Localization of Human Mcm10 Is Spatially and Temporally Regulated during the S Phase*

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Mcm10 (Dna43) is an essential protein for the initiation of DNA replication in Saccharomyces cerevisiae. Recently, we identified a human Mcm10 homolog and found that it is regulated by proteolysis and phosphorylation in a cell cycle-dependent manner and that it binds chromatin exclusively during the S phase of the cell cycle. However, the precise roles that Mcm10 plays are still unknown. To study the localization dynamics of human Mcm10, we established HeLa cell lines expressing green fluorescent protein (GFP)-tagged Mcm10. From early to mid-S phase, GFP-Mcm10 appeared in discrete nuclear foci. In early S phase, several hundred foci appeared throughout the nucleus. In mid-S phase, the foci appeared at the nuclear periphery and nuclear regions. In the late S and G2 phases, GFP-Mcm10 was localized to nucleoli. Although the distributions of GFP-Mcm10 during the S phase resembled those of replication foci, GFP-Mcm10 foci did not colocalize with sites of DNA synthesis in most cases. Furthermore, the transition of GFP-Mcm10 distribution patterns preceded changes in replication foci patterns or proliferating cell nuclear antigen foci patterns by 30–60 min. These results suggest that human Mcm10 is temporarily recruited to the replication sites 30–60 min before they replicate and that it dissociates from chromatin after the activation of the prereplication complex.

To maintain the integrity of the genome, eukaryotic DNA replication is tightly regulated to ensure that the initiation of replication occurs only once per cell cycle. Recent analyses using different systems show that eukaryotes share a common mechanism that coordinates the initiation of DNA replication from a large number of origins (1). The initiation of DNA replication consists of two steps, namely the formation of a prereplication complex (pre-RC) and the activation of pre-RC. The pre-RC is formed by the sequential assembly of the hexameric origin recognition complex (Orc), Cdc6, Cdt1, and the Mcm2-7 complex on chromatin in telophase (1–3). This process is also referred to as replication licensing. At the G1/S transition, the pre-RC is activated by S phase cyclin-dependent kinases and the Cdc7/Dbf4 kinase, which mediate the association of Cdc45 to a preformed pre-RC at each origin with programmed timing (4–6). Subsequent to Cdc45 loading, the DNA is unwound at the replication origin (7) and the single-stranded DNA-binding protein (RPA) and DNA polymerases are recruited (7, 8). Eukaryotes have several means to prevent the initiation of DNA replication. In mammalian cells, which ensures that the pre-RC is not reassembled until the segregation of chromosomes in mitosis (9, 10). Moreover, Cdt1 is regulated by its expression levels and by its interaction with geminin, which guarantees that it is only functional in the G1 phase (11, 12).

Mcm10 was first identified in Saccharomyces cerevisiae in screens for genes that are required for chromosomal DNA replication (13, 14). Studies in S. cerevisiae suggest that Mcm10 plays multiple roles in DNA replication. mcmt mutants suffer a defect in the initiation of DNA replication (14). In the mcm10-1 mutant, the replication forks stall when the replication machinery passes through origins that did not fire (14). This suggests that Mcm10 plays a unique role in DNA replication. Budding yeast Mcm10 has also been found to physically interact with the components of Orc and Mcm2-7 complexes (14). Moreover, it genetically interacts with Cdc45 and DNA polymerases δ and ε, which are necessary for the elongation steps of DNA replication (15, 16). These results suggest that budding yeast Mcm10 is involved in both the activation of the pre-RC and the elongation steps of DNA replication.

Mcm10 homologs have been identified in Schizosaccharomyces pombe, Caenorhabditis elegans, Drosophila, and Xenopus (17–19). Recently we identified human Mcm10 and found that human Mcm10 interacts with the mammalian Orc2 protein and the Mcm2-7 complex (20), similar to the budding yeast Mcm10. However, the budding yeast and vertebrate Mcm10 proteins seem to have different functions. In S. cerevisiae, removing Mcm10 from the chromatin in G1 phase releases Mcm2 from chromatin, which suggests that Mcm10 is required for pre-RC formation (15). In contrast, the human and Xenopus Mcm10 proteins bind chromatin after pre-RC formation (18, 21). In addition, the functions of Mcm10 seem to be controlled by different mechanisms between species. Human Mcm10 protein levels fluctuate during the cell cycle and decrease from anaphase to G1 phase (21). Human Mcm10 also specifically binds chromatin in S phase and dissociates from chromatin in G2/M phase, which is accompanied by protein phosphorylation (21). In contrast, in S. cerevisiae and S. pombe, Mcm10 remains bound to chromatin throughout the entire cell cycle and displays constant expression levels (15, 16, 22).

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Dynamics of Human Mcm10 during DNA Replication

Our previous studies suggest that human Mcm10 is involved in S phase progression, although the precise role played by human Mcm10 still remains unknown. To clarify the function of Mcm10 in mammalian cells, we established HeLa cell lines expressing green fluorescent protein (GFP)- and hemagglutinin (HA)-tagged Mcm10 and studied the dynamics of Mcm10 localization during the cell cycle. The subcellular localization of human Mcm10 changed during S phase progression. Mcm10 appeared to be recruited to the replication sites prior to DNA synthesis. These results indicate that human Mcm10 may participate in the activation of pre-RC. The implications of these results and the role Mcm10 may play in DNA replication are discussed.

EXPERIMENTAL PROCEDURES

Plasmid Construction—The cDNA for human Mcm10 was first subcloned into pETGFP (23) or pETHA11, which contains the GFP tag or HA epitope tag upstream of the multicloning sites, respectively. To construct pETHA11, two oligonucleotides with the sequences 5′-CCGGT CCAGA CCATG CTGAT CGTACC GACGG CCGAC ACTAC GCTTG CATGA-3′ and 5′-GATCT CAAGC TAGTC TAGTG TGCGA CTGCAG TAGTG ATGTC CGTGAG GAGGC AACAT GTC-3′ were annealed and cloned into the AgeI-BglII sites of pETGFP. Human Mcm10 cDNA was amplified by PCR using the primers 5′-ATTAC TCAGG CATGA GAGGG AACAT GAAAT TTAGC ATG-3′ and 5′-ATTAC CGCGG TTTTA AGGCT GTTCA GAAAT TTAGC ATG-3′ were digested with AgeI and DraI, filled with T4 DNA polymerase, and the resulting fragment containing the entire coding region for GFP-tagged Mcm10 or HA-tagged Mcm10 was cloned into the BamHI site of pETGFP (Clontech).

Cell Culture and Synchronization—HeLa cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum. For synchronization at the mitotic phase, exponentially growing cells were treated with 50 ng/ml nocodazole (Aldrich) for 4 h and collected by mitotic shake-off. For synchronization in the G1 phase, the mitotic cells were treated with 50 ng/ml nocodazole (Aldrich) by the addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside at 30 °C for 3 h. After centrifugation, the cell pellets were suspended in 1/10 culture volume of lysis buffer (20 mM sodium phosphate buffer, pH 7.4, 0.15 mM NaCl, 0.5% Triton X-100) and lysates were incubated with 1 μl of anti-Mcm10 rabbit antiseraum at 4 °C for 1 h, after which 10 μl of protein A-Sepharose Fast Flow (Amersham Biosciences) were added and incubated for an additional 1 h. The beads were washed five times with lysis buffer, and the precipitates were dissolved in 40 μl of 2× SDS-PAGE sample buffer.

Establishment of HeLa Cell Lines Expressing GFP-tagged Mcm10 Protein in Escherichia coli—His6-tagged Mcm10 was constructed by cloning the PCR-amplified human Mcm10 into the Sall-Nol sites of pET24a (Novagen). Expression of His6-tagged Mcm10 was induced in the E. coli strain BL21(DE3) (Stratagene) by the addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside at 30 °C for 3 h. After centrifugation, the cell pellets were suspended in 1/10 culture volume of lysis buffer (20 mM sodium phosphate buffer, pH 7.4, 0.15 mM NaCl, 0.5% Triton X-100, 8 mM urea). His6-tagged Mcm10 was purified over a TALON column (Clontech) according to the manufacturer’s instructions.

RESULTS

Establishment of HeLa Cell Lines Expressing GFP-tagged Mcm10—To better understand the dynamics of human Mcm10 localization during the cell cycle, we first tried to detect endogenous Mcm10 protein by indirect immunofluorescence. This was unsuccessful because of the poor reactivity of the anti-Mcm10 antibody in immunohistochemistry. Therefore, we established stable HeLa cell lines that express a GFP-tagged Mcm10 protein. The expression of GFP-Mcm10 was maintained by the bicistronic message encoding the hygromycin B-resistant gene (hyg) because the expression of transfected genes in mammalian cells is usually repressed by epigenetic mechanisms. Forty arbitrary clonal cell lines were expanded in the presence of hygromycin B and screened for GFP-Mcm10 expression by immunoblotting. Fig. 1a shows the immunoblot of the whole cell extracts made from 11 cell lines that were probed with the anti-Mcm10 antibody, which recognizes both the endogenous Mcm10 protein that migrates at 100 kDa and the GFP-tagged Mcm10 protein that migrates at 130 kDa. Each of these cell lines expressed GFP-Mcm10 at different levels relative to endogenous Mcm10 expression. G10-24 and G10-30 cells were initially considered to be the most desirable of the cell lines for further studies because they expressed the exog-
We first examined whether GFP-Mcm10 was expressed in stable cell lines. GFP-Mcm10 and the endogenous Mcm10 protein were monitored by immunoblotting analysis with a polyclonal antibody against Mcm10, which detects both the endogenous Mcm10 protein and GFP-tagged Mcm10. a, whole cell extracts from untransfected HeLa cells and stably transfected HeLa cells (G10-1 to G10-36) were subjected to immunoblotting as described previously (21). Lamin B1 was detected as a reference. b, HeLa cells and G10-1 cells were released from the metaphase block, and samples were harvested at the various time points indicated. DNA contents of cells were analyzed by flow cytometry. Positions of 2C and 4C DNA contents are indicated. c, whole cell extracts (wce) and Triton X-100-soluble fractions (sup) and insoluble fractions (ppt) were prepared from G10-1 cells at the indicated salt concentrations and subjected to immunoblotting. The GFP-Mcm10 protein as well as proteins were separated by low speed centrifugation and analyzed by immunoblotting. The GFP-Mcm10 protein as well as the endogenous Mcm10 remained in the insoluble chromatin fraction at 0.1 M NaCl but increasing the salt concentration to 0.3 M removed both proteins from the chromatin (Fig. 1c). We have previously reported that human Mcm10 is regulated by proteolysis and phosphorylation in a cell cycle-dependent manner (21). To verify that GFP-Mcm10 is regulated in the same manner as endogenous Mcm10, the cells were synchronized in metaphase with nocodazole followed by mitotic shake-off. The cells were then collected into the G1 phase, or accumulated at the G1/S boundary with aphidicolin. The cells were then fractionated into Triton X-100-soluble and -insoluble fractions, and the subcellular localization of GFP-Mcm10 was investigated by immunoblotting (Fig. 1d). In cells that had been synchronized to be in the G1 phase or at the G1/S boundary, most of the GFP-Mcm10 proteins were mainly found in the insoluble fraction. GFP-Mcm10 appeared to be more soluble at the G1/S boundary, which may be due to the effect of GFP-tag. At metaphase, however, the GFP-Mcm10 protein dissociated from the chromatin and was found in the soluble fraction. This pattern reflects that of endogenous Mcm10. Moreover, the expression of GFP-Mcm10 decreased in the early G1 phase, as did the expression of endogenous Mcm10. The down-regulation of GFP-Mcm10 could be explained by its proteasome-mediated proteolysis because the presence of proteasome inhibitors stabilized GFP-Mcm10 levels (data not shown). In addition, like endogenous Mcm10, GFP-Mcm10 in metaphase showed slower electrophoretic mobility, which could be due to its phosphorylation (21). Thus, the GFP-tagged protein appears to be regulated in the same fashion as its endogenous counterpart throughout the cell cycle. This suggests that GFP-Mcm10 is functional and equivalent to endogenous Mcm10 in living cells and that GFP-Mcm10 does not impair the function of endogenous Mcm10 as a dominant negative mutant.

The Intracellular Distribution of GFP-Mcm10 Changes throughout the Cell Cycle—We next examined the localization of GFP-Mcm10 in G10-1 cells. In an asynchronous population of G10-1 cells, GFP-Mcm10 was found to be concentrated in the nucleus, but weak fluorescence was also detected in the cytoplasm (Fig. 2a, left panels). The distribution of GFP-Mcm10 in the nucleus was relatively homogeneous (Fig. 2a, right panels). However, a different distribution of GFP-Mcm10 was observed when the cells were subjected to Triton X-100 extraction to remove soluble proteins (data not shown). In these cells, GFP-Mcm10 was observed as discrete foci in the nucleus. This distribution has also been observed for many DNA replication proteins, including PCNA (25), and replication foci (26). The fluorescence of GFP-Mcm10 in the cytoplasm disappeared after extraction. The distribution, number, and size of the GFP-Mcm10 foci in the individual cells varied. This is probably due to the fact that the cell population was asynchronous. Thus, to determine more precisely how the various staining patterns correlated with particular cell cycle phases, the cells were arrested at the G1/S boundary by aphidicolin treatment followed by mitotic shake-off and then released in aphidicolin-free medium. Three kinds of localization patterns were observed, each of which was observed at specific time points during the S phase (Fig. 2, b and c). The first pattern (type I) appeared in the first 3 h from the G1/S boundary. This pattern was characterized by hundreds of small foci distributed throughout the nuclei with the exception of the nucleoli. The second pattern (type II) appeared 3 h after the release into S phase. This pattern was characterized by foci concentrated around the nucleoli and in the nuclear periphery. At 3 h after release, weak fluorescence was still detected in the nucleoplasm. This disappeared 4.5 h after release. At 6 h after release, the number of foci decreased. The third pattern (type III) was observed toward the end of the...
S phase (6–7.5 h after release). This pattern was characterized by the appearance of GFP-Mcm10 in nucleoli. That GFP-Mcm10 was localized in the nucleoli during this phase was confirmed by the colocalization of nucleolin and GFP-Mcm10 (Fig. 2d). The physiological function of Mcm10 in nucleoli in late S phase is unknown. It may be that localization in the nucleoli serves to sequester Mcm10, thereby preventing it from reaching its target, which may help to ensure that a replicated chromosome does not reinitiate replication.

It is known that some fixation protocols alter the localization of proteins. To test the veracity of our confocal studies, we fixed cells with either 3.7% formaldehyde or ethanol:acetic acid (19:1). The same results were obtained. We also found that the same patterns were obtained when a different cell line was tested (data not shown), which indicates that these patterns are also not specific to one cell clone. Moreover, we examined the subcellular localization of GFP-Mcm10 and endogenous Mcm10 during S phase progression by immunoblotting after fractionating the cells into Triton X-100-soluble and -insoluble samples. Both GFP-Mcm10 and endogenous Mcm10 bound chromatin during the S phase and dissociated from chromatin in the G2 phase (Fig. 2e). This is consistent with our earlier observations (Fig. 1c). Also consistent with this is our observation that during mitosis, GFP-Mcm10 was absent from condensed chromosomes and had diffused into the cytoplasm (Fig. 2f).

**GFP-Mcm10 Is Recruited to Replication Sites before They Replicate**—We next investigated the spatial relationship between the GFP-Mcm10 foci and PCNA foci. The nuclear foci of PCNA have been well characterized and are known to colocalize with sites of newly synthesized DNA (24). Thus, synchronized cells were extracted prior to fixation, and PCNA was detected by indirect immunofluorescence (Fig. 3a). The cells arrested at the G1/S boundary by aphidicolin did not display any PCNA fluorescent labeling, whereas GFP-Mcm10 bound chromatin and appeared to be distributed throughout the nucleoplasm. Between 10 min and 1.5 h after release from the G1/S boundary, both the GFP-Mcm10 foci and the PCNA foci...
showed the type I pattern. However, the GFP-Mcm10 foci and the PCNA foci did not colocalize in most cases. At 3 h after release, the GFP-Mcm10 foci started to accumulate around the nucleoli and the nuclear periphery, whereas the PCNA foci were still distributed throughout the nuclei. Between 4.5 and 6 h, both the GFP-Mcm10 foci and the PCNA foci were concentrated around the nucleoli and in the nuclear periphery. At 7.5 h, GFP-Mcm10 was mainly concentrated in the nucleoli, whereas PCNA formed a few discrete foci around the nucleoli and in the nuclear periphery. Each of the patterns were enriched at specific time points, and the transition in the GFP-Mcm10 distributions preceded that of the PCNA distributions by 30–60 min (Fig. 3b).

To confirm the above results, we examined the correlation of the appearance of Mcm10 foci with that of the DNA replication foci. The DNA replication foci were detected by pulse labeling with the nucleotide analog BrdU and staining with an antibody against BrdU. Because the acid treatment denatures GFP and results in the loss of fluorescence, we established stable cell lines expressing HA-tagged Mcm10 employing the same strategy used to generate the GFP-tagged lines. We also confirmed that HA-Mcm10 was functionally equivalent to endogenous Mcm10 (data not shown). As with GFP-Mcm10, HA-Mcm10 foci did not colocalize with DNA replication foci, and the HA-Mcm10 distributions changed earlier than the replication focus distributions (Fig. 4a). In contrast, the PCNA foci mostly colocalized with the BrdU foci (Fig. 4b).

Mcm10 Is Reused for Consecutive Rounds of pre-RC Activation—As shown in Fig. 2, GFP-Mcm10 changes its localization during the progression of S phase, although only a small fraction of GFP-Mcm10 (10–15%) was soluble during the S phase. One possibility to explain this is that the transition from ear-

![Fig. 2—continued](image-url)
lier to later replicons involves the disassembly of Mcm10 into a nucleoplasmic pool of insoluble Mcm10 and the efficient reassembly at newly activated sites. Alternatively, it is possible that Mcm10 is temporarily stabilized at the replication sites and then degraded after pre-RC activation. To investigate which hypothesis is correct, we performed a pulse-chase experi-

Fig. 3. Subcellular distribution of GFP-Mcm10 during the progression of the S phase and its relationship with PCNA foci. a, synchronized cells were extracted with CSK buffer containing 0.1% Triton X-100, fixed with ethanol:acetic acid (19:1), and immunostained for PCNA. DNA was stained with DAPI. Colocalization of GFP-Mcm10 and PCNA was evaluated by computer merging. b, at each time point, the spatial distribution of GFP-Mcm10 or PCNA was sorted according to whether it fell into one of three patterns. The type I pattern is characterized by the presence of many foci distributed throughout the nuclei (open and closed circles). The type II pattern is characterized by the peripheral and perinucleolar staining (open and closed squares). The type III pattern is characterized by the nucleolar staining of GFP-Mcm10 (open triangles). The percentage of cells with each of these patterns was determined for each time point. The percentage of PCNA-negative cells was also scored (closed triangles).
iment to measure the half-life of human Mcm10 in the S phase.

As shown in Fig. 5a, anti-Mcm10 serum precipitated a 100-kDa Mcm10 protein, which was not precipitated with pre-immune serum. HeLa cells arrested at the G1/S boundary with aphidicolin were pulsed for 1 h and chased in aphidicolin-free medium. After the transfer to aphidicolin-free medium, the S phase progressed with a normal time course as monitored by BrdU incorporation (data not shown). The half-life of human Mcm10 was 6 h (which is close to the length of S phase), which suggests that most Mcm10 proteins assemble and disassemble both efficiently and sequentially throughout the S phase (Fig. 5b).

It is also important to determine how much Mcm10 is loaded onto replicons at the onset of DNA replication. Mcm10 is 40–60 times more abundant than Orc in budding yeast (16), whereas Mcm10 is present at only two molecules/origin in Xenopus egg cytosol (18). We estimated the copy number of Mcm10 in HeLa cells. Because Mcm10 protein levels fluctuate throughout the cell cycle, whole cell extracts were prepared from HeLa cells arrested at the G1/S boundary. The Mcm10 in the extracts was then calibrated by immunoblotting. His6-tagged Mcm10, which was overexpressed in E. coli, was used as a reference. As shown in Fig. 5c, 10⁴ cells contained 480 pg of Mcm10, which corresponds to 3.0 × 10⁵ molecules/cell. Given that the average size of replicons in mammalian cells is 200–300 kb (27), the human diploid genome contains 2.0–3.0 × 10⁴ replication origins. Because Mcm10 bound only a limited region of the genome in the S phase, Mcm10 seems to be present in excess over the number of replication origins.

**DISCUSSION**

Several lines of evidence suggest that human Mcm10 is involved in DNA replication, but little is known about its precise functions in this event. To address this point, we fluorescently tagged Mcm10 to study the localization dynamics of Mcm10 within mammalian cells. We established HeLa cell lines that stably express GFP-Mcm10 by using the bicistronic message encoding a selectable marker. GFP-tagged Mcm10

![Fig. 4. Subcellular distribution of HA-tagged Mcm10 during the progression of the S phase and its relationship with BrdU foci.](image-url)
showed the same cell cycle-dependent chromatin association, phosphorylation, and fluctuation of protein levels as the endogenous Mcm10 protein, which suggests that GFP-Mcm10 is functionally equivalent to endogenous Mcm10 protein in HeLa cells.

We found that GFP-Mcm10 exhibited several unique distribution patterns as the S phase progressed. From early to mid-S phase, GFP-Mcm10 appeared in discrete nuclear foci. In early S phase, several hundred foci were distributed throughout the nucleus but were excluded from the nucleoli. In mid-S phase, foci were distributed at the nuclear periphery and nucleoli. In late S phase, GFP-Mcm10 was localized in nucleoli. These spatial patterns of human Mcm10 and the temporal order of their appearance during the S phase are similar to those of replication foci. However, Mcm10 was not present in sites of DNA replication elongation, and the patterns of Mcm10 changed 30–60 min before the patterns of replication foci changed. These results suggest that Mcm10 is recruited to the chromosomal domains 30–60 min before they replicate and that it then dissociates after DNA replication has been initiated, although we cannot exclude the possibility that a minor population of human Mcm10 proteins that escape immunodetection are present at the replication forks. The data also suggest that human Mcm10 plays an important role in pre-RC activation than in the elongation steps. Our results are in good agreement with the recent study using a Xenopus egg cell-free system showing that Xenopus Mcm10 binds to origins after pre-RC formation and is required for the loading of Cdc45 onto chromatin, which suggests that the loading of Mcm10 is involved in pre-RC activation (18). Like Xenopus Mcm10, the fission yeast Mcm10 homolog Cdc23 functions after pre-RC formation and is necessary for Cdc45 loading, although Cdc23 binds to chromatin throughout the cell cycle (22).

It is noteworthy that there is a time lag between Mcm10 loading and the initiation of DNA replication. Recent studies indicate that the activation of pre-RC requires the chromatin

**Fig. 5.** Half-life and copy number of Mcm10 in the S phase. a, the anti-Mcm10 antibody precipitates a 100-kDa Mcm10 protein. HeLa cells growing asynchronously were pulse-labeled with [35S]methionine for 1 h, and the cell extracts were prepared. Following immunoprecipitation with preimmune serum (lane 1) or anti-Mcm10 antiserum (lane 2), the immunoprecipitated materials were analyzed on a 8% SDS-PAGE gel and detected with BAS2500 (Fuji Film). The arrowhead indicates the 100-kDa Mcm10 protein. b, pulse-chase experiment of human Mcm10 during the S phase. HeLa cells arrested at the G1/S boundary with aphidicolin were grown in the presence of [35S]methionine for 1 h and transferred to medium containing unlabeled methionine. The cell extracts were then prepared 0, 2, 4, and 6 h after the transfer. The immunoprecipitated materials were analyzed on a SDS-PAGE gel (upper panel, IP). As a loading control, the immunoprecipitated Mcm10 was detected by immunoblotting (upper panel, Western). The left and right halves of the upper panel are from the duplicate samples in the same experiments. The relative intensity of the bands was measured by BAS2500, and the results are presented as percentages of the maximum (lower panel). c, purified recombinant Mcm10 and extracts from HeLa cells arrested at the G1/S boundary were analyzed by immunoblotting with the anti-Mcm10 antibody (upper panel). Relative intensity of the bands was measured by densitometry (Aisin-cosmos) and plotted (lower panel). The open circles indicate the intensity of the bands of 200, 400, or 800 ng of purified Mcm10. The closed circles and dotted lines indicate the intensity of the bands obtained from 10^4 HeLa cells.
loading of checkpoint proteins, the loading of Cdc45, and the phosphorylation of replication factors by Cdc7 kinase and cyclin-dependent kinase (5, 6, 28–30). It would be interesting to study the timing of each event and to investigate whether there are unknown steps between Mcm10 loading and the start of DNA replication in mammalian cells that have not yet been characterized. Interestingly, the chromatin association of RPA occurs several minutes after Mcm10 loading in the Xenopus cell-free system (18). This may reflect the different mechanisms that regulate the replication initiation in early development and in somatic cells. It would also be interesting to elucidate the mechanism that regulates the loading of Mcm10. The results obtained from the Xenopus cell-free system showed that Mcm10 binds chromatin independently of Cdc7 and cyclin-dependent kinase (18, 29). Because the loading of Mcm10 seems to be one of the earliest steps of pre-RC activation, the way Mcm10 loading is regulated may relate to the mechanism that decides the order of firing of replication origins. Our previous analysis showed that human Mcm10 is down-regulated in the G1 phase by ubiquitin-mediated proteolysis (21). Thus, the mechanism that regulates Mcm10 loading may involve the stabilization of Mcm10 at the G1/S boundary.

It remains unknown how Mcm10 is involved in the activation of pre-RC. Recent studies indicate that fission yeast Cdc23 physically interacts with both Dbf1/Hsk1 kinase (the homolog of Cdc7/Hdb4) and the Mcm2-7 complex and that the interaction facilitates the phosphorylation of the Mcm2-7 complex by Dbf1/Hsk1. Thus, Mcm10 may activate pre-RC by recruiting Cdc7/Hdb4 kinase to the pre-RC at the G1/S boundary (31). Mcm10 also interacts with multiple replication factors, including Orc, Cdc45, DNA polymerases δ and ε (14–16, 19, 20). Thus, Mcm10 may facilitate the assembly of the replication complex or mediate the contact of other factors by its interaction with multiple replication proteins.

The pulse-chase experiments revealed that the half-life of human Mcm10 was 6 h, which is close to the length of S phase. Because most Mcm10 molecules were insoluble and changed their distribution as S phase progressed, Mcm10 proteins seem to transfer from earlier to later replicons by dissociating from chromatin and then efficiently reassociating with chromatin. It would be possible that the loading of Mcm10 on chromatin is rate-limiting for DNA replication.

Our results also revealed that Mcm10 is 10 times more abundant than the number of origins in HeLa cells. Because Mcm10 is localized in limited chromosomal domains throughout the S phase, Mcm10 seems to be present in excess over the number of replicons. One possible explanation for this result is that Mcm10 may assemble into a homocomplex. Recently, Cook et al. (32) reported that budding yeast Mcm10 forms a multisubunit homocomplex in vitro. The assembly of Mcm10 into homocomplexes may provide a structural basis for the multiple interactions of Mcm10 with several replication factors. Alternatively, Mcm10 may form multiple homocomplexes on DNA and change the topology of the DNA or the chromatin structure. It is also possible that Mcm10 multimer interacts with nuclear structure. It should be noted that G10-1 cells overexpressed GFP-Mcm10 at twice the levels of the endogenous Mcm10 protein without having any effect on the cell cycle progression. Because the level of endogenous Mcm10 expression in the G10-1 cells was almost the same as the level in wild type HeLa cells, this means that in the G10-1 cells, the amount of Mcm10 that bound to chromatin during the S phase was triple the amount in wild type cells. When Mcm10 is overexpressed, it may be bound to dormant origins via its interaction with Orc.

It would be interesting to examine whether Mcm10 homologs in other organisms show the same behavior as human Mcm10. Yeast Mcm10, at least, seems to be controlled by different mechanisms because yeast Mcm10 binds chromatin during the entire cell cycle and exhibits constant expression levels (15, 16, 22). Mammalian cells have several means of replication control involving geminin (11, 12) and dissociation of Orc1 from chromatin after origin firing (33) that are not observed in yeast. The control of Mcm10 loading may be an additional regulatory step to ensure the fidelity of DNA replication in higher eukaryotes.

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