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MCM2–7 Proteins Are Essential Components of Prereplicative Complexes that Accumulate Cooperatively in the Nucleus during G1-phase and Are Required to Establish, But Not Maintain, the S-phase Checkpoint

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A prereplicative complex (pre-RC) of proteins is assembled at budding yeast origins of DNA replication during the G1-phase of the cell cycle, as shown by genomic footprinting. The proteins responsible for this prereplicative footprint have yet to be identified but are likely to be involved in the earliest stages of the initiation step of chromosome replication. Here we show that MCM2–7 proteins are essential for both the formation and maintenance of the pre-RC footprint at the origin ARS305. It is likely that pre-RCs contain heteromeric complexes of MCM2–7 proteins, since degradation of Mcm2, 3, 6, or 7 during G1-phase, after pre-RC formation, causes loss of Mcm4 from the nucleus. It has been suggested that pre-RCs on unreplicated chromatin may generate a checkpoint signal that inhibits premature mitosis during S-phase. We show that, although mitosis does indeed occur in the absence of replication if MCM proteins are degraded during G1-phase, anaphase is prevented if MCMs are degraded during S-phase. Our data indicate that pre-RCs do not play a direct role in checkpoint control during chromosome replication.

INTRODUCTION

Budding yeast origins of DNA replication are bound throughout the cell cycle by the Origin-Recognition Complex (ORC) (Bell and Stillman, 1992; Diffley and Cocker, 1992; Diffley *et al.*, 1994; Santocanale and Diffley, 1996; Aparicio *et al.*, 1997; Tanaka *et al.*, 1997). During G1 phase, a larger prereplicative complex (pre-RC) is assembled around ORC, as evidenced by genomic footprinting (Diffley *et al.*, 1994). It is likely that pre-RCs play a key role in the earliest stages of chromosome replication, since the initiation site of bidirectional replication at the chromosomal origin ARS1 has been found to lie in the center of the pre-RC footprint (Bielinsky and Gerbi, 1999). The formation of pre-RCs represents a key step in establishing the "replication competence" of an origin and is inhibited outside of G1-phase by cyclin-dependent kinase activity (Dahmann *et al.*, 1995; Detweiler and Li, 1998), thereby ensuring that each origin is activated just once during S-phase, so that a single copy of the genome is made in each round of the cell cycle.

Until now, the protein components of the pre-RC, as defined by genomic footprinting, have remained poorly characterized. One candidate is the Cdc6 protein, which is essential for pre-RC formation at the end of mitosis and during G1-phase (Cocker *et al.*, 1996; Santocanale and Diffley, 1996; Detweiler and Li, 1997). However, Cdc6 is degraded early in G1-phase (Piatti *et al.*, 1995; Drury *et al.*, 1997), whereas pre-RCs persist at origins until initiation occurs, or until the origin is replicated passively during S-phase, by replication forks from neighboring origins (Santocanale *et al.*, 1999). Cdc6 is required for the six members of the MCM2–7 family to become associated with chromatin (Donovan *et al.*, 1997; Liang and Stillman, 1997; Weinreich *et al.*, 1999). MCM proteins are further candidates, therefore, for the proteins that make up the pre-RC; moreover, they play a key role in both the initiation and elongation stages of chromosome replication in budding yeast (Labib *et al.*, 2000; Yan *et al.*, 1993), and they have been shown to be components of "Replication Licensing Factor" in *Xenopus* egg extracts (Chong *et al.*, 1995; Madine *et al.*, 1995; Kubota *et al.*, 1997; Thommes *et al.*, 1997).

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Experiments involving chromatin immunoprecipitation have shown that Cdc6 is essential for associating MCM proteins with origin-containing DNA (Aparicio *et al.*, 1997; Tanaka *et al.*, 1997). However, these experiments, which have a resolution of around 500 bp, do not distinguish between the possibility that MCM proteins are bound to origins as components of pre-RCs, or instead are associated with other sequences adjacent to the origin. Furthermore, an allele of *CDC6* has been described, *CDC6-d1*, that supports partial pre-RC formation at ARS305 in the absence of bulk loading of MCM proteins onto chromatin (Perkins and Diffley, 1998). The role of MCM proteins in pre-RC formation, therefore, remains unclear.

Other proteins, such as Cdc45 and Sld3, are known to associate with early origins of DNA replication during G1 phase (Aparicio *et al.*, 1999; Kamimura *et al.*, 2001). It is unlikely that these proteins are core-components of pre-RCs, however, as they do not associate with late origins until S-phase, whereas pre-RC formation occurs at all origins at the end of mitosis. Furthermore, genomic footprinting of a cold-sensitive allele of *CDC45*, *cdc45–1*, has shown that cells accumulate at the restrictive temperature with the 2-μm origin of DNA replication in the prereplicative state (Owens *et al.*, 1997).

In addition to their role in the initiation of chromosome replication, it has also been suggested that pre-RCs may be the source of a checkpoint signal, during G1 phase and S-phase, that inhibits premature entry into anaphase (Kelly *et al.*, 1993; Maiorano *et al.*, 1996; Piatti *et al.*, 1995; van Brabant *et al.*, 2001). If pre-RC formation is blocked, by depletion of Cdc6, anaphase occurs in the absence of S-phase (Piatti *et al.*, 1995). Conversely, when the checkpoint is active–for example if cells are treated with the ribonucleotide-reductase inhibitor hydroxyurea, which reduces dNTP pools and inhibits the progression of DNA replication forks from early origins–pre-RCs are still present at late origins (Santocanale and Diffley, 1998). It has been proposed, therefore, that anaphase may not occur until pre-RCs are disassembled throughout the genome (van Brabant *et al.*, 2001).

Alternatively, the checkpoint could be activated by events downstream of pre-RC formation, dependent upon initiation. This may involve proteins present at replication forks, aspects of the DNA structure of forks, or the presence of RNA primers in Okazaki fragments. Li and Deshaies (1993) proposed that these possibilities could be distinguished, by comparing the effects of inactivating a candidate protein, either before or after the establishment of DNA replication forks, and then by testing whether chromosome segregation occurs (Li and Deshaies, 1993). For example, the effects of inactivating pre-RCs in G1 phase or in hydroxyurea-arrested cells could be compared. Previous experiments with *cdc6* mutants do not address this issue, as they allow us to examine only the effects of inhibiting pre-RC formation in G1-phase.

Here we use temperature-sensitive degron mutants and genomic footprinting to show, for the first time, that MCM proteins are essential components of the pre-RC in budding yeast. Degron mutants of MCM2–7 genes provide a tool that allows us to inactivate pre-RCs after their formation, either in G1-phase or during S-phase. We use these mutants to test the role of pre-RCs in the S-phase checkpoint.

MATERIALS AND METHODS

Strains and Media

The strains used in this study are based upon W303–1a and are listed in Table 1. The construction of strains carrying either a degron allele of one of the *MCM2–7* genes or a fusion of Mcm4 to green fluorescent protein (GFP) has been described previously, together with details of media composition and protocols for cell-cycle arrests (Labib *et al.*, 1999; Labib *et al.*, 2000).

Plasmid Construction

To make pKL153, a *Pvu*I fragment from pAFS91 (Straight *et al.*, 1997), containing the *TUB1*- GFP gene fusion, was subcloned into *Pvu*I digested pRS305 (Sikorski and Hieter, 1989). To direct integration of this plasmid to the *LEU2* locus, the plasmid was linearized with the restriction enzyme *Afl*II before transformation.

Other Techniques

Genomic footprinting at the chromosomal origin ARS305 was performed as described previously (Noton and Diffley, 2000; Perkins and Diffley, 1998). Protocols for microscopy and flow cytometry were as described (Labib *et al.*, 1999). The rabbit polyclonal antibody JD145, kindly provided by Corrado Santocanale, was used at a dilution of 1/1000 to detect Rad53 protein.

RESULTS

Complex Formation Is Essential for MCM Nuclear Localization during G1-phase

In budding yeast MCM2–7 proteins accumulate in the nucleus at the end of mitosis, when pre-RCs form, and are excluded from the nucleus as they are displaced from chromatin during S-phase (Dalton and Whitbread, 1995; Hennessy *et al.*, 1990; Labib *et al.*, 1999; Nguyen *et al.*, 2000; Yan *et al.*, 1993). Addition of an exogenous nuclear localization signal to any of the MCM2–7 proteins prevents nuclear exclusion of all the others (Nguyen *et al.*, 2000), indicating that they associate with each other between S-phase and the end of mitosis. By examining the localization of a fusion of Mcm4 to GFP (Mcm4-GFP), we have taken a converse approach to address whether MCM2–7 proteins also associate with each other during late mitosis and G1 phase, as cells pass through mitosis and into G1 phase in the absence of another member of the MCM2–7 family (Figure 1A). To do this, we used strains in which the only copy of a particular *MCM* gene was fused to the temperature-sensitive degron cassette (Labib *et al.*, 2000). Proteolysis of degron-fusion proteins involves recognition by the Ubr1 protein, followed by polyubiquitylation of lysine residues in the degron cassette, and is stimulated at high temperatures (Dohmen *et al.*, 1994). To improve the efficiency of degradation, and to provide a further level of regulation, we used strains in which the only copy of the *UBR1* gene is expressed from the *GAL1,10* promoter (Labib *et al.*, 2000).

Degron mutants of *MCM2, 3, 6, or 7*, together with a control strain, were grown at 24°C in the absence of *UBR1* expression. Cells were arrested in G2/M with the microtubule-depolymerising drug nocodazole, and the cultures were split in two. Expression of *UBR1* was induced for 45

Table 1. List of strains

Strain	Genotype	Derivation	Integrated plasmid
W303-1a	*MAT***a** *ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100*		
YKL52	*MAT***a** *ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 mcm7-td ubr1Δ::GAL-UBR1*	W303-1a	pKL61, pKL30,
YKL83	*MAT***a** *ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 ubr1Δ::GAL-UBR1*	W303-1a	pKL54
YKL97	*MAT***a** *ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 mcm2-td ubr1Δ::GAL-UBR1 MCM4-GFP*	W303-1a	pKL75, pKL54, pKL34
YKL99	*MAT***a** *ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 mcm7-td ubr1Δ::GAL-UBR1 MCM4-GFP*	YKL52	pKL97
YKL100	*MAT***a** *ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 mcm4-td ubr1Δ::GAL-UBR1*	YKL83	pKL40
YKL109	*MAT***a** *ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 mcm6-td ubr1Δ::GAL-UBR1 MCM4-GFP*	YKL122	pKL80
YKL113	*MAT***a** *ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 mcm3-td ubr1Δ::GAL-UBR1 MCM4-GFP*	W303-1a	pKL30, pKL97, pKL35
YKL122	*MAT***a** *ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 ubr1Δ::GAL-UBR1 MCM4-GFP*	YKL83	pKL34
YKL222	*MAT***a** *ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 mcm7-td ubr1Δ::GAL-UBR1 TUB1-GFP*	YKL52	pKL153
YKL223	*MAT***a** *ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 mcm4-td ubr1Δ::GAL-UBR1 rad9Δ::HIS3 TUB1-GFP*	*rad9*Δ (de la Torre-Ruiz, 1998)	pKL30, pKL40, pKL153
YKL224	*MAT***a** *ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 mcm7-td ubr1Δ::GAL-UBR1 rad9Δ::HIS3 TUB1-GFP*	*rad9*Δ (de la Torre-Ruiz, 1998)	pKL30, pKL61, pKL153
YKL225	*MAT***a** *ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 ubr1Δ::GAL-UBR1 TUB1-GFP*	YKL83	pKL153
YKL220	*MAT***a** *ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 mcm4-td ubr1Δ::GAL-UBR1 TUB1-GFP*	YKL100	pKL153

min in one half, and each culture was then shifted to 37°C for an additional 45 min. Proteolysis of the degron-fusion proteins occurred specifically in the cultures expressing Ubr1, without affecting the stability of the other MCM2–7 proteins (Figure 1) (Labib *et al.*, 2000). Cells were then released into fresh medium containing α-factor mating pheromone instead of nocodazole, so that they completed mitosis and arrested in the subsequent G1-phase (which was confirmed microscopically).

Mcm4-GFP was predominantly cytoplasmic in G2/M arrested cells and then accumulated in the nucleus as the control strain completed mitosis and entered G1-phase (Figure 1B, control), either in the presence or absence of Ubr1 protein (YPGal and YPRaff, respectively). Mcm4-GFP also accumulated in the nucleus of the *mcm2, 3, 6, 7* degron mutants, when cells passed through mitosis at 37°C in the absence of Ubr1 protein (Figure 1B, YPRaff). In contrast, Mcm4-GFP did not accumulate in the nucleus of cells lacking either Mcm2, 3, 6, or 7 proteins (Figure 1B, YPGal). This indicates that MCM2–7 proteins interact with each other during the transition between late mitosis and early G1-phase.

We also examined the effects of degrading Mcm2, 3, 6, or 7 in G1-arrested cells, after nuclear accumulation of MCM proteins and pre-RC formation had already occurred (Figure 2A). Cells were arrested in G1 phase at 24°C, and once again the cultures were split in two, before induction of *UBR1* expression in one half. Localization of Mcm4-GFP was examined both before and after shifting the cultures to 37°C for 1 hour. In all strains, Mcm4-GFP was nuclear at 24°C in G1-arrested cells (Figure 2B, stages 1 and 3). On shifting cells to 37°C in the absence of Ubr1 protein, Mcm4-GFP remained nuclear in all strains (Figure 2B, stage 4). However, degradation of either Mcm2, 3, 6, or 7 by shifting cells to 37°C in the presence of Ubr1 protein caused loss of Mcm4-GFP from the nucleus (Figure 2B, stage 2) without affecting the level of Mcm4-GFP protein (Figure 2C).

Taken together with the results of Nguyen *et al.*, the preceding experiments indicate that MCM proteins interact with each other throughout the budding yeast cell cycle. Moreover, our data show that this interaction is essential for nuclear accumulation to occur during late mitosis and G1-phase, when MCM2–7 proteins are loaded onto chromatin at origins of DNA replication.

MCM2–7 Proteins Are Essential for the Formation and Maintenance of PreRCs

We used *mcm* degron mutants to address directly the role of MCM proteins in pre-RC formation at ARS305. From the time of initiation, during early S-phase, until late mitosis, the origin is in the postreplicative state, characterized by three ORC-induced DNase I hypersensitive sites (sites 1–3, Figure 3, Control, stage 1). During G1 phase, the larger prereplicative complex at this origin is characterized by suppression of the three ORC-induced hypersensitive sites in the genomic footprint, together with an extended region of protection from DNase I digestion adjacent to the ORC binding site and induction of a G1-specific hypersensitive site (Figure 3, Control, stage 2, (Noton and Diffley, 2000; Perkins and Diffley, 1998)).

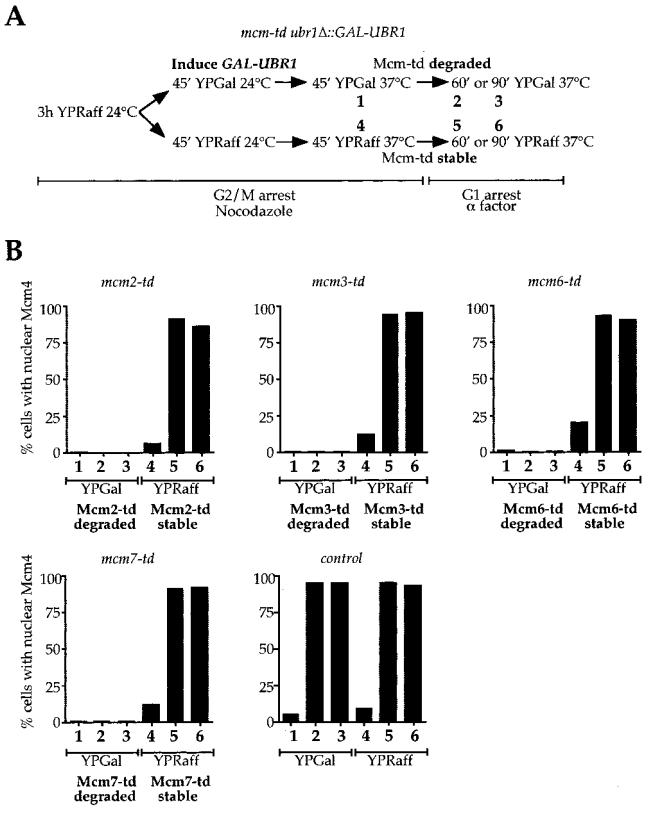

Figure 1. Nuclear accumulation of Mcm4-GFP at the end of mitosis requires other members of the MCM2–7 family. (A) Experimental protocol. (B) The proportion of cells with nuclear Mcm4 was determined at each stage of the experiment indicated in (A). At least a hundred cells were counted for each sample. *mcm2-td* = YKL97, *mcm3-td* = YKL113, *mcm6-td* = YKL109, *mcm7-td* = YKL99, *control* = YKL122.

To test whether MCM2–7 proteins are required for pre-RC formation at ARS305, we grew *mcm4-td*, *mcm7-td*, and a control strain in the absence of *UBR1* expression at 24°C, and we arrested cells in G2/M with the microtubule depolymerising drug nocodazole. Expression of *UBR1* was then induced for 30 min, and cells were shifted to 37°C for 45 min to degrade the degron-fusion proteins Mcm4-td and Mcm7-td. At this stage, ARS305 was in the postreplicative state in all three strains (Figure 3, stage 1). Cells were then released into fresh medium at 37°C for 2 hours, in the presence of α-factor mating pheromone, so that they completed mitosis before arresting in the subsequent G1 phase. In the control strain, pre-RC formation could be observed at ARS305 (Figure 3, Control, stage 2). In the absence of Mcm4 or Mcm7, however, pre-RC formation did not occur, and instead the origin remained in the postreplicative state (Figure 3, *mcm4-td* and *mcm7-td*, stage 2). This shows that MCM function is essential for pre-RC formation to occur as cells pass through mitosis and into G1 phase.

To provide stronger evidence that MCM2–7 proteins are components of the pre-RC at ARS305, rather than simply being required for its formation, we tested the effects of degrading Mcm4 or Mcm7 in G1 cells, after pre-RC forma-

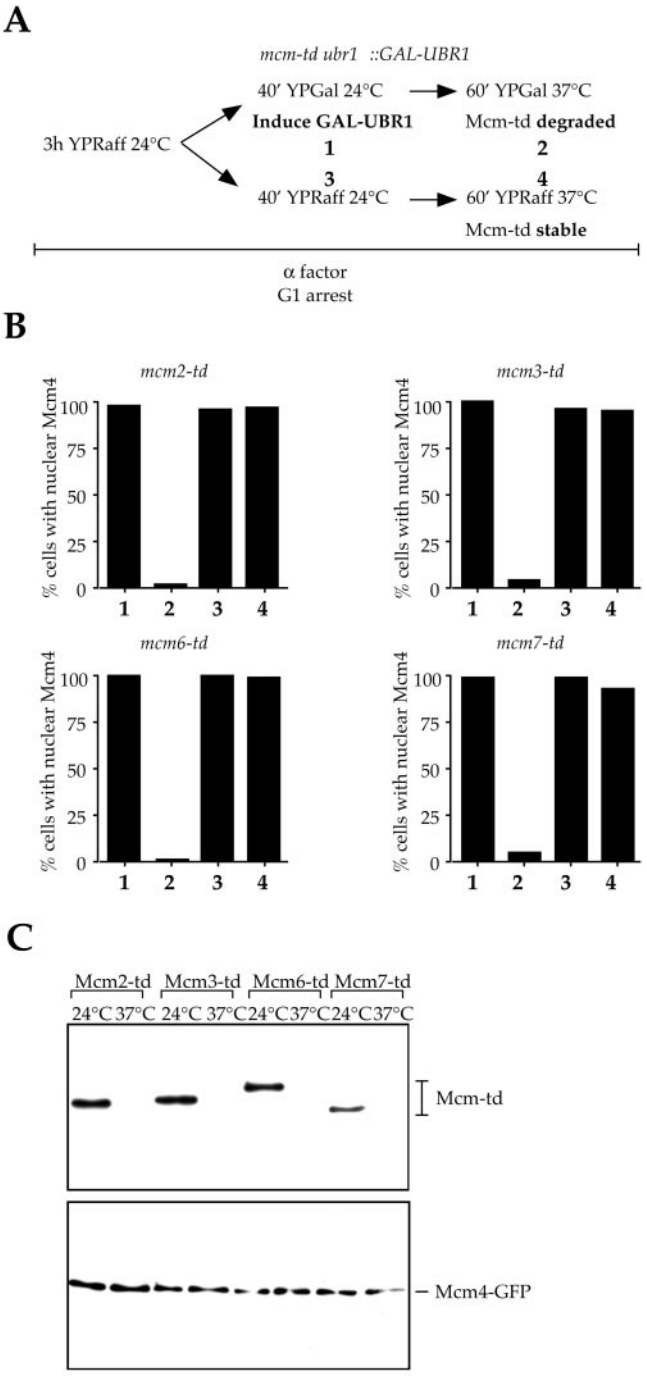

Figure 2. Maintenance of nuclear Mcm4 after pre-RC formation requires the other members of the MCM2–7 family. (A) Experimental protocol. (B) The proportion of cells with nuclear Mcm4 was determined at each stage of the experiment indicated in (A). At least a hundred cells were counted for each sample. (**C**) Protein extracts were prepared from stages 1 and 2 and were used for immunoblot analysis, with the use of 12CA5 antibody to detect the HA-tagged Mcm-td proteins and the anti-GFP antibody 3E1 to detect Mcm4-GFP. The strains used were those described in the legend to Figure 1.

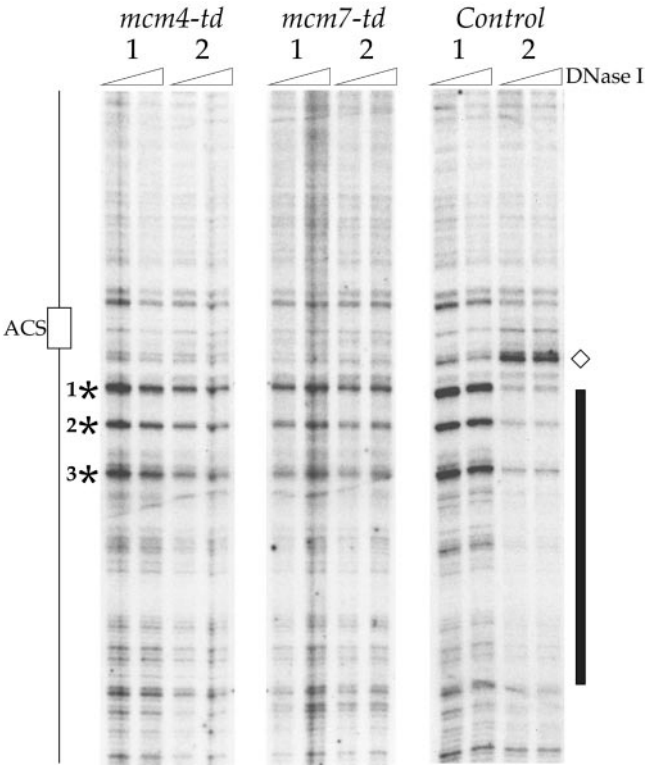

Figure 3. MCM2–7 proteins are essential for pre-RC formation. See text for details. Genomic footprints are shown of the chromosomal origin ARS305. On the left, the ORC-induced hypersensitive sites 1–3 are marked with asterisks, and the position of the ARS consensus sequence (ACS) is marked with a box. On the right, the position of the G1-specific DNase I hypersensitive site is marked with a diamond, and the thick black line denotes the region of protection from DNase I digestion in the prereplicative footprint. For each of the three strains shown (*mcm4-td* = YKL100, *mcm7-td* = YKL52, *control* = YKL83), stage 1 corresponds to G2/M-arrested cells at 37°C, and stage 2 denotes cells that have subsequently passed through mitosis at 37°C, before arresting in G1 phase.

tion had already occurred. The same three strains as above were grown at 24°C in the absence of *UBR1* expression, and cells were arrested in G1 phase with α-factor. At this stage of the experiment, pre-RC formation at ARS305 could be observed in all three strains (Figure 4, stage 1). Expression of *UBR1* was then induced for 30 min, and the cultures were split in two. One half was shifted to 37°C for 1 hour to induce degradation of Mcm4-td and Mcm7-td proteins (Figure 4, stage 2). As a control, the other half of each culture was left at 24°C for the same period of time (Figure 4, stage 3). G1-arrest was maintained throughout the experiment. Degradation of either Mcm4 or Mcm7 caused the origin to revert from the prereplicative to the postreplicative state (Figure 4, compare stages 2 and 3 for each strain). All three features of the pre-RC at ARS305 were lost upon degradation of an MCM protein: the G1-specific hypersensitive site disappeared, the three ORC-induced hypersensitive sites reappeared, and the region of protection from DNase I digestion, adjacent to the ORC-binding site, was also lost (Figure 4).

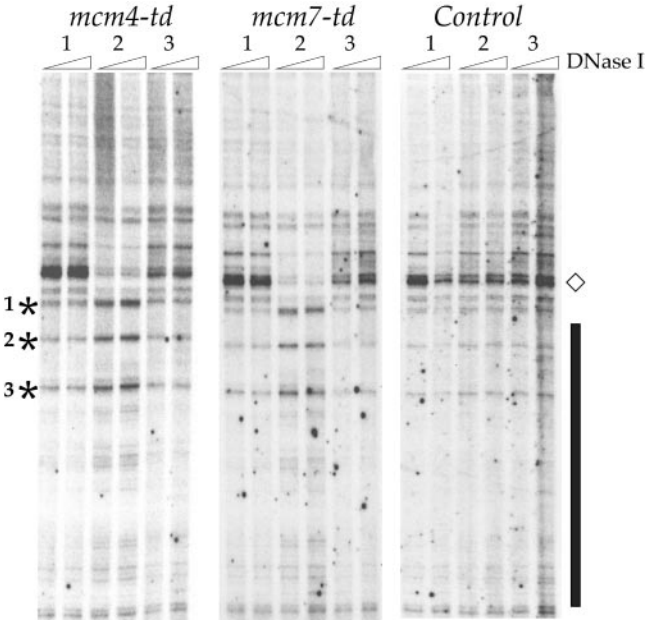

Figure 4. MCM2–7 proteins are required for maintenance of the pre-RC during G1-phase. Genomic footprints of ARS305 are labeled as in Figure 3. Stage 1 corresponds to G1-arrested cells at 24°C, stage 2 denotes cells shifted subsequently to 37°C for 1 h, and stage 3 represents cells maintained at 24°C during this same period, as a control. The strains are as described above for Figure 3.

These experiments show that MCMs are essential for formation and maintenance of the pre-RC at ARS305, suggesting that the pre-RC footprint may represent, in fact, the MCM-binding site at budding yeast origins of replication, adjacent to ORC.

PreRCs and Checkpoint Inhibition of Mitosis during S-phase

The preceding experiments show that *mcm* degron mutants provide a tool with which we can degrade preexisting pre-RCs at origins of DNA replication. This allows us to test the role of pre-RCs in the S-phase checkpoint, after the approach suggested by Li and Deschaies (1993). First, we examined the effects of degrading an MCM protein before the establishment of DNA replication forks. Cultures of *mcm4-td*, *mcm7-td*, and a control strain were grown at 24°C in the absence of *UBR1* expression, and G1-arrested cells, lacking pre-RCs (*mcm4-td* and *mcm7-td*) or containing pre-RCs (control), were generated at 37°C, exactly as described above for the experiment in Figure 3. Cells were then released from G1 arrest at 37°C into fresh medium, and samples were taken every 20 min to follow DNA content and progression through mitosis. One half of the control culture was released from G1 arrest into medium containing 0.2 M hydroxyurea (HU), as a positive control for activation of the checkpoint that inhibits mitosis in response to incomplete S-phase.

The control strain completed S-phase and mitosis rapidly in the absence of HU, before entering the next cell cycle (Figure 5, control). In the presence of HU, S-phase was blocked, and activation of the checkpoint prevented an-

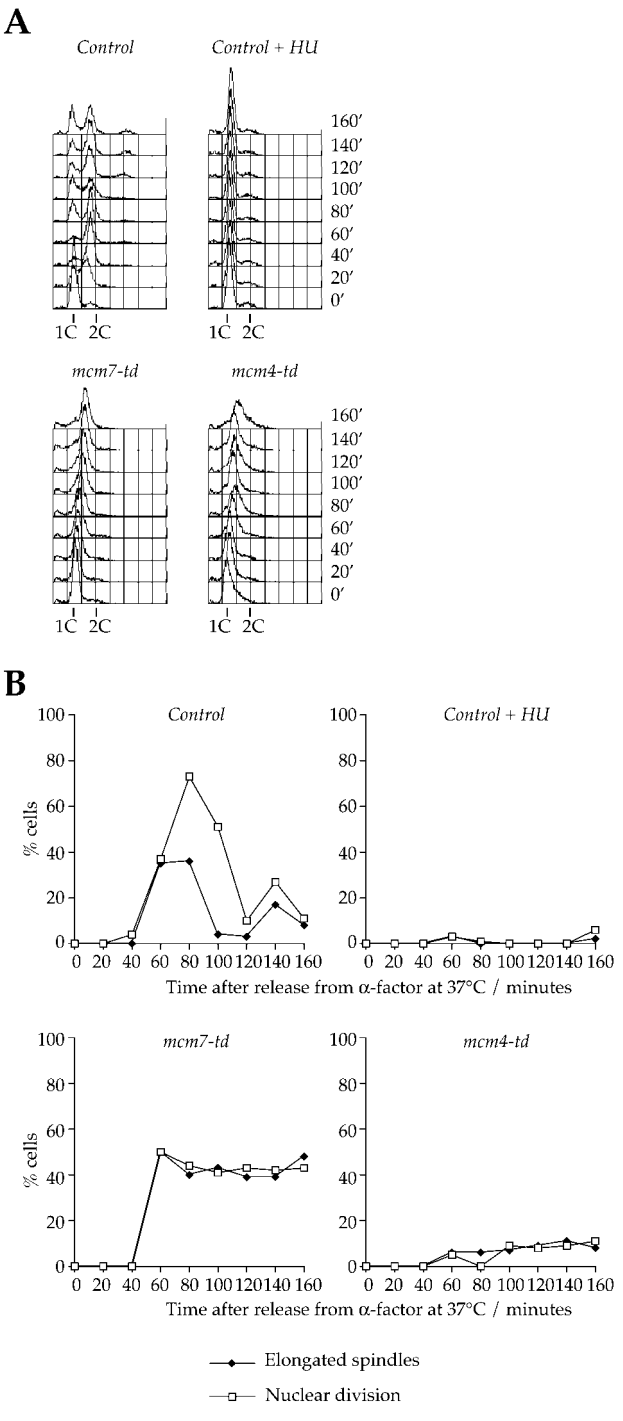

Figure 5. Degradation of pre-RCs in G1-phase promotes nuclear division in the absence of chromosome replication. See text for details. (A) Cells were released from G1 arrest at 37°C for the indicated times, and DNA content was measured by flow cytometry. (B) Progression through mitosis was assayed at each stage of the experiment by determining the proportion of cells with divided nuclei (open squares), and the proportion of cells with elongated spindles (filled diamonds). Spindle elongation was assayed with the use of a fusion of GFP to the α-tubulin protein Tub1. *Control* = YKL225, *mcm7-td* = YKL222, *mcm4-td* = YKL226.

aphase (Figure 5, control +HU). Degradation of Mcm7-td protein prevented S-phase, but it did not block the subsequent anaphase, showing that checkpoint activation was defective in the absence of pre-RCs (Figure 5, *mcm7-td*). A smaller proportion of cells with divided nuclei was seen after degradation of Mcm4-td protein (Figure 5B), probably reflecting the slightly leakier nature of the *mcm4-td* allele (compare the flow cytometry profiles of *mcm4-td* and *mcm7-td* in Figure 5A). These data are consistent with a previous report of an allele of *MCM3*, *mcm3–10*, for which a proportion of cells undergo nuclear division without completing chromosome replication (Toyn *et al.*, 1995).

These experiments show that anaphase can occur in the absence of S-phase, due to a failure in checkpoint activation, if prereplicative MCM2–7 complexes are degraded before establishment of DNA replication forks. We then examined the effects of degrading an MCM protein after the establishment of forks from early origins of DNA replication. The same three strains as before were arrested in G1 phase in the absence of *UBR1* expression, before releasing into fresh medium containing 0.2 M HU. We have shown previously that early origins of replication are activated efficiently in *mcm* degron mutants under such conditions (Labib *et al.*, 2000). Expression of *UBR1* was then induced for 45 min and the cultures shifted to 37°C in the continued presence of HU to induce degradation of Mcm4-td and Mcm7-td proteins. Cells were then released into fresh medium at 37°C that lacked HU, and progression through S-phase and mitosis was monitored every 20 min.

The control strain completed S-phase rapidly upon release from HU and then proceeded through a synchronous round of nuclear division (Figure 6, control). Degradation of Mcm4-td or Mcm7-td prevented continued DNA synthesis (Figure 6A), as we have reported previously (Labib *et al.*, 2000), due to a defect in DNA replication fork progression during the elongation phase of chromosome replication. However, nuclear division did not occur in cells lacking Mcm4 or Mcm7 proteins, either before or after release from HU, indicating that the checkpoint remained intact (Figure 6B, C). This was confirmed by examination of the phosphorylation status of the Rad53 protein kinase, an important transducer of the checkpoint signal, which remained in its active, hyperphosphorylated form upon release from HU in the absence of Mcm4 or Mcm7 (Figure 6D). This indicates that loss of MCM proteins, after the establishment of replication forks, actually promotes checkpoint inhibition of anaphase, by inhibiting the progression of replication forks during the elongation phase of chromosome replication. Just as HU blocks the progression of replication forks, and so anaphase, by a Rad9-independent mechanism (Weinert *et al.*, 1994), so too the inhibition of nuclear division, seen when elongation is blocked by MCM depletion, is also independent of Rad9 function (Figure 7).

Taken together, the preceding experiments indicate that MCM2–7 proteins do not play a direct role in checkpoint control during S-phase, either in pre-RCs or during elongation. Instead, pre-RCs play an indirect role, insofar as they are essential for initiation and the establishment of replication forks. Inactivation of MCM proteins during S-phase prevents entry into anaphase by inhibiting the progression of DNA replication forks.

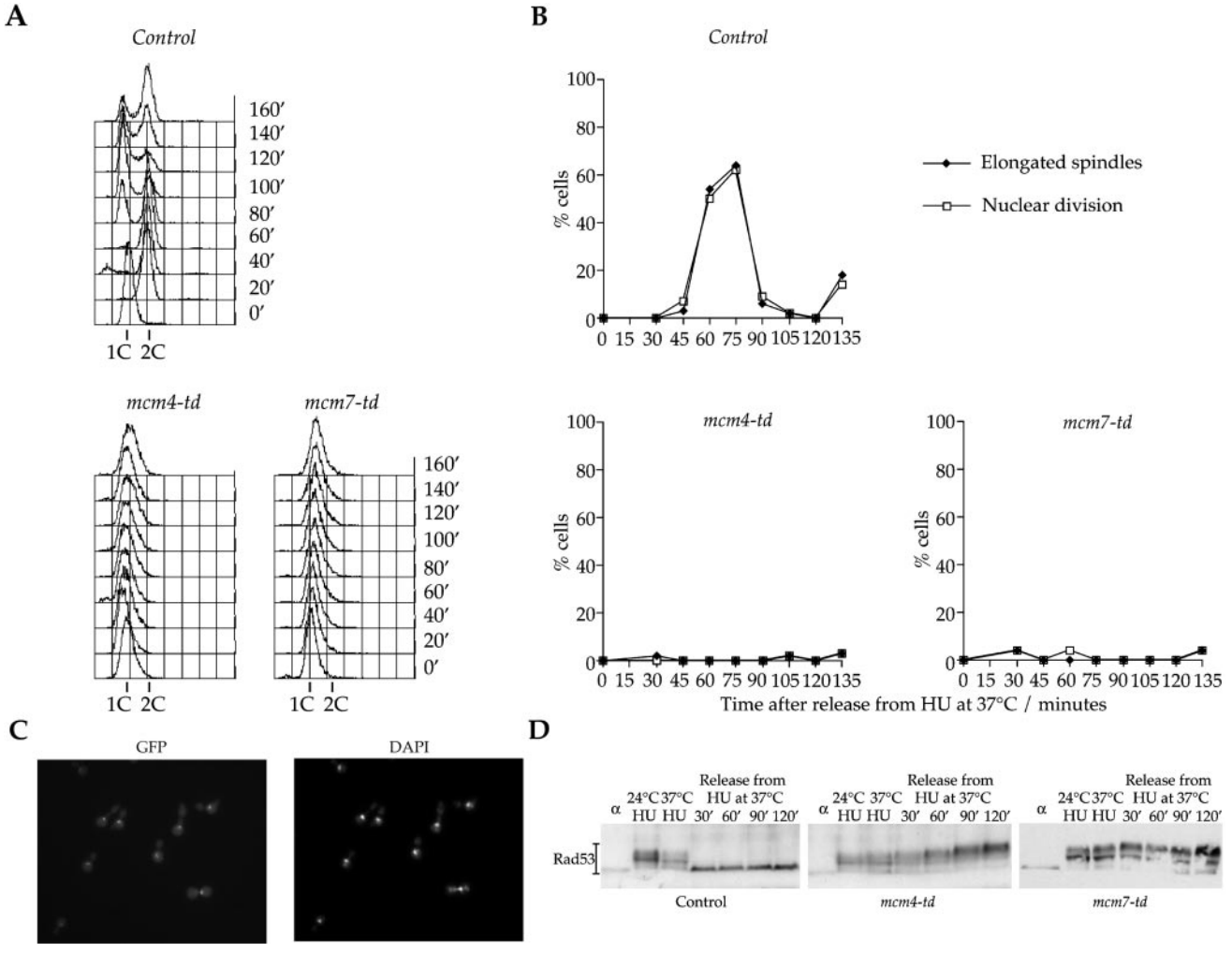

Figure 6. Degradation of MCM2–7 proteins during S-phase inhibits progression through anaphase. See text for details. The strains used were those described above for Figure 5. (A) Cells were released from HU-arrest at 37°C for the indicated times, and DNA content was measured by flow cytometry. (B) Progression through mitosis was assayed as described in Figure 5. (C) Examples of *mcm4-td* cells are shown 135 min after release from HU at 37°C, with short spindles (GFP) and undivided nuclei (DAPI). (D) The phosphorylation status of the Rad53 protein kinase was determined throughout the experiment by immunoblotting. The hypophosphorylated form migrates as a single band with high mobility, whereas hyperphosphorylated forms are retarded and migrate with lower mobility.

DISCUSSION

The pre-RC is assembled over and around the ORC-binding site at origins of DNA replication in budding yeast. PreRC formation and maintenance require both Cdc6 (Cocker *et al.*, 1996; Santocanale and Diffley, 1996) and the MCM2–7 complex (this study). As Cdc6 is required for the association of MCM2–7 proteins with chromatin, and as pre-RC formation cannot occur in cells containing Cdc6 but not MCM proteins, our data suggest that the prereplicative footprint represents the binding site of MCM2–7 proteins alongside ORC.

MCM2–7 proteins have been estimated to be between 20 and 100 times more abundant than ORC, Cdc6, or the number of origins of DNA replication (Lei *et al.*, 1996; Donovan *et al.*, 1997), and a significant proportion is associated with chromatin during G1-phase. The reason for the high relative-abundance of MCM proteins remains unclear. It is interesting to note that a mutant allele of *CDC6*, *CDC6-d1*, supports the formation of a partial prereplicative footprint at ARS305 but does not support the loading of wild-type levels of MCM2–7 proteins onto chromatin (Perkins and Diffley, 1998). The partial pre-RC induced by Cdc6-d1 protein produces suppression of ORC-induced hypersensitive sites 1 and 2 (see Figures 1 and 2) but does not cause suppression of the third ORC-induced hypersensitive site or protection of the adjacent region from DNase I digestion (Perkins and Diffley, 1998). Because all aspects of the pre-RC footprint at ARS305 are MCM-dependent, it is possible that the partial pre-RC and the full pre-RC differ quantitatively in the number of MCM2–7 complexes bound to the origin. For example, the partial pre-RC may contain a single MCM2–7 complex, and generation of the full pre-RC at ARS305 may require the binding of multiple MCM2–7 complexes. It is worth noting

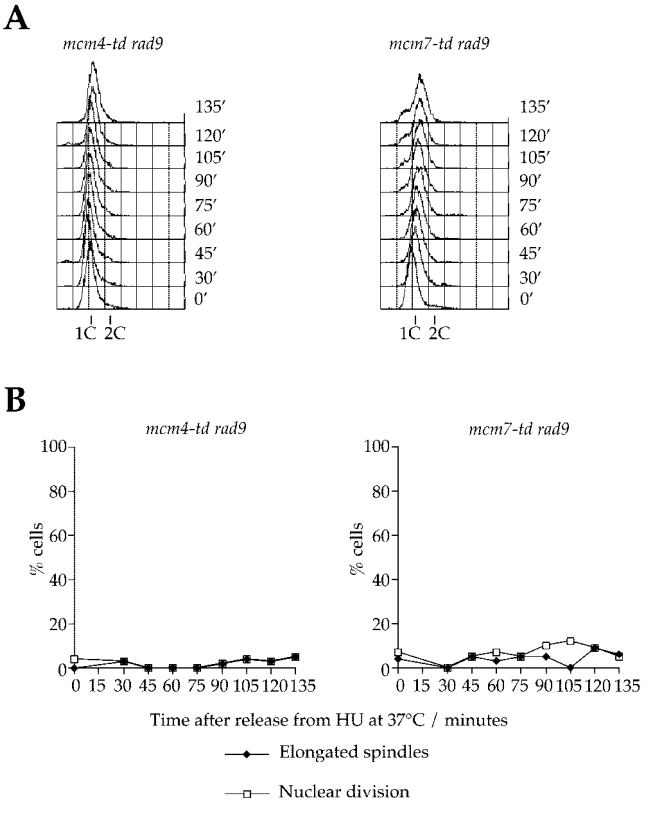

Figure 7. Checkpoint arrest of anaphase, upon degradation of MCM2–7 proteins during S-phase, is Rad9-independent. See text for details. (A) Cells were released from HU-arrest at 37°C for the indicated times, and DNA content was measured by flow cytometry. (B) Progression through mitosis was assayed as described above for Figure 5. *mcm4-td rad9*Δ = YKL223, *mcm7-td rad9Diffley@icrf.icnet.uk*Δ = YKL224.

that, at a very late and normally silent origin such as ARS301, the pre-RC footprint involves suppression of two ORC-induced hypersensitive sites without significant protection of adjacent regions (Santocanale and Diffley, 1996). Perhaps one important difference between early origins, such as ARS305 and very late origins, such as ARS301, is that the former are bound by more MCM2–7 complexes than the latter. It is likely, however, that other factors also contribute to the determination of origin-timing, as proximity to a telomere delays activation of a normally-early origin without changing the prereplicative footprint (Santocanale and Diffley, 1998).

Our data, together with those of Nguyen *et al.* (Nguyen *et al.*, 2000), indicate that budding yeast MCM2–7 proteins interact with each other in vivo, during late mitosis, G1-phase, and after S-phase. Furthermore, we show that this interaction is essential for nuclear accumulation of MCM proteins during the period of the cell cycle when they are assembled into pre-RCs, as previously reported for fission yeast (Pasion and Forsburg, 1999). We have also shown previously that Mcm2, 3, 4, 6, and 7 proteins are required during S-phase for the elongation phase of chromosome replication (Labib *et al.*, 2000). Taken together, these experiments reinforce the notion that the active form of MCM2–7 proteins, throughout the cell cycle, is likely to be a heterohexamer.

Our experiments show that, once replication forks have been established from early origins, MCM2–7 proteins, and therefore pre-RCs, are not required to inhibit anaphase in response to incomplete chromosome replication. Inhibition of the progression of DNA replication forks, either by HU treatment or by MCM-depletion after initiation, blocks entry into anaphase. In both cases, hyperphosphorylation of Rad53 is maintained, and anaphase is inhibited in a Rad9-independent manner. It appears that stalling of replication forks, rather than presence of MCM2–7 proteins, or pre-RCs, is important for the checkpoint inhibiting mitosis in response to incomplete replication.

Several studies, however, have reported that other replication proteins, such as RF-C (Sugimoto *et al.*, 1997; Noskov *et al.*, 1998; Reynolds *et al.*, 1999; Shimada *et al.*, 1999) or the budding yeast Dpb11 protein and its fission yeast homologue Cut5 (Saka and Yanagida, 1993; Saka *et al.*, 1994; Araki *et al.*, 1995; McFarlane *et al.*, 1997; Wang and Elledge, 1999) are required to maintain checkpoint inhibition of mitosis in HU-arrested cells, suggesting that these proteins may indeed play a role in checkpoint control. But it remains to be shown that activation of early origins and replication fork establishment have occurred normally in these experiments. Failure to establish replication forks, due to the combination of HU and the defective nature of a particular conditional allele chosen for such an experiment could cause entry into anaphase without the test protein having a direct role in checkpoint control.

It is worth noting that mitosis occurs with very similar timing, both in wild-type cells, and also in cells that segregate their chromosomes in the absence of DNA replication (this study, Piatti *et al.*, 1995; Tercero *et al.*, 2000). It is likely, therefore, that the timing of anaphase in budding yeast is determined by a second mechanism, distinct from the checkpoint that blocks mitosis in response to incomplete chromosome replication.

We favor the view that some aspect of the structure of replication forks may be sensed by checkpoint proteins, leading to the generation of the checkpoint signal. It has been argued that this may involve detection of the RNA primer present at the beginning of Okazaki fragments (Michael *et al.*, 2000), but this view is not consistent with experiments implicating RF-C in checkpoint control, as RF-C acts after primer formation, and it is not clear how mutation of RF-C would affect the presence or absence of RNA primers in Okazaki fragments.

Our experiments suggest one approach to addressing these issues in the future, by making degron mutants of other replication proteins such as primase and RF-C and by comparing the effects of degrading these proteins in HU-arrested cells after confirming that establishment of replication forks from early origins has indeed taken place.

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