated goat anti-rat secondary antibody (Vector Laboratories), as described previously (29). To determine the mitotic index, cells were fixed in methanol/acetic acid (3:1), spread onto slides, and stained with Giemsa (Invitrogen).

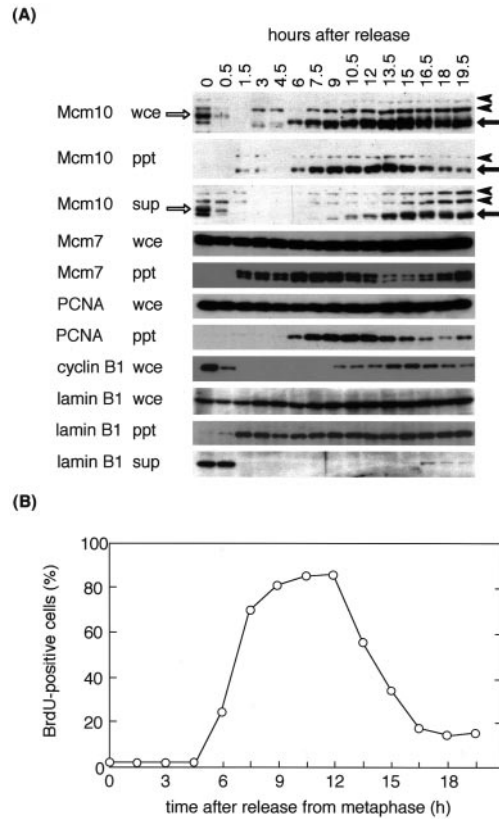
**Fractionation of Cellular Proteins and Immunoblotting**—Aliquots of  $2.0 \times 10^6$  HeLa cells were washed three times with ice-cold phosphate-buffered saline and suspended in 250  $\mu$ l of cytoskeleton buffer (CSK buffer: 10 mM PIPES, pH 6.8, 100 mM NaCl, 300 mM sucrose, 3 mM MgCl<sub>2</sub>, 1 mM EGTA, 1 mM dithiothreitol, 0.1 mM ATP, 0.2 mM Na<sub>3</sub>VO<sub>4</sub>, 10 mM NaF, 0.25 mM phenylmethylsulfonyl fluoride, 0.4  $\mu$ g/ml aprotinin, 0.4  $\mu$ g/ml leupeptin, 0.2  $\mu$ g/ml antipain, and 0.2  $\mu$ g/ml pepstatin A) containing 0.1% Triton X-100 at 4°C for 10 min, and subjected to low-speed centrifugation (3000 rpm for 3 min). Supernatants were further purified by centrifugation at 15,000 rpm for 5 min to obtain Triton X-100-soluble fractions. After washing Triton-extracted nuclei once in 500  $\mu$ l of CSK buffer containing 0.1% Triton X-100, insoluble pellets were collected by low speed centrifugation. For whole-cell extracts,  $1 \times 10^6$  HeLa cells were washed three times with ice-cold phosphate-buffered saline, resuspended in 125  $\mu$ l of CSK, and boiled with SDS-PAGE sample buffer.

For immunoblotting, samples were separated on 8% SDS-polyacrylamide gel and transferred electrophoretically to 0.45  $\mu$ m polyvinylidene difluoride membranes (Millipore). After incubation of membranes with primary antibodies in phosphate-buffered saline containing 5% (w/v) nonfat dry milk for 12 h at 4°C, membranes were incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibodies (Vector Laboratories) in the same buffer and re-washed. Protein bands were detected using the chemiluminescent reagent SuperSignal (PIERCE) according to the manufacturer's instructions. Kaleidoscope prestained standards (Bio-Rad) were employed as molecular mass standards. Antiserum against Mcm10 was generated in rabbit, as described previously (28). Antibodies against human Mcm7, lamin B1, PCNA, p53, and cyclin B1 were purchased from Santa Cruz Biotechnologies, while mouse actin antibody was obtained from Chemicon. Peroxidase-conjugated secondary antibodies were purchased from Vector Laboratories.

**Northern Blot Analysis**—HeLa cells were lysed in ISOGEN (Nippon Gene) every 4 h after release from metaphase. Poly(A)<sup>+</sup> RNA and proteins were purified from the same lysate according to the manufacturer's protocols. Poly(A)<sup>+</sup> RNA (2  $\mu$ g) was subjected to Northern blot analysis, as specified (28).

**$\lambda$  Phosphatase Treatment**—Triton X-100-soluble fraction collected from  $2 \times 10^4$  HeLa mitotic cells was incubated with 400 units of  $\lambda$  phosphatase (New England Biolabs) in 15  $\mu$ l of phosphatase buffer (50 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 5 mM dithiothreitol, 0.01% Brij 35, and 2 mM MnCl<sub>2</sub>) containing  $1 \times$  Complete (EDTA-free, Roche Molecular Biochemicals) protease inhibitors at 4°C for 30 min. In control experiments, phosphatase was inhibited by the addition of 50 mM NaF and 10 mM Na<sub>3</sub>VO<sub>4</sub>. The reaction was stopped by the addition of SDS-PAGE sample buffer. Products were separated by SDS-PAGE and analyzed by immunoblotting.

**Proteasome Inhibition**—The specific inhibitors, ALLM, MG132, and proteasome inhibitor I (PSI) were purchased from Calbiochem and dissolved in dimethyl sulfoxide. Clasto-lactacystin  $\beta$ -lactone from MBL was dissolved in dimethyl sulfoxide before use. HeLa cells were treated with either protease inhibitors (20  $\mu$ M) or dimethyl sulfoxide for 12 h. Cells were harvested, and whole cell extracts were prepared. To examine the effects of proteasome inhibitors in early G<sub>1</sub> phase, MG132 was added 0–2 h after release from metaphase, and whole cell extracts were prepared 3 h after release.

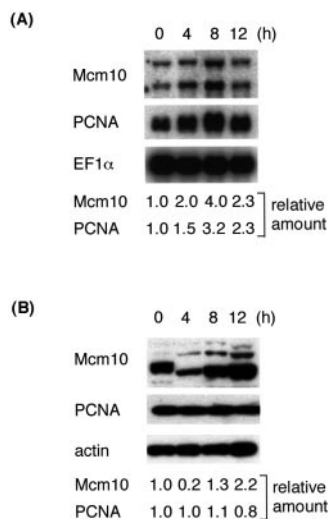


**FIG. 1. Mcm10 decreases in late M/early G<sub>1</sub> phase and rebinds chromatin at G<sub>1</sub>/S boundary.** A, HeLa cells were synchronized at metaphase with nocodazole treatment. Cells were released in nocodazole-free medium and harvested for the preparation of whole cell extracts (*wce*), Triton X-100-soluble fractions (*sup*), and Triton X-100-insoluble fractions (*ppt*) at indicated time points. Mcm10, Mcm7, PCNA, cyclin B1, and lamin B1 in each fraction were detected by SDS-PAGE and immunoblotting. Closed arrows depict Mcm10 with faster electrophoretic mobility, while open arrows indicate mitotic Mcm10 with slower mobility. Arrowheads specify 125- and 133-kDa bands, which may be ubiquitinated Mcm10. B, DNA synthesis was monitored by BrdUrd incorporation and immunofluorescent staining as described under "Experimental Procedures."

## RESULTS

**Mcm10 Is Loaded onto Chromatin at G<sub>1</sub>/S Boundary**—We initially investigated the subcellular localization of Mcm10, following fractionation of HeLa cells into Triton X-100-soluble, chromatin-bound, and nuclease-unextractable samples. Human Mcm10 localized mainly to the nuclease-resistant fraction during S phase and dissociated from the fraction after completion of DNA replication. Here, we estimate the subcellular localization of Mcm10, using Triton X-100 extraction. HeLa cells were lysed with cytoskeleton buffer containing Triton X-100 in the presence of ATP, and soluble and insoluble fractions were separated. This procedure permeabilized nuclei and allowed the elution of weakly bound proteins from chromatin, while prereplication complex and replication machinery remained stably bound.

We initially investigated the stage at which human Mcm10 rebound chromatin. HeLa cells were synchronized at metaphase by nocodazole treatment and released into nocodazole-free medium. At indicated timepoints, whole cell extracts and Triton X-100-soluble and insoluble fractions were prepared, and subcellular localization of Mcm10 and other proteins was analyzed by immunoblotting (Fig. 1A). Mcm10 existed in a soluble form at metaphase (Fig. 1A, indicated by open arrows). SDS-PAGE analyses revealed a slight mobility shift of Mcm10 in metaphase extracts (Fig. 1A, indicated by open and closed

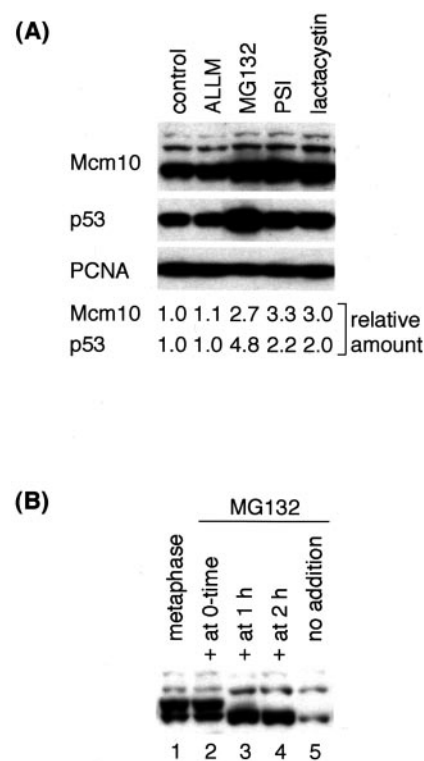


**FIG. 2. Mcm10 mRNA is present in early G<sub>1</sub>.** HeLa cells were released from metaphase and harvested for isolation of poly(A)<sup>+</sup> RNA and total proteins at indicated time points after release. **A**, Poly (A)<sup>+</sup> RNA (2  $\mu$ g) was analyzed by Northern blotting. The blot was probed with human Mcm10, PCNA, and elongation factor 1 $\alpha$  (EF1 $\alpha$ ) cDNA probes. The radioactivities of individual bands were measured with BAS2500 (Fuji Film) and normalized against the radioactivity of EF1 $\alpha$ . Expression levels of Mcm10 and PCNA were calculated relative to the amounts at zero time. **B**, protein fractions were prepared from the cell lysates, and levels were analyzed by immunoblotting as described under "Experimental Procedures." The intensities of individual bands were measured using Luminas Imager densitometry (Aisin Cosmos R&D) and normalized against actin intensity.

arrows), suggesting that the protein was subjected to modification. Expression levels of Mcm10 decreased in late M phase, remained low in early G<sub>1</sub> phase, and started to increase 6 h after release at the point of initiation of DNA replication (Fig. 1B). The level of Mcm10 in the insoluble fraction remained fairly constant during S phase (7.5–13.5 h). Mcm10 dissociated from the insoluble fraction in G<sub>2</sub> phase, concomitant with an increase in cyclin B1 levels. It is noteworthy that the 117-kDa band of Mcm10 was accompanied by 125- and 133-kDa bands (Fig. 1A, arrowheads), which may represent ubiquitinated Mcm10, as described below. The insoluble fraction mainly contained the 125 kDa protein, whereas both 125 and 133 kDa proteins were detected in the soluble fraction.

Conversely, Mcm7, a component of the prereplicative complex, was free in soluble fraction at metaphase, a part of which was loaded onto chromatin 1.5 h after release. At this time-point, lamin B1 was insoluble, indicating that the nuclear membrane was reassembled. Levels of Mcm7 associated with chromatin peaked at G<sub>1</sub>/S boundary and decreased during the progression of S phase, as demonstrated previously (28). In contrast, a fraction of PCNA protein (involved in the elongation steps of DNA replication) was associated with chromatin only in S phase. The time-course of loading and dissociation of Mcm10 was similar to that of PCNA. Levels of PCNA and Mcm7 proteins were fairly constant during the entire cell cycle.

**Mcm10 Is Stabilized by Proteasome Inhibitors**—To investigate whether low expression of Mcm10 results from periodic transcription, Northern blot hybridization was employed to determine mRNA levels (Fig. 2A). Since the transcripts of most proteins involved in DNA replication are regulated by E2F transcription factors, transcription should be down-regulated in early G<sub>1</sub>. The mRNA levels of Mcm10 and PCNA were measured at indicated timepoints after release from metaphase. Mcm10 transcripts resolved into a doublet, as observed previously (28). Mcm10 mRNA increased 2-fold at 4 h after release, reached maximum levels at 8 h (4-fold) and subse-



**FIG. 3. Proteasome inhibitors stabilize Mcm10.** **A**, HeLa cells were treated with either dimethyl sulfoxide (control) or 20  $\mu$ M each ALLM, MG132, PSI, and clasto-lactacystin  $\beta$ -lactone for 12 h. Whole cell extracts were prepared, and Mcm10, p53, and PCNA levels were analyzed by immunoblotting. The intensities of individual bands were measured using Luminas Imager densitometry and normalized against PCNA intensity. **B**, MG132 was added at 0, 1, and 2 h after release from metaphase block, and whole cell extracts were prepared 3 h after release (lanes 2–4). Mcm10 was detected by immunoblotting. As a control, whole cell extracts were prepared from metaphase cells (lane 1) or treated with dimethyl sulfoxide for 3 h after release (lane 5).

quently decreased. Similar to the pattern of Mcm10 mRNA expression, the mRNA levels of PCNA exhibited a 3-fold change during the cell cycle. In contrast, Mcm10 protein levels observed 4 h after release showed an 80% decrease, with a subsequent increase (Fig. 2B). These results suggest that Mcm10 protein expression is not mainly regulated at the transcription level, but depends on degradation.

We observed 125- and 133-kDa bands associated with the 117 kDa Mcm10 protein in cell extracts (Fig. 1A). From the apparent molecular mass of these proteins, we speculated that these bands represent ubiquitinated Mcm10. The ubiquitin-proteasome pathway is responsible for degradation of cell cycle-regulating proteins, including cyclin B1 and securin in late M phase (30, 31). Therefore, we next determined whether Mcm10 degradation is dependent on the 26 S proteasome by examining the effects of proteasome inhibitors on the steady-state level of Mcm10. After HeLa cells were treated with proteasome inhibitors for 12 h, Mcm10 levels were determined by immunoblotting (Fig. 3A). Treatment with proteasome inhibitors (MG132, PSI, and lactacystin) resulted in a 2.7–3.3-fold increase in Mcm10 expression. Since MG132 and PSI are potent inhibitors of other proteases, including calpain and calpactins, cells were also treated with ALLM, which is more inhibitory for calpain than proteasome. ALLM had no effect on the Mcm10 level. As a positive control, the levels of p53, a well known substrate of ubiquitin-mediated proteolysis, were determined. The treatment with proteasome inhibitors also increased p53 levels, although p53 was more stabilized by MG132 than by PSI or lactacystin. In contrast, proteasome inhibitors did not affect PCNA levels.



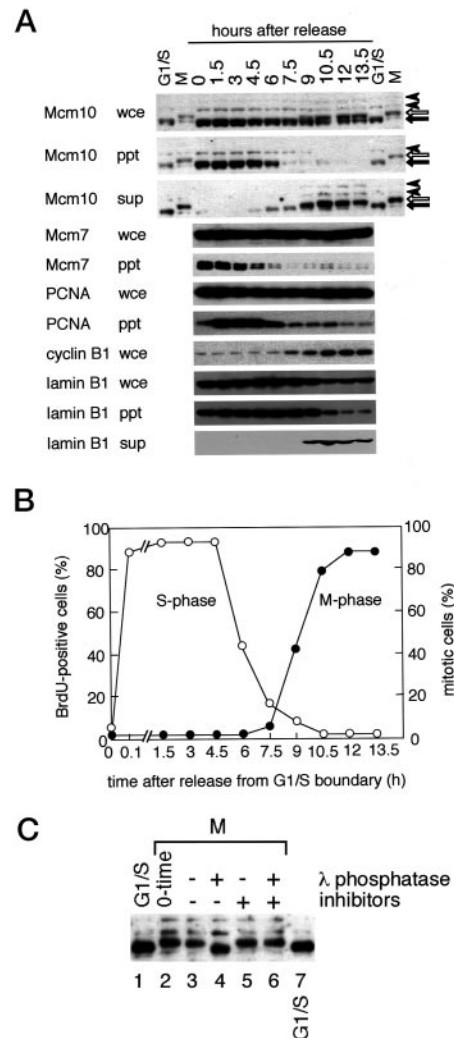
To investigate whether the down-regulation of Mcm10 in late M/G<sub>1</sub> phase was due to proteasome-mediated degradation, we synchronized HeLa cells at metaphase and examined the effects of MG132 on Mcm10 expression (Fig. 3B). When MG132 was added to cells during metaphase, cells were not released into G<sub>1</sub> phase, since degradation of cyclin B1 by proteasome is essential for mitosis progression (Fig. 3B, lane 2). Therefore, MG132 was added 1–2 h after release, and cell lysates were prepared 3 h after release (Fig. 3B, lanes 3–4). The protein levels of Mcm10 in early G<sub>1</sub> phase increased in the presence of MG132, suggesting that proteasome-mediated proteolysis is involved in the regulation of Mcm10 protein expression.

**Mcm10 Shows Altered Mobility in Soluble Fraction**—As illustrated in Figs. 1 and 3, Mcm10 displayed slower electrophoretic mobility at metaphase. To investigate whether this change is correlated with dissociation from chromatin, the electrophoretic mobility of Mcm10 during G<sub>2</sub>/M transition was investigated. HeLa cells were arrested at G<sub>1</sub>/S boundary by aphidicolin treatment following mitotic shake-off and resuspended in aphidicolin-free medium. Nocodazole was re-added 3 h after release from G<sub>1</sub>/S boundary to arrest cells at metaphase. Cell lysates were prepared at indicated times, and Mcm10 was detected by immunoblotting (Fig. 4A). As references of electrophoretic mobility, extracts from G<sub>1</sub>/S boundary and metaphase were loaded on the same gel. Mcm10 in insoluble fractions migrated faster, with no change in electrophoretic mobility. Levels of Mcm10 in soluble fractions increased after 6 h and migrated slower, as observed in metaphase extract. Interestingly, the change in electrophoretic mobility was associated with an increase in cyclin B1 and mitotic index (Fig. 4B). Lamin B1 in the soluble form also increased concomitantly with cyclin B1 increase, concordant with the theory that phosphorylation of lamin proteins by cdc2 kinase is essential for the disassembly of nuclear lamina structure. As illustrated in Fig. 1, Mcm7 dissociated from chromatin during S phase progression. In contrast, Mcm10 and PCNA remained coupled to chromatin throughout S phase. Remarkably, most Mcm10 proteins bound chromatin when cells were arrested at G<sub>1</sub>/S boundary with aphidicolin (zero time), whereas substantial levels of PCNA proteins associated with chromatin after removal of aphidicolin.

We speculate that Mcm10 may be phosphorylated upon dissociation from nuclease-resistant nuclear structures, since several cell cycle-regulating proteins undergo this modification in G<sub>2</sub>/M phase by mitotic kinases (32). Treatment of metaphase extract with  $\lambda$  phosphatase converted the slow migrating form in metaphase extracts to a faster migrating form in S phase extract (Fig. 4C). This modification was blocked by phosphatase inhibitors. The hyperphosphorylation of Mcm10 was not an artifact resulting from prolonged arrest at metaphase, since this process was also detected during cell release from G<sub>1</sub>/S boundary in the absence of nocodazole (data not shown).

#### DISCUSSION

The function of Mcm10 in DNA replication is poorly understood, although several lines of evidence indicate that Mcm10 has multiple roles in DNA replication in budding yeast. In this report, we characterize the human Mcm10 protein in all stages of the cell cycle, and demonstrate that: 1) human Mcm10 protein levels fluctuate during the cell cycle and decrease in late M/G<sub>1</sub> phase, 2) human Mcm10 binds chromatin specifically in S phase, and 3) dissociation of human Mcm10 from chromatin in G<sub>2</sub>/M phase is accompanied by protein phosphorylation. Interestingly, yeast Mcm10 displays different behavior to human Mcm10. Yeast Mcm10 binds chromatin during the entire cell cycle, with constant expression levels (25, 26). No modifications of yeast Mcm10 have been reported to date. We therefore as-



**FIG. 4. Phosphorylation of Mcm10 in G<sub>2</sub>/M phase.** A, HeLa cells were synchronized at G<sub>1</sub>/S boundary with aphidicolin treatment after mitotic shake-off. Cells were released in aphidicolin-free medium and harvested for the preparation of whole cell extracts (wce), Triton X-100 soluble fractions (sup), and Triton X-100-insoluble fractions (ppt) at indicated time points. Mcm10, Mcm7, PCNA, cyclin B1, and lamin B1 were detected by immunoblotting. As a reference of electrophoretic mobility, whole cell extracts from cells synchronized at metaphase (M) or G<sub>1</sub>/S boundary (G<sub>1</sub>/S) were loaded onto the same gel. Closed arrows indicate S phase Mcm10 with faster electrophoretic mobility. Open arrows indicate mitotic Mcm10 with slower electrophoretic mobility. Arrowheads indicate 125- and 133-kDa bands, which may represent ubiquitinated Mcm10. B, DNA synthesis was monitored by BrdUrd incorporation and immunofluorescent staining. Mitotic index was determined by Giemsa staining. Open circles indicate the percentage of BrdUrd-positive cells, while closed circles indicate the percentage of mitotic cells. C, mitotic extract was treated with buffer alone (lane 3),  $\lambda$  phosphatase (lane 4), phosphatase inhibitors (50 mM NaF and 10 mM Na<sub>3</sub>VO<sub>4</sub>, lane 5), or  $\lambda$  phosphatase in the presence of phosphatase inhibitors (lane 6), and Mcm10 was detected by immunoblotting. Whole cell extracts from HeLa cells arrested at G<sub>1</sub>/S boundary (lanes 1 and 7) and untreated mitotic extracts (lane 2) were separated on the same gel.

sume that Mcm10 function may be controlled by different mechanisms between species. Although the overall mechanism of DNA replication is well conserved among eukaryotes, different regulatory mechanisms for each replication factor are observed between yeast and higher eukaryotes. For example, yeast Orc stably binds to origins throughout the entire cell cycle (3, 4), whereas the interactions of *Xenopus* and mammalian Orc with chromatin appear to vary (33). Cdc6 is mainly regulated by degradation in yeast (19), but modulated via localization in mammalian cells (20). Thus, it is likely that mam-

malian cells have evolved a different regulatory system for Mcm10.

Our present analyses reveal that human Mcm10 levels decrease in late M/G<sub>1</sub> phase. The loss of Mcm10 in late M/G<sub>1</sub> may be explained either by down-regulation of transcription or specific degradation. The rapid loss of Mcm10 at the end of mitosis was not mainly regulated at the transcriptional level, since Mcm10 mRNA levels increased in G<sub>1</sub> phase. However, steady-state levels of Mcm10 increased after treatment with proteasome inhibitors, and down-regulation of Mcm10 in late M/G<sub>1</sub> was not observed in the presence of proteasome inhibitor. Furthermore, two proteins were detected in immunoblotting, with similar expression patterns as that of Mcm10 protein. The apparent molecular weights of these two proteins were consistent with monoubiquitinated and diubiquitinated Mcm10. These data suggest that a significant fraction of Mcm10 is degraded by proteasome-mediated proteolysis in late M/G<sub>1</sub> phase. Cell cycle-regulated proteolysis in anaphase depends on the anaphase-promoting complex (APC), a multisubunit ubiquitin ligase (E3) (34). APC transfers ubiquitin onto cyclin B1, cyclin A, geminin, and securin in anaphase and regulates exit from mitosis (30, 31, 35). APC targets contain destruction boxes necessary for ubiquitination-mediated proteolysis (30). Mcm10 contains a sequence (REQLAYLES, amino acids 719 to 727) homologous to the cyclin A destruction box (RAALAVLKS). Recently, a new recognition sequence (KEN box; K-E-N) in Cdc20 and Cdc6 was identified as an APC target (36, 37). Mcm10 additionally contains a putative KEN box (amino acids 71–73), which is another candidate for ubiquitination. In addition, a computer analysis using PEST-FIND program allowed the identification of two putative PEST domains at the amino-terminal end of Mcm10 (amino acids 34–68 and 71–99), which are hypothesized as targets for degradation by 26 S proteasome (38).

Since Mcm10 is regulated by ubiquitin-dependent proteolysis, it would be interesting to evaluate how ubiquitination is controlled in the cell cycle. One possibility is that replication factors interact with Mcm10 at G<sub>1</sub>/S boundary and protect the protein from polyubiquitination. Alternatively, ubiquitination may be coupled with phosphorylation. As discussed below, Mcm10 was dephosphorylated in late M phase, possibly providing a good substrate for ubiquitination. Remarkably, the monoubiquitinated form was dominant in insoluble fraction. Monoubiquitination requires E1 and E2 activities only, while polyubiquitination requires E3 activity (39). Therefore, the absence of the polyubiquitinated form in S phase may be due to the lack of APC activity in S phase.

Dissociation of Mcm10 from chromatin was consistent with changes in electrophoretic mobility. Studies on Mcm10 modification revealed that  $\lambda$  phosphatase converted the slower migrating form in the metaphase extract to the faster migrating form in S phase extract. It seems that Mcm10 is phosphorylated by protein kinase(s) in G<sub>2</sub>/M phase, similar to Orc1 (40, 41), Orc2 (42), Mcm2, and Mcm4 (21, 43). One possible kinase candidate is cdc2/cyclin B1, since the mobility shift of Mcm10 to a slower migrating form occurred in conjunction with an increase in cyclin B1. Mcm10 has several potential target sites for cdc2 kinase at its amino-terminal end. It is therefore likely that phosphorylation of Mcm10 by cdc2 kinase causes dissociation from chromatin, similar to the mechanism for Mcm2–7 complex (21). The issues of whether phosphorylation is essential for dissociation of Mcm10 from chromatin and cdc2 kinase directly phosphorylates Mcm10 require clarification.

The role of mammalian Mcm10 in DNA replication is still obscure. Results presented in this report reveal that human Mcm10 is regulated by proteolysis and phosphorylation in a

cell cycle-dependent manner. Consequently, a substantial amount of Mcm10 binds chromatin exclusively in S phase. Our data further suggest that Mcm10 is involved in S phase progression and is not a component of the prereplicative complex, inconsistent with previous observations in yeast. Homesley *et al.* (25) reported that the yeast Mcm2–7 complex is released from chromatin when the temperature-sensitive *mcm10–43* mutant dissociates under non-permissive temperature conditions, suggesting that binding of Mcm10 to chromatin is essential for the loading of the Mcm2–7 complex (25). Yeast Mcm10 may have an additional function in pre-replicative complex formation. We cannot exclude the possibility that a small fraction of human Mcm10 that escaped degradation in late M/G<sub>1</sub> phase, may be involved in the formation of prereplicative complex. However, further experiments are necessary to clarify these hypotheses. The functions of human Mcm10 during S phase progression remain unresolved so far. We have discovered that human Mcm10 interacts with the mammalian Orc and Mcm2–7 complex (28). Mcm10 may react with those proteins to release Mcm2–7 complex from origins for triggering DNA replication. Recent findings in yeast indicate that Mcm10 genetically interacts with elongation factors including DNA polymerase  $\delta$ ,  $\epsilon$  and Cdc45 (26). It is possible that Mcm10 facilitates the assembly of the replication complex or stabilizes replication machinery.

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