consequences of the presence or absence of 4.5*S* RNA for Ffh-FtsY complex formation, an extension of these results suggests that the catalytic activity of 4.5*S* RNA could serve as a built-in regulator for the SRP targeting cycle. As the M domain also contains the signal sequence binding site of Ffh (*22*), signal sequence binding could induce minor changes in RNA conformation (on the order of 3 kcal mol^{-1}, i.e., breaking or forming only a few hydrogen bonds), which in turn could lead to changes in kinetics controlling Ffh-FtsY complex formation. Indeed, the structure of SRP RNA bound to the Ffh M domain shows that SRP RNA is closely juxtaposed to the signal sequence binding pocket, which underscores the feasibility of direct crosstalk between bound signal sequences and the RNA (*24, 25*). It is therefore an appealing hypothesis that protein targeting by a SRP and its receptor could be controlled through conformational changes in the RNA in addition to nucleotide occupancy of the GTPase domains. According to this view, SRP RNA would play a much more active role than previously presumed in regulating the interaction between the two GTPases.

The catalytic properties shown here for 4.5*S* RNA may not be unique to this system but may be a paradigm for other ribonucleoprotein assemblies, such as spliceosomes and ribosomes, that go through a dynamic cycle (*26, 27*). Like SRP-mediated protein targeting, these biological processes depend on the coordinated formation and dissociation of complex ribonucleoprotein assemblies. Perhaps the RNA components in these complexes affect conformational changes in a manner analogous to 4.5*S* RNA and thus provide new means for regulation by modulating the kinetic parameters that govern complex formation and disassembly.

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28. Fluorescence spectra were obtained and reactions carried out in 50 mM Hepes (pH 7.5), 150 mM KOAc, 1.5 mM $Mg(OAc)_2$, 0.01% (v/v) Nikkol detergent, and 2 mM dithiothreitol at 25°C (Ac, acetyl). *Escherichia coli* Ffh, 4.5S RNA, and FtsY were prepared as described (*5*). Ffh/4.5S RNP was assembled using a twofold molar excess of RNA over Ffh and used without further purification (*6*).

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14 February 2000; accepted 27 March 2000

Uninterrupted MCM2-7 Function Required for DNA Replication Fork Progression

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Little is known about the DNA helicases required for the elongation phase of eukaryotic chromosome replication. Minichromosome maintenance (MCM) protein complexes have DNA helicase activity but have only been functionally implicated in initiating DNA replication. Using an improved method for constructing conditional degron mutants, we show that depletion of MCMs after initiation irreversibly blocks the progression of replication forks in *Saccharomyces cerevisiae*. Like the *Escherichia coli* dnaB and SV40 T antigen helicases, therefore, the MCM complex is loaded at origins before initiation and is essential for elongation. Restricting MCM loading to the G_1 phase ensures that initiation and elongation occur just once per cell cycle.

The six MCM proteins of the MCM2-7 family are assembled at the end of mitosis into prereplicative complexes (pre-RCs) at origins of DNA replication and are essential for origin activation to occur as cells enter the S phase [reviewed in (*1*, *2*)]. A subcomplex of three human MCM proteins, MCM4, -6, and -7, has limited DNA helicase activity in vitro (*3*, *4*), and an archaeal protein related to the MCM2-7 family also functions as a DNA helicase (*5*, *6*), suggesting that MCM proteins may be required for strand unwinding during the initiation step of chromosome replication. After initiation, MCM proteins become redistributed away from origins (*7*), but previous genetic analysis indicated that MCM proteins are not required for chromosome replication to be completed after origin activation (*8*, *9*), which supports arguments against an essential role for MCM proteins in the progression of replication forks.

To clarify the in vivo role of MCM proteins during chromosome replication, we generated a novel set of conditional *mcm* mutants. Initially, we used the "heat-inducible degron" method of Dohmen, Wu, and Varshavsky (*10*), whereby the target gene is altered such that an NH_2-terminal extension (the degron) is added to the protein of interest, specifically reducing the half-life of the fused product at 37°C. However, we found that degradation of such fusion proteins at 37°C is inefficient and inactivation of protein function is incomplete. For example, when a *mcm7-td* mutant is shifted to 37°C, the level of Mcm7-td protein declines slowly and can still be detected after 2 hours at 37°C [Fig. 1A, (i)], and cells arrest with a DNA content between 1C (the amount of DNA in a haploid cell in the G_1 phase) and 2C (the amount of DNA in a haploid cell in the G_2 phase) [Fig. 1B, (i)]. To improve the method, we took advantage of the fact that expression of *UBR1*, the gene encoding the degron recognition factor, can be regulated by the galactose-inducible *GAL1-10* promoter without inhibiting cell growth. In the absence of *UBR1* expression, the level of Mcm7-td protein is unchanged after shifting to 37°C [Fig. 1A, (ii)] and colony formation is not inhibited (*11*). However, when cells are grown in the presence of galactose to induce high levels of *GAL-UBR1* expression, Mcm7-td protein is present at 24°C but is very rapidly de-

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graded upon shifting to 37°C and disappears by 30 min [Fig. 1A, (iii)]. This causes cells to accumulate with a DNA content close to 1C [Fig. 1B, (ii)].

This approach has produced efficient temperature-sensitive (t.s.) mutants for more than 80% of genes for which we were able to make degron fusions. These include degron mutants, such as *mcm3-td*, that are able to grow at 37°C in the presence of wild-type levels of Ubr1. We used our improved method to make t.s. mutants of all six budding yeast *MCM* genes (the *mcm5-td* strain grows poorly at all temperatures and is not analyzed further in this study). All the fusion proteins are efficiently degraded at 37°C in the presence of high levels of Ubr1 (Fig. 1, A and C; data for Mcm4-td is given below), without causing degradation of other members of the MCM complex (Fig. 1C), and the mutant strains arrest with a DNA content close to 1C at the restrictive temperature (*12*). If cells are synchronized in G_1 phase with α factor mating pheromone at 24°C before degradation of the mutant Mcm-td protein, DNA replication does not occur upon release from G_1 arrest, in contrast to a control strain lacking the degron fusion (Fig. 1D). This indicates that all MCM proteins are equally important for entry into the S phase.

To establish whether MCM proteins are specifically required for initiation or may also play a role in fork progression, we determined whether they are necessary for chromosome replication to be completed after the activation of early origins. Because late origins are not essential for the completion of chromosome replication in budding yeast (*13*, *14*), a requirement for MCM proteins after the activation of early origins would suggest a role in elongation. We synchronized *mcm3-td* and *MCM3* strains in G_1 at 24°C (Fig. 2A, I) and released cells into medium containing hydroxyurea, so that replication forks stalled after the activation of early origins of DNA replication (Fig. 2, A and B, II). The cultures were then split in two, and *UBR1* expression was induced in one-half (Fig. 2A, VI) before shifting to 37°C to induce degradation of Mcm3-td (Fig. 2A, VII). The other half of each culture was shifted to 37°C in the absence of *UBR1* expression (Fig. 2A, IV) to prevent Mcm3 degradation in the *mcm3-td* strain. Cells were then released from the hydroxyurea arrest at 37°C. The *MCM3* strain completed the S phase with similar kinetics in the absence [Fig. 2C, (i)] and in the presence [Fig. 2C, (ii)] of *UBR1* expression. In contrast, although the *mcm3-td* strain completed the S phase as rapidly as the *MCM3* strain in the absence of *UBR1* expression [Fig. 2C, (i)], degradation of Mcm3-td prevented the resumption of chromosome replication upon release from hydroxyurea arrest at 37°C [Fig. 2C, (ii)]. Figure 2D shows that, in similar experiments, all five *mcm-td* mutants were unable to complete the S phase when released from hydroxyurea arrest at 37°C after the proteolysis of an individual MCM protein. Taken together, these experiments indicate that all MCM proteins are equally important for chromosome replication to continue after the activation of early origins of DNA replication.

To test whether MCM proteins are also required for DNA synthesis to continue after replication has resumed from stalled forks, we released cultures of *mcm2-td*, *mcm4-td*, and a control strain from hydroxyurea arrest at the permissive temperature of 24°C. Incubation was continued until cells were halfway through the S phase (Fig. 2E), and the cultures were then shifted to 37°C. Whereas the S phase was rapidly completed in the control strain and cell number increased twofold, DNA synthesis ceased in the *mcm2-td* and *mcm4-td* strains upon shifting cells to 37°C (Fig. 2E) and cell number did not increase (*15*). These results indicate that MCM proteins are required for DNA synthesis throughout the S phase and are not required solely for replication fork progression to resume upon release from hydroxyurea arrest.

To demonstrate directly a role for MCM proteins in the progression of replication forks, we used an assay based on density substitution, which allows the detection of elongation defects at specific chromosomal loci (*16*). Chromosomal DNA of *MCM4* and *mcm4-td* strains was fully substituted with the dense isotopes ^{13}C and ^{15}N, and cells were then transferred to medium that contained the light isotopes ^{12}C and ^{14}N and were synchronized in the G_1 phase. Upon release into fresh light-isotope medium containing hydroxyurea, restriction fragments with the early origins ARS306 and ARS607 moved from the heavy-heavy (HH) peak to the heavy-light (HL) peak, whereas

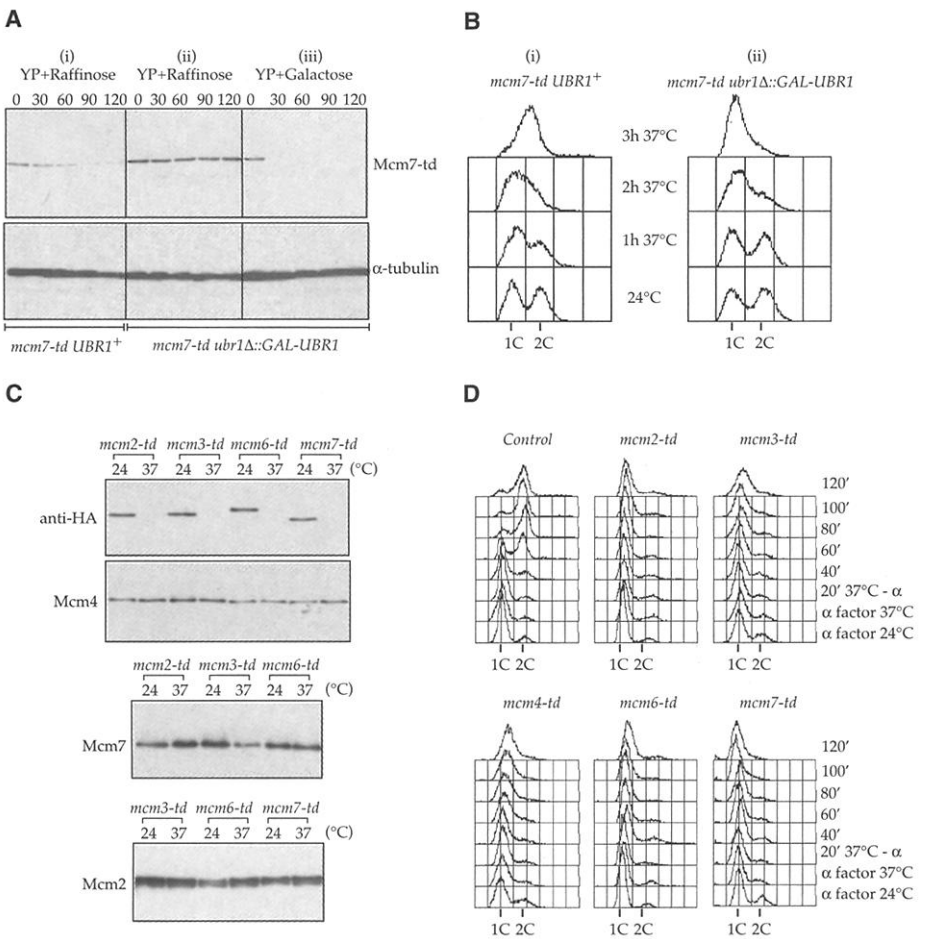

Fig. 1. An improved method for the generation of t.s. mutants that efficiently inactivate protein function (*11*). (**A**) Asynchronous cultures of *mcm7-td UBR1*$^+$ and *mcm7-td ubr1Δ::GAL-UBR1* strains were grown in yeast extract, peptone, and raffinose (YP+Raffinose) medium (*GAL-UBR1* OFF) or YP+Galactose (*GAL-UBR1* ON) at 24°C. Cells were then shifted to 37°C and samples were taken at the indicated times (in minutes) to prepare protein extracts. (**B**) The DNA content of *mcm7-td UBR1*$^+$ and *mcm7-td ubr1Δ::GAL-UBR1* strains was determined by flow cytometry after shifting to 37°C in YP+Galactose. (**C**) Immunoblot analysis of the indicated strains, before and after shifting to 37°C in YP+Galactose for 1 hour [for details of antibodies, see (*11*)]. (**D**) The indicated strains were grown in YP+Galactose at 24°C, before arresting in G_1 with α factor. Cells were then shifted to 37°C for 1 hour before releasing into fresh medium at 37°C lacking α factor. DNA content was monitored by flow cytometry.

DNA at the end of each replicon remained in the HH peak (Fig. 3). This shows that initiation occurred at both origins and that forks subsequently stalled before reaching the end of the replicon. Cells were then shifted to 37°C to induce degradation of Mcm4-td before release at 37°C into fresh medium lacking hydroxyurea. As shown in Fig. 3, A and B, part (i), the end of both replicons in the *MCM4* strain was replicated within 45 min of release from hydroxyurea arrest. In contrast, the end of both replicons in the *mcm4-td* strain remained in the HH peak, showing that replication fork progression was defective. The data are quantitated in Fig. 3, A and B, part (ii), showing that the majority of the forks from the origins do not reach the end of the replicon in the absence of Mcm4 function, even 90 min after release from hydroxyurea arrest. Therefore, MCM proteins are indeed required for the elongation of nascent DNA molecules to proceed at replication forks.

As the S phase proceeds, MCM proteins are displaced from chromatin (*7*, *17*–*20*) and Cdc28 kinase activity then prevents their reassociation with origins until the end of the subsequent mitosis (*21*). Similarly, displacement of MCM proteins from replication forks may block elongation irreversibly, such that the maintenance of the association of MCM proteins with forks must not be interrupted until termination, when opposing forks collide. We investigated this possibility by comparing the effects of transiently degrading an MCM protein at different stages of the cell cycle. We arrested *MCM4* and *mcm4-td* strains at 24°C in the G_1 phase, the early S phase, or the G_2-M phase before shifting cells to 37°C for 1 hour to induce degradation of Mcm4 protein in the *mcm4-td* strain (Fig. 4A). The cultures were then returned to 24°C for 2 hours more to allow resynthesis of Mcm4-td (Fig. 4A). At each stage of the experiment, viability was measured by plating cells at 24°C. As shown in Fig. 4B, viability of the *MCM4* control strain remained high throughout the experiment. Viability of the *mcm4-td* strain also remained high when Mcm4-td was degraded in G_2-M arrested cells. Degradation of Mcm4-td at 37°C in G_1-arrested cells caused a dramatic loss of viability upon plating, but viability was restored to high levels if Mcm4-td protein was remade at 24°C while the G_1 arrest was maintained. Cells were subsequently able to progress through the S phase with similar kinetics to the *MCM4* control strain upon release from G_1 arrest [Fig. 4C, (i) and (ii)]. Therefore, the ability to reform pre-RCs after degrading an MCM protein is retained during the G_1 phase.

In S phase–arrested cells, degradation of Mcm4-td after the activation of early origins caused a profound loss of viability. Viability was not restored by resynthesis of Mcm4-td protein at 24°C (Fig. 4B), and DNA synthesis did not resume upon release from the S phase arrest [compare Fig. 4C, (iii) and (iv)]. Thus,

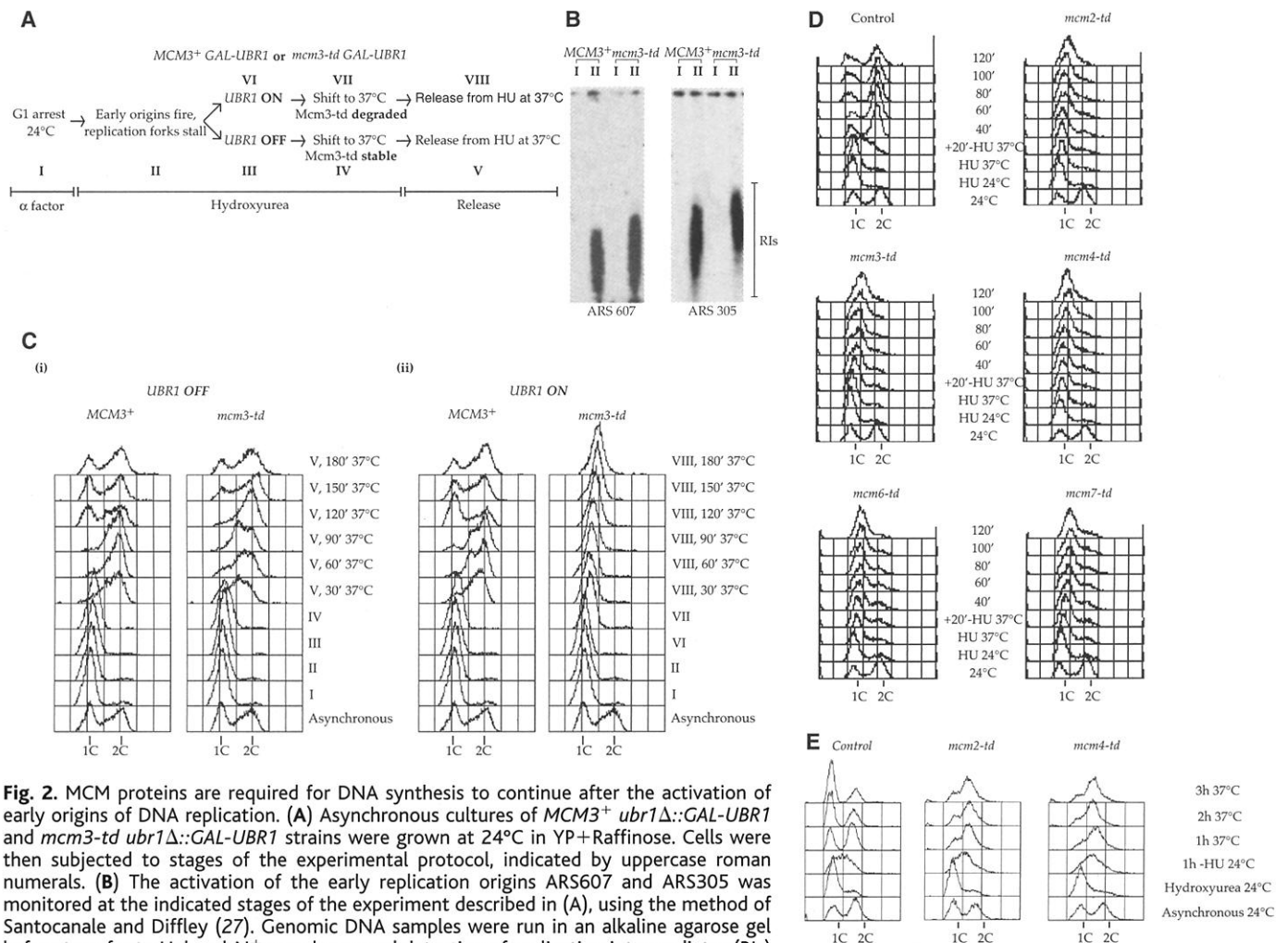

Fig. 2. MCM proteins are required for DNA synthesis to continue after the activation of early origins of DNA replication. (**A**) Asynchronous cultures of *MCM3*$^+$ *ubr1Δ::GAL-UBR1* and *mcm3-td ubr1Δ::GAL-UBR1* strains were grown at 24°C in YP+Raffinose. Cells were then subjected to stages of the experimental protocol, indicated by uppercase roman numerals. (**B**) The activation of the early replication origins ARS607 and ARS305 was monitored at the indicated stages of the experiment described in (A), using the method of Santocanale and Diffley (*27*). Genomic DNA samples were run in an alkaline agarose gel before transfer to Hybond-N$^+$ membrane and detection of replication intermediates (RIs) at the indicated origins by Southern hybridization. (**C**) DNA content was determined by flow cytometry at the indicated stages of the experiment described in (A). (**D**) All MCM proteins are required to complete the S phase after release from hydroxyurea arrest. Cells were grown in YP+Galactose, and an *ubr1Δ::GAL-UBR1* strain was used as the control. (**E**) MCM proteins are essential throughout the S phase and are not required only for replication to resume after hydroxyurea arrest. Cells were grown in the medium YP+Galactose, and α factor was added upon shifting to 37°C so that cells completing the S phase and mitosis would accumulate with a 1C DNA content in the subsequent cell cycle.

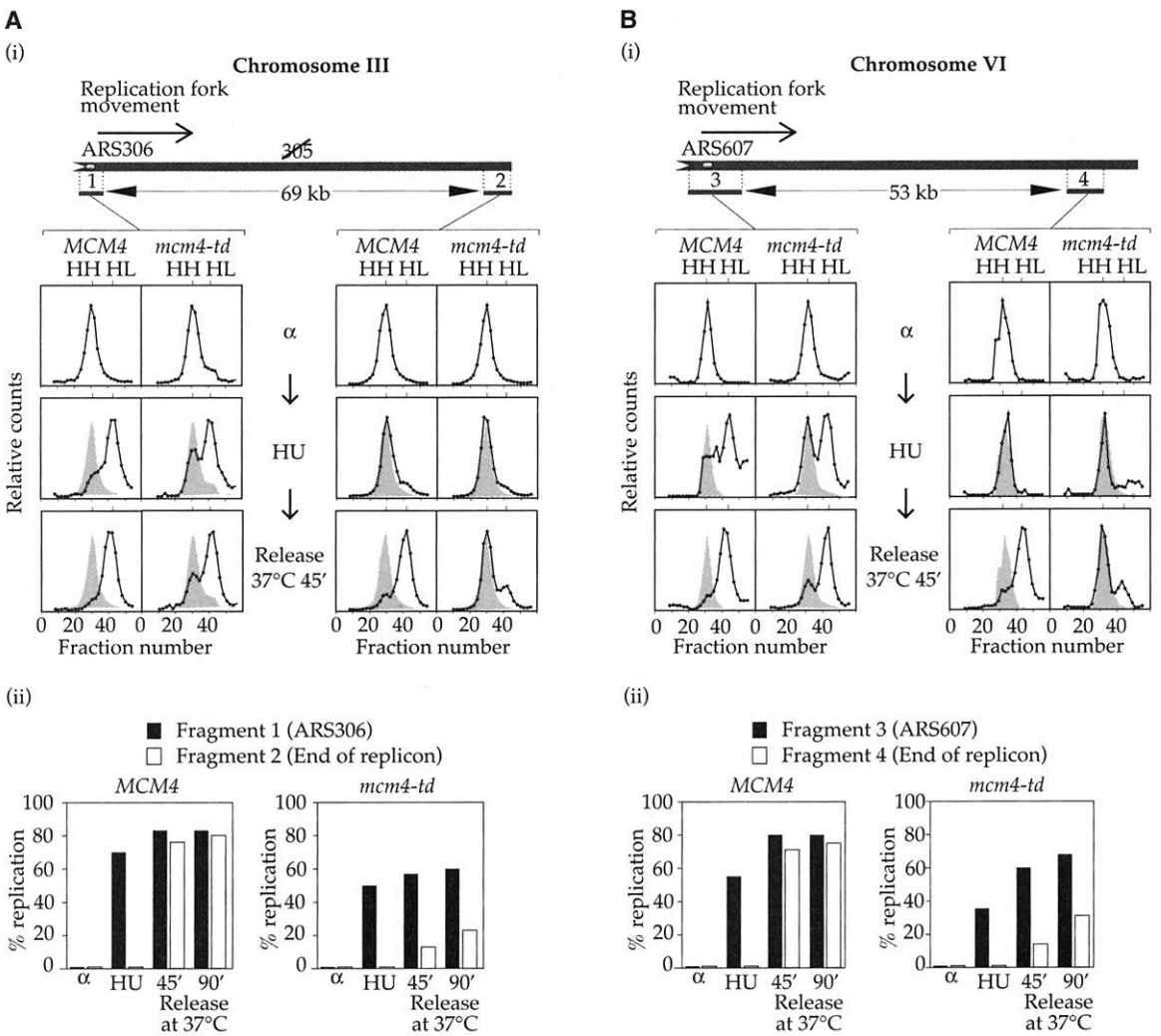

Fig. 3. MCM proteins are required for replication fork progression. See text and (*11*) for details. (**A**) (i) Analysis of the last replicon at the left end of chromosome III. ARS305 has been deleted in these strains, so the replicon covers the 74 kb from ARS306 to the end of the chromosome. Replication of the indicated fragments was determined by Southern hybridization of restriction fragments separated in a CsCl gradient. Shaded peaks indicate the position of the HH peak for each fragment at the start of the experiment. (ii) Quantification of the data from the same experiment. (**B**) (i) Analysis of the right end of chromosome VI. ARS607 is the last active origin, so the replicon covers the 71 kb until the end of the chromosome. (ii) Quantification of the data from the same experiment.

the effects of degrading Mcm4 within the S phase are irreversible. These data suggest that the maintenance of MCM proteins at stalled replication forks is essential for the subsequent resumption of fork progression. One likely reason for this in budding yeast is that displaced MCM proteins will be excluded from the nucleus by Cdc28 kinase activity (*22*, *23*). It is also possible that displaced MCM proteins cannot be reloaded at replication forks, even in other eukaryotic cells where MCM proteins remain in the nucleus throughout the cell cycle.

Our experiments show that MCM proteins provide a single, unique opportunity for DNA synthesis to occur at replication forks during the elongation phase of chromosome replication. Although DNA helicase activity has only been found in association with subcomplexes of human MCM4, -6, and −7, and it remains possible that MCM proteins have another biochemical activity yet to be determined, our data indicate that all budding yeast MCM proteins are essential for elongation, suggesting that the active form may be a heterohexamer. It is likely that MCM proteins are normally displaced from chromatin during termination, and it remains to be determined how displacement is prevented when replication fork progression is stalled in other ways, such as at natural pause sites in the genome (*24*–*26*) or at the sites of damaged nucleotides. It now appears that MCM2-7 proteins play a unique role in ensuring that both initiation and elongation occur only once during the S phase, thereby limiting chromosome replication to a single round in each cell cycle.

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28. We thank L. Johnston and A. Varshavsky for providing plasmids, K. Bousset for advice concerning density transfer experiments, and T. Lindahl for helpful comments on the manuscript. Supported by the Imperial Cancer Research Fund, and a postdoctoral fellowship from the Human Frontier Science Programme (J.A.T.).

12 January 2000; accepted 22 March 2000

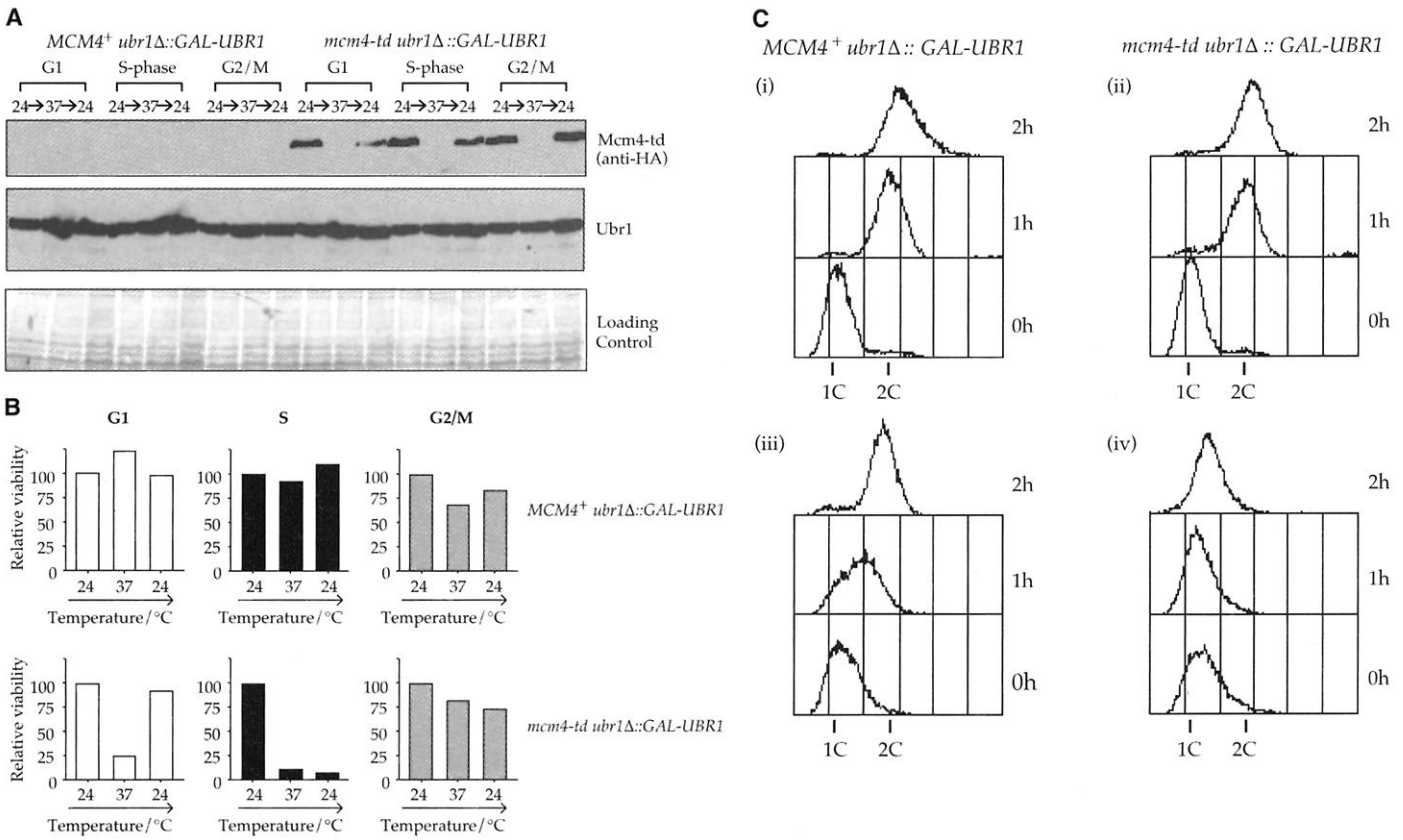

Fig. 4. Inactivation of an MCM protein after the activation of early origins of DNA replication blocks S-phase progression irreversibly. Cells were grown in the medium YP+Galactose. (**A**) Samples were taken throughout the experiment to make protein extracts, and the levels of Mcm4-td and Ubr1 were determined by immunoblot analysis [for details of antibodies, see (*11*)]. The loading control shows Ponceau S staining of a portion of the membrane used to detect Mcm4-td, between the 200 and 97 kD markers. (**B**) Cell viability was determined during the experiment described in (A) by plating cells on YPD (YP+dextrose) plates and counting the number of colonies after 2 days of incubation at 24°C. (**C**) At the end of the experiment described in (A), cells were released from α factor arrest [(i) and (ii)] and hydroxyurea arrest [(iii) and (iv)], and incubation was continued in fresh medium at 24°C for the indicated times. DNA content was monitored by flow cytometry.

Essential Role for Cholesterol in Entry of Mycobacteria into Macrophages

John Gatfield and Jean Pieters*

Mycobacteria are intracellular pathogens that can invade and survive within host macrophages, thereby creating a major health problem worldwide. The molecular mechanisms involved in mycobacterial entry are still poorly characterized. Here we report that cholesterol is essential for uptake of mycobacteria by macrophages. Cholesterol accumulated at the site of mycobacterial entry, and depleting plasma membrane cholesterol specifically inhibited mycobacterial uptake. Cholesterol also mediated the phagosomal association of TACO, a coat protein that prevents degradation of mycobacteria in lysosomes. Thus, by entering host cells at cholesterol-rich domains of the plasma membrane, mycobacteria may ensure their subsequent intracellular survival in TACO-coated phagosomes.

Macrophages play an important role in the host defense against bacteria because of their capacity to phagocytose and to degrade microbes in lysosomes. Mycobacteria, which are highly successful pathogens, resist delivery to lysosomes and instead survive within a specialized vacuole, the mycobacterial phagosome (*1–3*). The bacteria survive intracellularly because they are able to actively recruit and retain TACO (for tryptophane aspartate–containing coat protein) at the mycobacterial phagosome, where it prevents fusion of phagosomes with lysosomes (*4*). TACO lacks a putative transmembrane domain, which suggests that it is a peripherally associated protein. To analyze the association of TACO with the phagosomal membrane, murine macrophages were infected with *Mycobacteria bovis* Bacille Calmette-Guérin (BCG). Subjection of isolated phagosomes to conditions known to release membrane-associated proteins, such as high salt (1 M NaCl), sodium carbonate (pH 11), and Triton X-100 (1%), did not solubilize TACO (Fig. 1A), although some background release was apparent because of the fragility of isolated phagosomes. However, extraction of the phagosomes in 0.02% digitonin, a cholesterol-sequestering reagent (*5*), resulted in complete solubilization of TACO, to the same degree as disruption of the phagosomes in SDS or urea (Fig. 1A). Similarly, digitonin, but not Triton X-100, disrupted TACO association with membranes from uninfected cells

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