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A Lesion in the DNA Replication Initiation Factor Mcm10 Induces Pausing of Elongation Forks through Chromosomal Replication Origins in *Saccharomyces cerevisiae*

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We describe a new minichromosome maintenance factor, Mcm10, and show that this essential protein is involved in the initiation of DNA replication in *Saccharomyces cerevisiae*. The *mcm10* mutant has an autonomously replicating sequence-specific minichromosome maintenance defect and arrests at the nonpermissive temperature with dumbbell morphology and 2C DNA content. Mcm10 is a nuclear protein that physically interacts with several members of the MCM2-7 family of DNA replication initiation factors. Cloning and sequencing of the *MCM10* gene show that it is identical to *DNA43*, a gene identified independently for its putative role in replicating DNA. Two-dimensional DNA gel analysis reveals that the *mcm10-1* lesion causes a dramatic reduction in DNA replication initiation at chromosomal origins, including ORI1 and ORI121. Interestingly, the *mcm10-1* lesion also causes replication forks to pause during elongation through these same loci. This novel phenotype suggests a unique role for the Mcm10 protein in the initiation of DNA synthesis at replication origins.

DNA replication is a fundamental process of all dividing cells. During the eukaryotic cell cycle, DNA replication occurs exactly once and is initiated only upon the completion of mitosis. Strict regulation of timing appears to be mediated through the coordinated activities of numerous proteins. Due in large part to studies of viral DNA replication, the enzymatic activities at the replication fork were elucidated many years ago (33). In contrast, *trans*-acting factors which regulate the initiation of DNA replication have been described only recently. In an effort to gain a comprehensive understanding of the factors involved in this essential process, we have sought to identify gene products that influence the initiation of DNA replication.

Saccharomyces cerevisiae provides an excellent eukaryotic model for identifying proteins involved in DNA replication. Many replication initiation factors currently under investigation are conserved in mammalian cells and were found initially in yeast. Members of the origin recognition complex (ORC) (20, 22) were originally identified biochemically through their binding to the consensus sequence of autonomously replicating sequences (ARSs), which function as DNA replication origins (2). Our screen for yeast minichromosome maintenance (*mcm*) mutants (39) has also been fruitful in identifying replication initiation factors, such as those of the MCM2-7 family (9, 29, 52). The MCM2-7 proteins are a family of six conserved proteins that are ubiquitous in eukaryotes. Their essential role in the initiation of DNA synthesis not only has been demonstrated by in vivo studies in a number of organisms, including *S. cerevisiae* (55), *Schizosaccharomyces pombe* (40), and *Drosophila melanogaster* (51), but also is supported by in vitro studies in *Xenopus laevis* (8, 34, 38). The MCM2-7 proteins interact with one another, and possibly other proteins, to form large complexes (35). Despite their structural similarity, each of these proteins is indispensable for cell viability in *S. cerevisiae* (26, 28, 54), consistent with the idea that each of these six proteins is a subunit of a larger complex that is essential for the initiation of DNA synthesis. Although there is no evidence for the direct interaction between the MCM2-7 protein complexes and replication origins, these proteins have been shown to bind chromatin prior to the onset of DNA synthesis and to be released from chromatin after the onset of DNA synthesis (56). Furthermore, a defect in any one of these proteins results in a dramatic reduction in the frequency of initiation events at replication origins (55).

The *mcm* mutants affect the maintenance of minichromosomes in a manner that is dependent on the exact nature of the ARS: the activities of different ARSs, as measured by plasmid stability, are affected to different extents in a given *mcm* mutant. This mutant phenotype is referred to as the ARS-specific Mcm^- defect and appears to be unique to DNA replication mutants defective in the initiation step of DNA synthesis. All *mcm* mutants are characterized by an ARS-specific Mcm^- defect (21, 39, 54). Strains mutated in Abf1 or in the essential subunits of the ORC also display an ARS-specific Mcm^- phenotype (19, 47). Interestingly, ARSs that are exquisitely sensitive to the *mcm* mutations appear to be relatively insensitive to the *abf1* mutations and vice versa, suggesting that individual origins have customized regulatory mechanisms for origin usage (39, 47). These observations also suggest that the defined patterns of ARS specificity exhibited by different replication initiation mutants reflect their involvement in independent mechanisms that regulate the initiation of DNA synthesis at replication origins.

The screen for minichromosome maintenance-defective mutants has also uncovered other genes that may play a role in the regulation of DNA replication (39). For example, *MCM1* encodes a global transcription factor that regulates the expression of diverse genes (45, 46). While it is likely that Mcm1 regulates DNA synthesis by modulating the expression of DNA replication genes, as suggested by the putative Mcm1 binding sites found upstream of *MCM3*, *CDC46/MCM5*, and *CDC47/*

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TABLE 1. Plasmids used in this study

Plasmid	Description	Source or reference
pYCMCM10	Original clone containing *MCM10*	6
pBTM116	2μm *TRP1-lexA*(1-202)	S. Fields
pGAD2F	2μm *LEU2 GAL4*(768-881)	S. Fields
pBTM.MCM2	pBTM with *LEXA-MCM2* gene fusion	35
pBTM.MCM3	pBTM with *LEXA-MCM3* gene fusion	35
pBTM.CDC46	pBTM with *LEXA-CDC46* gene fusion	35
pBTM.MCM6	pBTM with *LEXA-MCM6* gene fusion	Y. Kawasaki
pBTM.CDC47	pBTM with *LEXA-CDC47* gene fusion	44
pBTM.CDC54	pBTM with *LEXA-CDC54* gene fusion	Y. Kawasaki
pGAD.MCM10	pGAD2F with *GAL4-MCM10* gene fusion	This study
YCp101	*ARS1 LEU2 CEN5*	54
YCp120	*ARS120 URA3 LEU2 CEN5*	54
YCp121	*ARS121 LEU2 CEN5*	54
YCp131	*ARS131 LEU2 CEN5*	54
YCpH2B	*ARSH2B URA3 LEU2 CEN5*	54
YCpHML	*ARSHMLα URA3 LEU2 CEN5*	54
YCpHO	*ARSHO URA3 CEN5*	54
pQE31	*ampR*; parent vector for pMCM10.His	Qiagen
pMCM10.His	pQE with Mcm10.His coding sequence	This study
pEG(KT)	2μm *URA3 GAL1*-GST	D. Mitchell
pEG(KT)MCM2	2μm *URA3 GAL1*-GST-*MCM2*	35
pEG(KT)MCM3	2μm *URA3 GAL1*-GST-*MCM3*	35
pEG(KT)MCM4	2μm *URA3 GAL1*-GST-*CDC54*	Y. Kawasaki
pEG(KT)MCM5	2μm *URA3 GAL1*-GST-*CDC46*	Y. Kawasaki
pEG(KT)MCM6	2μm *URA3 GAL1*-GST-*MCM6*	Y. Kawasaki
pEG(KT)MCM7	2μm *URA3 GAL1*-GST-*CDC47*	Y. Kawasaki

MCM7, direct evidence for Mcm1 playing such a role is elusive (1). In the current study, we have begun to elucidate the function of a new minichromosome maintenance factor, Mcm10. We have characterized the *mcm10* mutant phenotype and compared it to that of other replication initiation mutants. We have cloned and analyzed the *MCM10* gene sequence and studied the interaction of Mcm10 with members of the MCM2-7 protein family. Finally, we have examined chromosomal replication origin usage to find direct evidence of a DNA replication defect in the *mcm10* mutant.

MATERIALS AND METHODS

Strains and plasmids. *Escherichia coli* JM109 [*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi*Δ(*lac-proAB*) F′ (*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15)] was used to propagate plasmids and was used to express the Mcm10.His protein. *E. coli* JM101 [*supE thi*Δ(*lac-proAB*) F′ (*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15)] was used to propagate minichromosomes. Yeast strains used in this study include 8534-8C (*MAT*α *ura3-52 leu2-3,112 his4-34*), mcm10-2C (*MAT***a** *his3 his4 leu2-3,112 ura3-52 mcm10-1*), mcm10-1D (*MAT***a** *leu2-3,112 his mcm10-1*), mcm10-14 (*MAT***a** *lys2-801 his mcm10-1*), EGY40 (*MAT***a** *ura3-52 trp1 his3 leu2*), and BJ2168 (*MAT***a** *pep4-3 prc1-407 prb-1122 ura3-52 trp1 leu2*). The *mcm10-1* mutation was isolated by Richard Surosky as described by Gibson et al. (21). Plasmids used in this study are described in Table 1.

Media, chemicals, and enzymes. Rich (yeast extract-peptone-dextrose) and complete media were prepared as described elsewhere (24). Plates containing 5-fluoroorotic acid were made according to the method of Boeke et al. (3). DNA sequencing was performed with the sequencing kit from United States Biochemical Corp. (Cleveland, Ohio). Restriction enzymes were purchased from New England Biolabs (Beverly, Mass.) or Bethesda Research Laboratories Life Technologies, Inc. (Gaithersburg, Md.). DNA primers were purchased from Ransom Hill Bioscience, Inc. (Ramona, Calif.). Random hexanucleotides and deoxyribonucleotides were purchased from Pharmacia (Uppsala, Sweden).

Plasmid loss rates. Yeast strains were transformed with the appropriate plasmids (15). Mitotic plasmid stability assays were performed as described previously (21) except that growth in nonselective media occurred over 10 to 15 generations. Loss rates per generation were determined by the formula $1 - (F/I)^{1/N}$, where I is the initial percentage of plasmid-bearing cells and F is the percentage of plasmid-bearing cells following growth over N generations.

2D DNA gels. Two-dimensional (2D) DNA gel electrophoresis was performed according to the neutral-neutral method (4). To control for shearing of replication bubbles during extraction from cells, comparisons between ORI1 and ORI121 were made by using a single DNA preparation. DNA from each preparation was analyzed in at least two separate 2D gels to assess the consistency of results. For analysis of ORI1, genomic DNA was digested to completion with *Nco*I or *Eco*RI. For ORI121, *Bam*HI and *Eco*RI were used. Typically, between 30 and 50 μg of DNA was loaded in each gel. Probes for detecting ORI1 or ORI121 were prepared by isolating the *Nco*I or *Eco*RI fragment of ARS1 or the *Bam*HI-*Eco*RI fragment of ARS121, followed by radiolabelling (16). Radiolabelled ARS1 or ARS121 DNA probe (12.5×10^6 cpm) was added per 10 ml of hybridization solution and allowed to hybridize for 24 h at 42°C. For better separation of Y-form and linear DNA of smaller-molecular-size fragments (<1.5 kb), the second-dimension gel was run at an angle of 95 to 100° to the direction of electrophoresis at a voltage of 5 V/cm for 6 h with a 2% agarose gel (31).

Preparation of anti-Mcm10 antiserum. Mcm10 was purified for antibody production by expression of plasmid pMCM10.His in *E. coli*. In this plasmid, six histidine residues replace the first 54 amino acids of Mcm10. Expression from the *lacZ* promoter produces a histidine-tagged protein, designated Mcm10.His. Mcm10.His was expressed at high levels in *E. coli* JM109 and purified by means of immobilized metal affinity chromatography over an Ni_2^+-nitrilotriacetic acid resin column (Qiagen, Inc., Chatsworth, Calif.). Anti-Mcm10 antiserum was raised in two rabbits injected three times at 2-week intervals with 0.2 mg of Mcm10.His. Antisera were collected and stored at −20°C. Affinity purification of the anti-Mcm10 antisera was achieved by incubating crude antisera with Mcm10.His immobilized on an Immobilon-P membrane (Millipore Corp., Bedford, Mass.). The resulting membrane was incubated for 16 h at 4°C with a 20% solution of crude antiserum in a mixture of 150 mM NaCl, 10 mM Tris (pH 8.0), 1 mM EDTA, 1% lowfat milk, and 0.1% Tween 20. The Mcm10-specific antibodies were eluted with 100 mM glycine, pH 2.5, followed by neutralization to pH 8.0 with Tris buffer. This purification procedure was repeated three times in succession to produce the purified anti-Mcm10 antibodies used in subsequent studies.

Western blot analysis. Proteins from strain BJ2168 were extracted by glass bead lysis in the presence of protease inhibitors and separated by molecular weight on polyacrylamide gels. Proteins were transferred to Immobilon-P membranes and incubated with purified anti-Mcm10 antibodies as described elsewhere (25). Affinity-purified goat anti-rabbit immunoglobulin G (IgG)–horseradish peroxidase conjugate was obtained from Bio-Rad Laboratories (Richmond, Calif.) and visualized with Renaissance chemiluminescence reagents (DuPont, Boston, Mass.).

Flow cytometry. Yeast cells were grown at 23°C and shifted to 37°C for 4 h. The final concentration of cells was 1.5×10^8 (wild type) and 8×10^7 (*mcm10-1* mutant). Cells were stained with propidium iodide as published previously (27).

Indirect immunofluorescence. Indirect immunofluorescence was carried out by a modification of the Kilmartin and Adams method (30). Primary antibodies used were affinity-purified rabbit anti-Mcm10 antibodies and rat anti-yeast tubulin antibodies (Yol1/34; John Kilmartin, Cambridge, United Kingdom). Secondary antibodies used were fluorescein isothiocyanate-conjugated goat anti-rat IgG antibodies (Cappel, Durham, N.C.) and rhodamine-conjugated goat anti-rabbit IgG antibodies (Boehringer Mannheim, Indianapolis, Ind.).

Two-hybrid analysis. PCR-amplified *MCM10* gene was cloned into pGAD2F (18) to allow production of the Gal4-Mcm10 fusion protein for two-hybrid analysis. pGAD.MCM10 complements the temperature sensitivity of the *mcm10-1* mutant. Other two-hybrid system plasmids have been described elsewhere (35). Plasmids were transformed into yeast strain EGY40 and selected on medium lacking tryptophan, uracil, and leucine. Colonies thus isolated were patched onto medium lacking the same amino acids and containing X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside).

GST fusion protein affinity chromatography. Glutathione *S*-transferase (GST) and GST-Mcm fusion proteins expressed in yeast were conjugated to glutathione-Sepharose 4B (Pharmacia) as described elsewhere (35). The protein-conjugated beads were incubated with yeast protein extracts at 4°C for 2 h with gentle shaking. The beads were then washed four times with 10 ml of ice-cold buffer containing 0.1 M NaCl. Bound proteins were eluted from the beads with 500 μl of ice-cold elution buffer containing 0.5 M NaCl. Eluates were analyzed on Western blots with anti-Mcm10 antibodies.

RESULTS

Mcm10 is required for stable plasmid maintenance. We have isolated several DNA replication initiation mutants which lose minichromosomes in an ARS-specific manner. In a given *mcm* mutant, plasmids with certain ARSs have high loss rates while other ARSs allow maintenance of the minichromosome at rates close to that of an isogenic wild-type strain (39, 54). To elucidate the pattern of minichromosome maintenance in the *mcm10* mutant, we determined loss rates in the mutant strain mcm10-2C at room temperature (approximately 23°C) and at 30°C. The results in Fig. 1 indicate that at room temperature the minichromosome maintenance phenotype of the *mcm10*

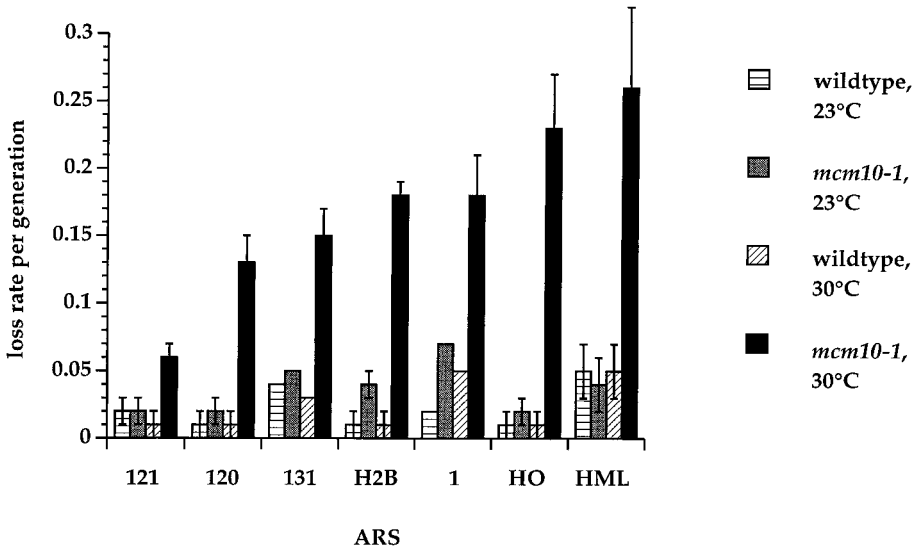

FIG. 1. Minichromosome loss rates in wild-type (8534-8C) and *mcm10-1* (mcm10-2C) strains at 23 and 30°C. The maximum theoretical loss rate is 0.5 per generation. The values are averages derived with two independent transformants and were obtained in three or four independent experiments. Measurements that have negligible variations from experiment to experiment are shown without error bars.

mutant strain resembles that of the wild type. While the loss rates appear to be slightly elevated relative to wild-type rates, there is little variation in loss rates of plasmids with different ARSs. At 30°C, however, a striking, ARS-specific pattern of minichromosome maintenance emerges. Loss rates of all minichromosomes increase in the *mcm10* mutant. Moreover, plasmids containing some ARSs, such as ARS1, are lost at greater rates than plasmids containing other ARSs, such as ARS121. The ARS-specific Mcm^- phenotype suggests that the *mcm10-1* mutation affects DNA replication initiation at ARSs on the minichromosomes.

***mcm10* mutant cells have a cell division cycle arrest phenotype at the restrictive temperature.** Growth of the *mcm10* mutant strain is normal at 12 and 23°C. At 30°C, the mutant strain shows a slight growth defect, with a doubling time of 120 min compared to 75 min in the wild-type strain. At 37°C, while the wild-type strain forms colonies in 2 days, the *mcm10* mutant fails to grow (data not shown).

To investigate a possible cell division cycle arrest phenotype of the *mcm10* strain, we examined the morphology and the position of 4′,6-diamidino-2-phenylindole (DAPI)-stained nuclei of mutant and wild-type cells microscopically. At 23°C, *mcm10* mutant cells in all stages of the cell cycle were observed (data not shown), consistent with the normal growth rate observed in the mutant at the permissive temperature. After 4 h at 37°C, however, the *mcm10* mutant strain has arrested premitotically; over 95% of cells contain a large bud and a single nucleus (Fig. 2C and D). The isogenic wild-type strain grows well at 37°C, as revealed by the presence of cells in all stages of the cell cycle (Fig. 2A and B).

We also assessed the status of nuclear DNA replication in arrested mutant cells. Nuclear DNA was stained with propidium iodide. Fluorescence-activated cell sorting analysis of stained cells shows that at the permissive growing temperature of 23°C, both wild-type (8534-8C) and *mcm10* mutant cells are present in two main populations (Fig. 3). One population of cells contains 1C DNA and is therefore in the G_1 phase of the cell cycle. The other has 2C DNA content and is therefore completing S phase or undergoing mitosis. At 30°C, the wild-type cells contain mostly either 1C or 2C DNA (Fig. 3). In the *mcm10* mutant, in addition to cells containing either 1C or 2C DNA, a significant portion of the cell population has a DNA content between 1C and 2C and is therefore undergoing DNA replication (Fig. 3). This result suggests that at 30°C, *mcm10* cells have a prolonged S phase which presumably contributes to the observed 60% increase in the length of the cell cycle. After 4 h at 37°C, the wild-type strain shows a normal distribution of cells in different stages of the cell cycle (Fig. 3). In contrast, few of the *mcm10* mutant cells contain 1C DNA; instead, the vast majority of cells show a nuclear DNA content of approximately 2C DNA (Fig. 3). A significant portion of the cell population has a DNA content of slightly greater than 2C. Such a population of cells is expected if mitochondrial DNA continues to replicate after cessation of chromosomal DNA replication at the nonpermissive temperature.

The *mcm10-1* mutant is allelic to another DNA replication mutant, the *dna43-1* mutant. *MCM10* was cloned by complementation of the temperature-sensitive phenotype of *mcm10-1*. A YCp50-based yeast genomic library (48) was used to transform the *mcm10-1* strain, and viable transformants were selected on chloramphenicol-uracil medium at 37°C. A dozen viable transformants grew up among approximately 17,400 transformants after 5 days of growth at 37°C. The plasmid DNA that complemented the temperature-sensitive phenotype of the *mcm10-1* strain was recovered from four of the viable transformants. All four plasmids contained an identical 7.6-kb insert in the YCp50 vector. Subcloning of the 7.6-kb insert indicated that a 2.2-kb *Kpn*I-*Xba*I fragment complemented the temperature-sensitive phenotype of *mcm10-1*.

Sequencing of the 2.2-kb *Kpn*I-*Xba*I fragment indicated that it contains an open reading frame that had already been identified as *DNA43* in an independent screen for DNA replication mutants (49). *DNA43* is located on chromosome IX, 32 centimorgans distal to the centromere from *his5*. Significantly, Solomon and colleagues (49) showed that at the restrictive temperature, while RNA and protein synthesis continues in the *dna43-1* mutant cells, DNA synthesis ceases after one generation. Additionally, they showed that *DNA43* encodes an essential gene product. Both *dna43-1* and *mcm10-1* are recessive conditional lethal mutations. Genetic analysis indicates that

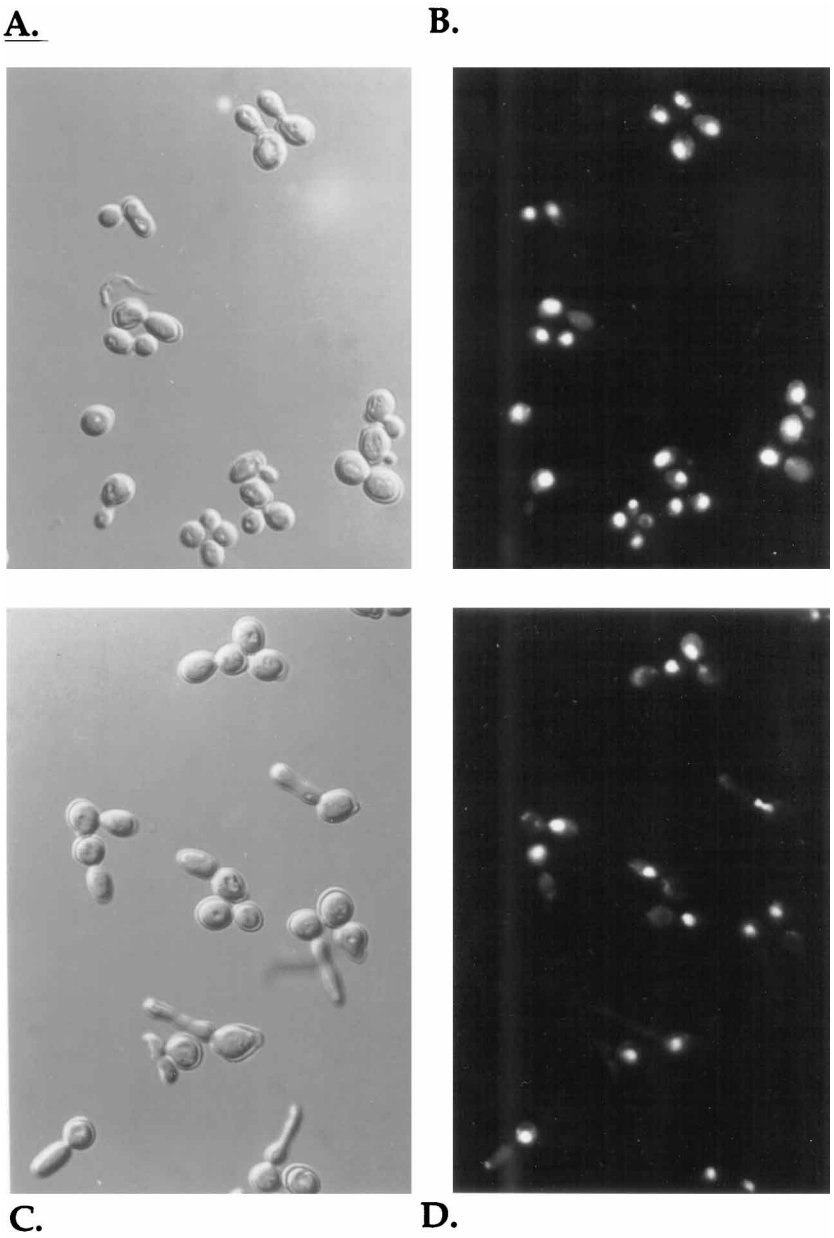

FIG. 2. The *mcm10-1* mutant arrests premitotically at 37°C. Wild-type (8534-8C [A and B]) and *mcm10-1* mutant (mcm10-2C [C and D]) strains were grown at 23°C for several hours until early log phase and then shifted to 37°C for 4 h. (A and C) Nomarski microscopy reveals cell morphology. (B and D) DAPI staining of the DNA reveals the morphology and location of the nucleus.

mcm10-1 and *dna43-1* are noncomplementing alleles of the same gene. Tetrads of *mcm10/dna43* diploids comprise four heat-sensitive spores. A search in the SGD database found that *MCM10/DNA43* encodes a conceptual protein of 571 amino acids (open reading frame product YIL150c) with a predicted molecular mass of 65.8 kDa and an isoelectric point of 9.7, in contrast to the 59.6-kDa molecular mass reported by Solomon et al. No significant homologies were detected between Mcm10 and any other sequences in the database.

Mcm10 is localized in the nucleus throughout the cell cycle. Antiserum from rabbits injected with the histidine-tagged Mcm10 truncation protein cross-reacts with numerous proteins in yeast extract (Fig. 4, lane 1). Antibodies were affinity purified against Mcm10.His immobilized on a polyvinylidene difluoride filter. These purified antibodies react predominantly with a single yeast protein of approximately 70 kDa, close to the predicted molecular mass of 65.8 kDa of Mcm10 (Fig. 4, lane 2). To verify that the single band is indeed Mcm10, Mcm10 was overproduced by expression from the Gal1-10 promoter in yeast cells. Immunoblotting analysis shows that an overproduced 70-kDa Mcm10 protein was indeed recognized by the purified antibodies (data not shown).

The location of the Mcm10 protein in different stages of the cell cycle was determined by indirect immunofluorescence of Mcm10 in a population of asynchronously growing wild-type cells (Fig. 5). Light microscopy of cells in various stages of the cell cycle is shown in Fig. 5A. Specific stages of the cell cycle can be determined by the position of the DAPI-stained nucleus

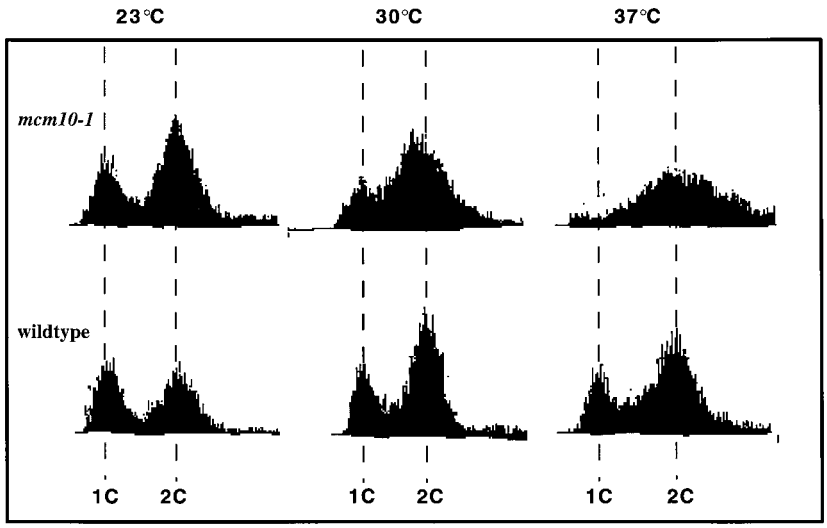

FIG. 3. Flow cytometry reveals a nuclear DNA content of approximately 2C in *mcm10-1* cells arrested at 37°C. Wild-type (8534-8C) or *mcm10-1* (mcm10-2C) cells were grown at 23°C until early log phase and then incubated for 4 h at 23, 30, or 37°C. Cells were prepared for flow cytometric analysis as described in Materials and Methods.

of each cell (Fig. 5B) and the morphology of the spindles revealed by immunostaining of tubulin (Fig. 5D). As seen in Fig. 5C, the anti-Mcm10 antibodies detect a protein that is present in the nucleus at all stages of the cell cycle.

Mcm10 interacts with several members of the MCM2-7 family. The *mcm10-1* arrest phenotype shares a notable similarity with the arrest phenotype of established replication initiation mutants, including *mcm2-1* and *mcm3-1* strains isolated in this lab. Additionally, the *mcm* mutants, including *mcm10-1*, exert their most dramatic effects on a similar subset of minichromosomes. Furthermore, previous work in our lab has demonstrated that many *MCM* gene products in yeast extracts interact with each other to form complexes that appear larger than 700 kDa as determined by sedimentation through glycerol gradients (35). We speculated that since Mcm10 is involved in initiating DNA replication, it might interact with other initiation factors. Therefore, we used the two-hybrid system (18) and GST–Mcm2-7 affinity chromatography (35) to examine the interaction of Mcm10 with members of the MCM2-7 family. The MCM2-7 family includes Mcm2 and Mcm3, identified by the *mcm* screen; Cdc46/Mcm5, Cdc47/Mcm7, and Cdc54/Mcm4, identified by a screen for *cdc* mutants that fail to enter S phase (43); and Mcm6, which was cloned as the *S. cerevisiae* homolog of *S. pombe* Mis5 (28, 50). *MCM6* is an essential gene that encodes a conceptual protein of 1,017 amino acids (open reading frame product YGL201c) (28). It contains all three conserved domains characteristic of the MCM2-7 family (32).

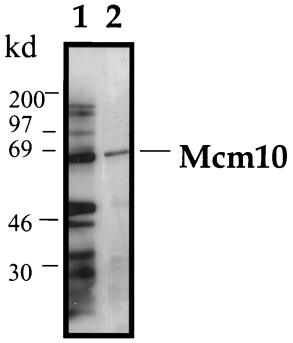

FIG. 4. Preparation of monospecific anti-Mcm10 antibodies. Western blots of yeast extracts were probed with antiserum from rabbit (lane 1) or affinity-purified Mcm10-specific antibodies (lane 2).

In the yeast two-hybrid system, if two proteins interact, they reconstitute transcriptional activity detected by expression of the *lacZ* reporter gene. Cells expressing the reporter gene form blue colonies on X-Gal culture plates. A chimeric construct encoding the Gal4 transcriptional activation domain fused N terminally to Mcm10 was cloned in vector pGAD2F. Recombinants of the coding regions of members of the MCM2-7 family fused to the DNA binding domain of LexA were cloned in vector pBTM116 (Fig. 6A). Except for pBTM.MCM6, which was not tested, each of the constructs (pBTM.MCM2, pBTM.MCM3, pBTM.CDC46, pBTM.CDC47, pBTM.CDC54, and pGAD.MCM10) complements a null or a temperature-sensitive allele of the respective genes; hence, the fusion proteins are functional.

As shown in Fig. 6A, plasmids encoding LexA-Mcm2, LexA-Mcm6, or LexA-Cdc47 produce a strong blue color in cells which also produce the Gal4-Mcm10 fusion protein. However, no such blue color could be detected in cells expressing the Gal4-Mcm10 protein together with any of the other LexA-Mcm fusions. Because negative results are not proof of an absence of interactions, these observations suggest that Mcm10 interacts with at least three members of the MCM2-7 family of DNA replication initiation factors: Mcm2, Mcm6, and Cdc47/Mcm7.

As an independent biochemical method for detection of the physical interaction between Mcm10 and members of the MCM2-7 family, we constructed GST fusions of each of the MCM2-7 proteins for affinity chromatography. Each of these GST-MCM fusions complemented the corresponding *mcm* mutant for function except GST-Mcm6, which was not tested. Each of the GST-Mcm fusion proteins expressed in yeast was bound to glutathione-Sepharose resin. Yeast protein extracts (Fig. 6B, lane 1) were incubated with the resin before it was extensively washed with buffer containing 0.1 M NaCl. Proteins were eluted from each column coupled with GST (lane 2) GST-Mcm2 (lane 3), GST-Mcm3 (lane 4), GST-Mcm4 (lane

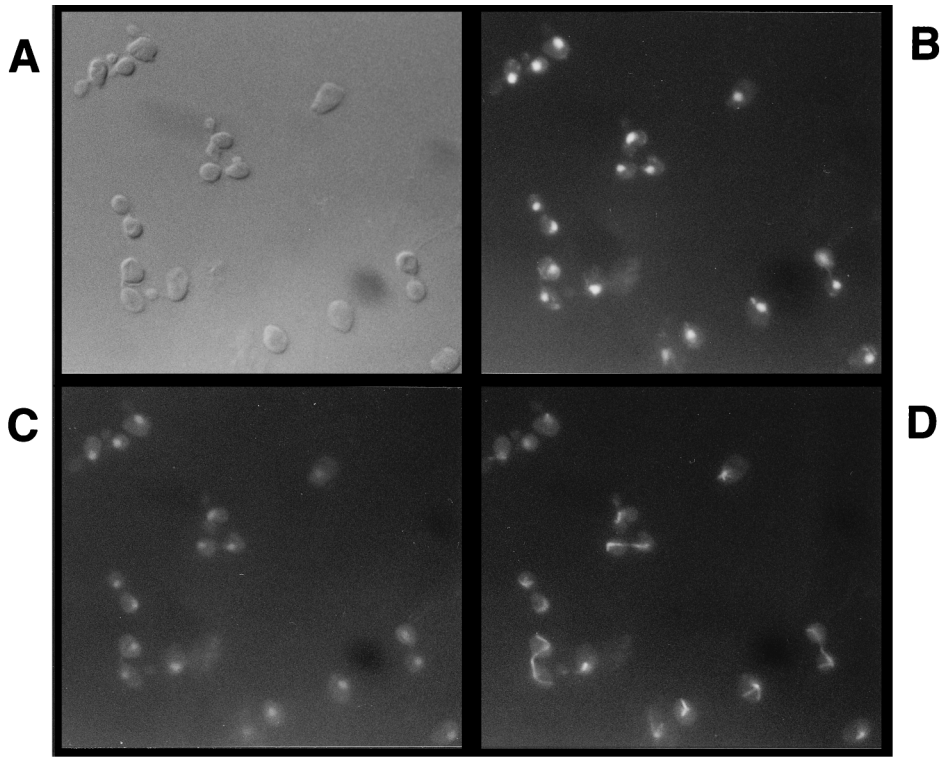

FIG. 5. Indirect immunofluorescence of Mcm10 protein shows that the Mcm10 protein is constitutively nuclear. Wild-type (8534-8C) cells in log phase at 30°C were prepared. (A) Nomarski microscopy; (B) DAPI staining; (C) affinity-purified anti-Mcm10 antibodies; (D) Yol1/34 antitubulin antibodies.

5), GST-Mcm5 (lane 6), GST-Mcm6 (lane 7), and GST-Mcm7 (lane 8) with 0.5 M NaCl. Western blots of each of these protein fractions were probed with Mcm10-specific antibodies. As shown in Fig. 6B, Mcm10 was eluted from columns coupled with GST-Mcm3, GST-Mcm4, and GST-Mcm6. Thus, interactions between Mcm10 and five of the six MCM2-7 proteins, Mcm2, Mcm3, Mcm4, Mcm6, and Mcm7, could be detected by either two-hybrid analysis or GST fusion affinity chromatography or both.

The *mcm10-1* mutation disrupts DNA replication initiation at chromosomal origins. The data presented above are consistent with the hypothesis that the *MCM10* gene product is important for initiating DNA replication. We anticipated that if Mcm10 is a DNA replication initiation factor, then the *mcm10* mutant should show a decrease in the frequency of chromosomal initiation events in vivo. Therefore, we examined chromosomal replication origin usage in the *mcm10* strain by using 2D DNA gels (4). We chose to analyze cells growing at 30°C since plasmid stability assays had shown that at this temperature minichromosome loss rates vary in an ARS-specific manner (Fig. 1). Moreover, if ARS usage on the minichromosome reflects respective origin usage on the natural chromosome, then ORI121 should be used more frequently than ORI1 to initiate DNA replication.

Wild-type (8534-8C) and *mcm10* mutant (mcm10-2C) strains were grown at 30°C for several generations. Chromosomal DNA was harvested from cultures in early log phase. The DNA was digested with restriction enzymes to derive 3-kb linear ORI121 fragments, with a centrally located origin (53), or to derive 5-kb linear ORI1 fragments, with an off-center origin (17) (see Fig. 9A). Restriction-digested genomic DNA was then analyzed by electrophoresis in two dimensions, followed by Southern blotting; DNA fragments containing ORI1 or ORI121 were visualized by hybridization with probes corresponding to the entire length of the relevant restriction fragment.

The results of 2D gel analysis are shown in Fig. 7. As anticipated from the minichromosome maintenance data, ORI121 (Fig. 7A) is more active than ORI1 (Fig. 7C). In the wild-type strain, a strong bubble arc is visible for both origins. The bubble arc indicates that DNA replication has initiated from within the restriction fragment. Both ORI1 and ORI121 are used as initiation origins in the vast majority of cells, in agreement with results obtained by others (17, 53). In the *mcm10* mutant, in contrast, replication initiation at the same sites is severely reduced (Fig. 7B and D). Examination of restriction fragments containing ORI121 (Fig. 7B) reveals a weak bubble arc and a strong Y arc. This pattern indicates that ORI121 is functioning as an active origin of DNA replication in only a minor portion of the cells in S phase. DNA replication is presumably initiated at sites outside of the ORI121 DNA fragment, and the ORI121 fragment is replicated by elongation from these neighboring sites. The ORI1 restriction fragments give rise to an even weaker bubble signal (Fig. 7D). The predominant Y arc indicates that ORI1 is used very infrequently to initiate DNA replication at 30°C in the *mcm10* strain.

The *mcm10-1* mutation retards replication elongation in the region of the ARS. Shorter exposures of the 2D gel autoradiographs consistently reveal an accumulation of signal in discrete locations within the Y arcs at or near replication origins in the mutant. Each elongation arc, formed by ORI1 or ORI121 fragments, contains two spots of increased intensity (Fig. 8B and D) not observed in the wild-type strain (Fig. 8A and C). The greater intensity reflects the accumulation of replication intermediates of a particular size, as has been documented in several other studies (5, 12, 23, 37). The presence of a com-

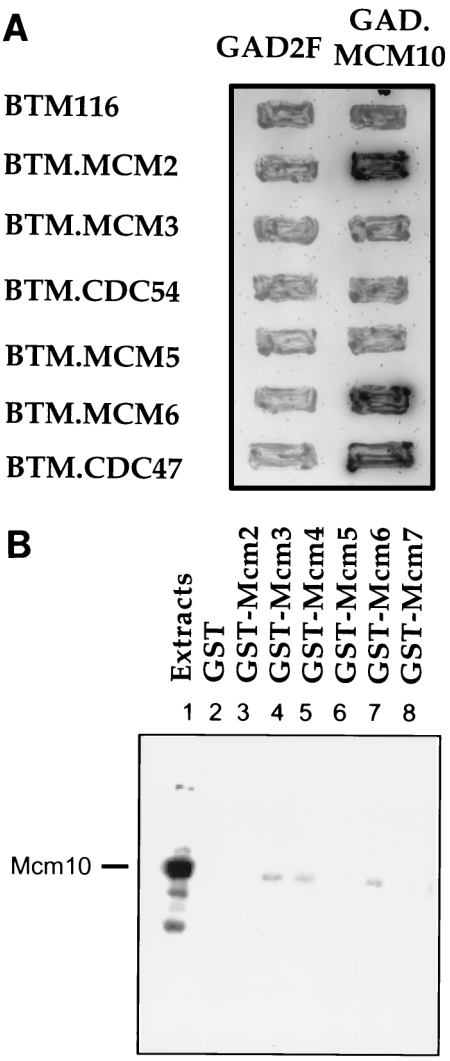

FIG. 6. Physical interactions between Mcm10 and members of the MCM2-7 family of proteins. (A) Two-hybrid analysis. BTM116 and BTM.MCM denote plasmids that express LexA and LexA–Mcm2-7 proteins, respectively. GAD2F and GAD.MCM10 denote plasmids that express Gal4 and Gal4-Mcm10 proteins, respectively. Yeast transformants of EGY40 carrying each pair of the plasmids and the *lacZ* reporter gene were assayed for β-galactosidase expression by colony color on X-Gal selective plates. (B) GST–Mcm2-7 fusion affinity column chromatography. The Western blot contains input yeast extracts and proteins eluted in buffer B–0.5 M NaCl from glutathione-Sepharose 4B columns coupled with GST or GST-Mcm2 through GST-Mcm7 as indicated. The Western blot was probed with Mcm10-specific antibodies.

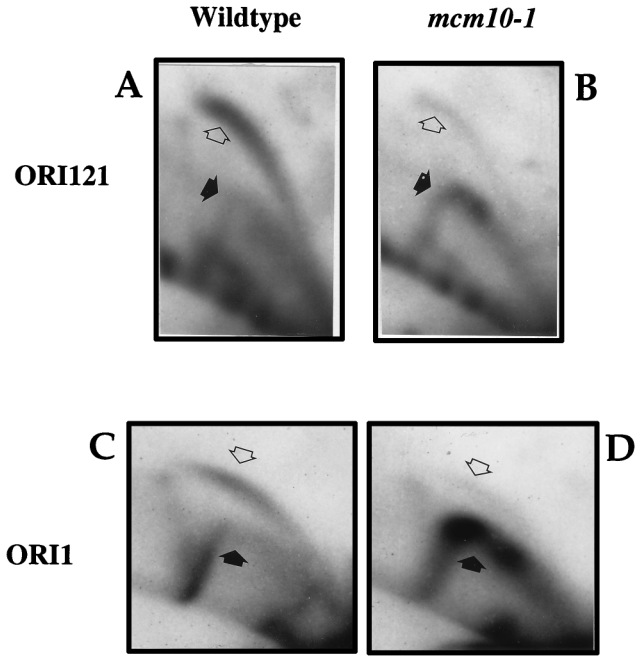

FIG. 7. 2D DNA gel analysis of replication origins reveals decreased origin usage at ORI121 and ORI1 in the *mcm10-1* mutant. DNA was prepared from wild-type (8534-8C) or *mcm10-1* mutant (mcm10-2C) cells grown to early log phase at 30°C. (A and B) Replication origin usage at ORI121; (C and D) replication origin usage at ORI1. In all panels, the first dimension was run left to right and the second dimension was run top to bottom. Open arrows, bubble arcs; closed arrows, Y arcs.

plete Y arc and the absence of a double-Y signal reflects a pause, not a complete halt, of replication fork progression (23). In contrast to the diffuse elongation pause signal observed in a region near tRNA genes as a result of tRNA transcription (12), the pause signals that we observe near the chromosomal replication origins in the *mcm10* mutant are quite compact, indicating that the elongation fork may be pausing within a small segment of the DNA. Furthermore, pausing of elongation due to transcriptional interference generates a single spot in 2D gel analysis, since forks stall only when they oppose the direction of gene transcription. In contrast, we observe two pause signals in each Y arc formed by ORI1 and ORI121 DNA fragments in the *mcm10* mutant. Although two pause signals could result from the pausing of elongation forks emanating from one direction at two independent sites or from both directions at a single site, we prefer a single-pause-site hypothesis for the reason presented below.

Two pause signals representing two populations of pause intermediates (PI) are expected if replication forks elongating from either direction pause at a single site that is asymmetrically located within the restriction fragment (Fig. 8E and F). In one population (PI1), the fork enters from the right and pauses at or near the ARS. In the second population (PI2), the fork enters from the left and, again, pauses at or near the ARS. The spacing of the pause signals in both ORI1 (Fig. 8F) and ORI121 (Fig. 8E) is consistent with this hypothesis. The approximate masses of the observed PI support the idea that pausing occurs at or near the ARS. In the case of ORI121, the arc of elongation intermediates begins at 3 kb and ends, upon complete replication, at 6 kb (Fig. 8B and E). A high percentage of replicating molecules has accumulated with sizes of roughly 4.5 and 5 kb (open triangles in Fig. 8B; PI1 and PI2 in Fig. 8E). For ORI1, the mass of the Y arc begins at approximately 5 kb and ends at about 10 kb (Fig. 8D). Replicating molecules have accumulated with sizes of roughly 7 and 9 kb (open triangles in Fig. 8D; PI1 and PI2 in Fig. 8F). While the exact position(s) of pausing cannot be determined from these gels, the data suggest that in both cases, pausing occurs within a 1-kb region that overlaps with the autonomously replicating sequence at ORI1 or ORI121.

Pausing of elongation forks at ORI1 occurs within a region of 300 bp that includes the minimal functional ARS1 sequence. The functional ARS1 is a 117-bp sequence consisting of an essential A domain and three important elements (B1, B2, and B3) in the B domain (41). To improve resolution of the location of the pause site(s), genomic ORI1 DNA was cleaved into two fragments (I and II) with *Eco*RI (Fig. 9A). Fragment I,

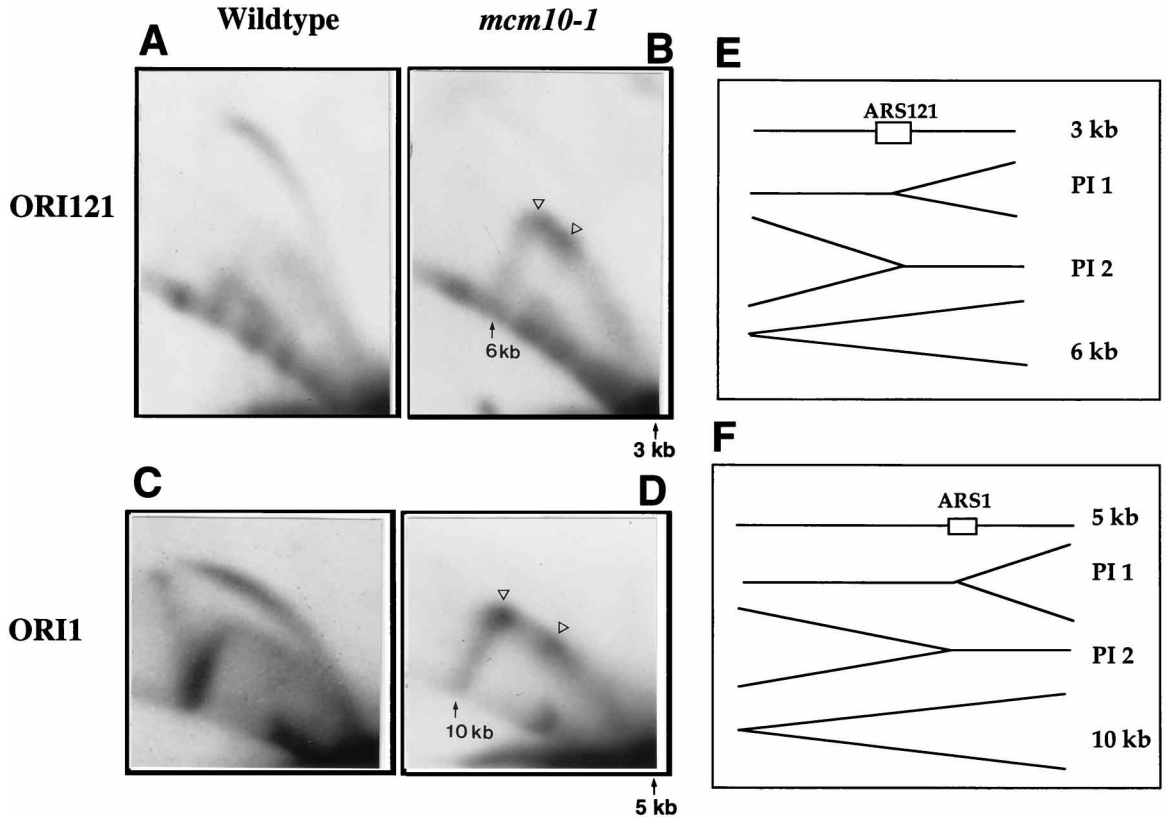

FIG. 8. DNA replication forks pause during elongation through two chromosomal regions containing replication origins in the *mcm10* mutant. (A to D) Short exposures of identical gels shown in Fig. 7. Southern blots were probed with ^{32}P-labelled *Bam*HI-*Eco*RI ARS121 fragment (A and B) or ^{32}P-labelled *Nco*I ARS1 fragment (C and D). Triangles indicate pause signals. (E and F) Cartoons depicting the approximate locations of ARS121 in the *Bam*HI-*Eco*RI fragment containing ORI121 (E) and ARS1 in the *Nco*I fragment containing ORI1 (F).

1,453 bp, contains a centrally located ARS1 such that the B3 element is positioned at 751 bp from one end. Fragment II, 3,089 bp, does not contain an ARS. If pause signals are the results of the stalling of replication forks emanating from both directions at ARS1, then two predictions can be made. First, no pause signals should be detected within fragment II. Second, two clustered pause signals, which may or may not be resolved by 2D gel electrophoresis, should be detected within fragment I.

2D gels of *Eco*RI-restricted genomic yeast DNA were probed with the 1.4-kb (Fig. 9B) and 3.1-kb (Fig. 9C) *Eco*RI fragments to detect replication intermediates in regions I and II, respectively. A single pause signal is detected within region I, as expected if a single pause site is located near the center of the fragment (Fig. 9B). In corroboration, no pause signals could be detected in region II, which is devoid of an ARS. These results support the hypothesis that the *mcm10* mutation not only prevents initiation of DNA synthesis at replication origins but also impedes the progression of elongation forks through these replication origins.

DISCUSSION

The phenotype of the *mcm10* mutant suggests a DNA replication initiation defect. In eukaryotic cells, the initiation of DNA replication is orchestrated by a vast network of gene products. Many of the relevant genes have been identified through screens for cell division cycle (*cdc*) and minichromosome maintenance (*mcm*) mutants. Additionally, the biochemical isolation of the ORC has added to the repertoire of factors known to play a part in the initiation process. However, our understanding of how DNA replication initiates remains incomplete and must be broadened by identifying additional gene products required for the process. To this end, we have characterized the *mcm10* mutant. The data presented in this paper implicate the *MCM10* gene product in initiating DNA replication, possibly in conjunction with the MCM2-7 proteins or their protein complexes.

The *mcm10* mutant arrest phenotype is remarkably similar to that of several known initiation mutants. While the *mcm10* mutant grows normally at 23°C, it arrests following 4 h of incubation at 37°C with a distinct, uniform morphology (Fig. 2). Cells arrest premitotically, with a large bud and a single nucleus. The significance of the elongation of the bud is not clear. Temperature-sensitive strains containing lesions in either Mcm3, Cdc46/Mcm5, or Cdc47/Mcm7 arrest with a dumbbell morphology similar to that of the arrested *mcm10* strain (21, 42), as does a strain depleted of Mcm2 (54). Examination of the nuclear DNA content in the arrested *mcm10* strain shows approximately 2C DNA and indicates that prior to arresting in the cell cycle, the *mcm10* mutant may replicate nearly all of its DNA at the restrictive temperature. This nearly 2C DNA content is again similar to the results obtained with other initiation mutants. In these mutants, DNA replication initiating from a subset of the chromosomal origins is apparently sufficient to replicate the majority of the genomic DNA prior to arrest. Other studies have also suggested that the

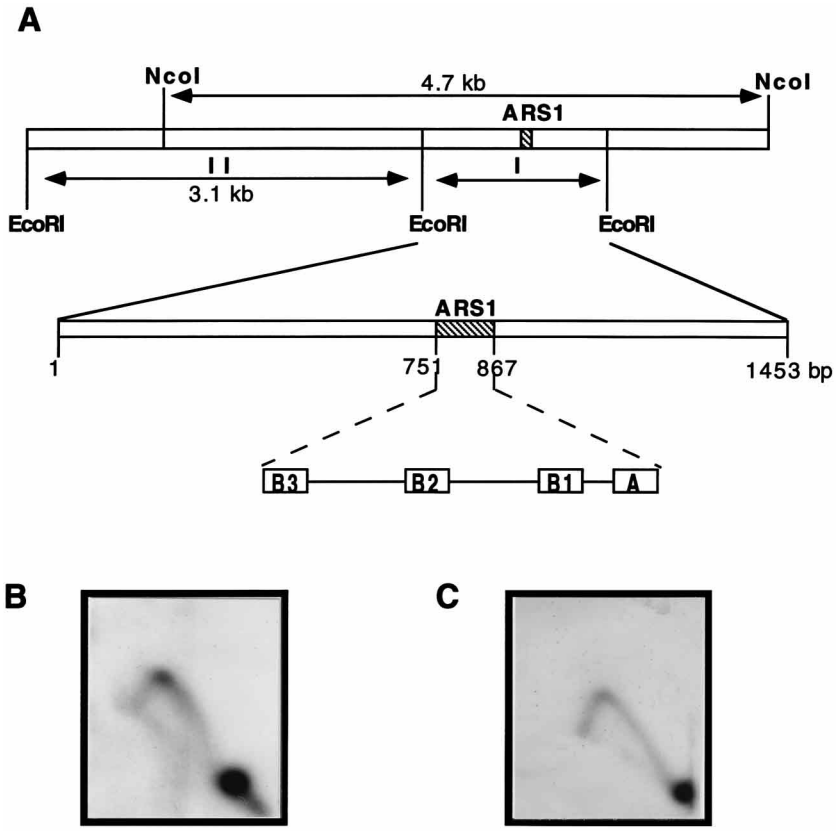

FIG. 9. Mapping of pause sites in the region near ARS1 on the chromosome. (A) Map of relevant restriction sites in the region near ARS1. Arrows indicate the restriction fragments that were analyzed by 2D gels in Fig. 8C and D (*Nco*I fragment) and in panels B (1.4-kb *Eco*RI fragment I) and C (3.1-kb *Eco*RI fragment II). Boxes A, B1, B2, and B3 represent the elements of a functional ARS1. (B and C) 2D gels of *Eco*RI-restricted genomic DNA probed with fragments I and II, respectively. Replication intermediates were enriched by benzoylated naphthoylated DEAE-cellulose chromatography (14).

entire yeast genome is replicated by initiation at only a fraction of all origins (10).

The minichromosome maintenance assay reveals that at 30°C the *mcm10* mutant perturbs minichromosome maintenance in an ARS-specific manner (Fig. 1). The plasmid containing ARS121 is the most stable, followed by that containing ARS120. The other plasmids tested, containing ARS1, ARS131, ARSHO, ARSH2B, or ARSHML, are consistently less stable. Based upon the idea that plasmid ARS activity reflects chromosomal origin activity (11, 55) and on 2D gel data (discussed below), we infer that the *mcm10* mutant uses only a subset of chromosomal replication origins in vivo, resulting in a prolonged S phase and thus an increase in the length of the cell cycle in the *mcm10* mutant at 30°C.

The Mcm10 protein interacts with several members of the MCM2-7 family. Initiation of DNA synthesis involves the interaction of a large number of proteins, many of which exist as subunits of large complexes. The ORC, a protein complex of six subunits, is believed to be constitutively bound to replication origins in *S. cerevisiae* (2), and the MCM2-7 proteins are believed to mediate the transition of replication origins from an inactive prereplicative state to an active, replicative state (13). While the MCM2-7 proteins can form large complexes, it is unclear whether these large complexes are functional and whether they contain all six members of the MCM2-7 proteins and/or other proteins (35). Additional proteins may interact with the MCM2-7 proteins to induce the replicative state at replication origins. As reported in this paper, Mcm10 interacts with multiple members of the MCM2-7 family in the two-hybrid system and in affinity chromatography (Fig. 6). Indirect immunofluorescence shows that Mcm10 is a nuclear protein (Fig. 5). Thus, it is likely that Mcm10 interacts with members or complexes of the MCM2-7 proteins in the nucleus to regulate the initiation of DNA synthesis at replication origins.

Mcm10 is required for the initiation of DNA replication at chromosomal replication origins. The results described above provide strong evidence that Mcm10 has a role in initiating DNA replication. We therefore used 2D gel analysis to determine directly whether the *mcm10* mutant is defective in the initiation of chromosomal DNA replication. Indeed, at ORI121 and at ORI1, the frequency of initiation events is reduced significantly in the mutant compared to that in the wild-type (Fig. 7). Furthermore, the *mcm10-1* mutation exerts a more dramatic effect on initiation at ORI1 than on ORI121. Given the dramatic reduction in initiation frequencies at the two origins examined in the *mcm10* mutant, we conclude that initiation of DNA synthesis at replication origins requires the *MCM10* gene product. In sharp contrast to the *abf1* mutants (47), *mcm10* affects the activities of the same set of ARSs in a hierarchy similar to that observed in the *mcm2*, *mcm3* (55), and *mcm5* (7) mutants or when Mcm2 is limiting (35). The similar ARS specificities exhibited by the *mcm* mutants and the physical interactions between Mcm10 and members of the MCM2-7 proteins support the hypothesis that Mcm2-7 and Mcm10 function in the same pathway in regulating origin usage in the initiation of DNA replication. Finally, because the

MCM10 gene product is essential (49), we speculate that the essential function of this protein lies in initiating DNA replication.

The *mcm10-1* mutation causes pausing of DNA replication at or near the ARS. In vivo analysis of DNA replication initiation in the *mcm10* mutant revealed a decrease in the frequency of initiation events at the two origins examined, similar to that observed for other initiation mutants. However, we observed a striking difference in the 2D gel patterns derived from the *mcm10* mutant strain. Unlike other mutants examined in other studies (19, 36, 55), the *mcm10-1* lesion induces distinct pause signals in the elongation arcs of both chromosome segments examined (Fig. 8). Several lines of evidence suggest that these pause signals are the result of the pausing of replication forks from opposite directions at or near the ARS during elongation through ORI1 and ORI121. The presence of two pause signals and the spacing of the pair of signals in each of the two origin fragments studied support this interpretation. More convincingly, we have shown that separation of the two pause signals near ORI1 on 2D gels can be manipulated by changing the relative position of ARS1 within the DNA fragment (Fig. 9). Furthermore, we have mapped the pause signals in the ORI1 region within a 300-bp sequence that includes ARS1. Assuming that the recessive *mcm10-1* mutation results in a loss of Mcm10 function, the pausing suggests that Mcm10 must be present to allow unimpeded progression of DNA replication forks through the ARS-containing regions of the chromosomes.

What might be the role of Mcm10 in the initiation of DNA replication? We envision that Mcm10 may mediate the initiation of DNA synthesis at replication origins through interaction with the MCM2-7 proteins. The loss of Mcm10 function not only prevents replication initiation at chromosomal origins but also presents a block to replication fork movement through the same regions. This block could result from the prolonged binding of the replication initiation complex which is normally discharged from replication origins upon activation of initiation. In this hypothesis, the failure to initiate DNA synthesis at replication origins in the *mcm10* mutant could also be the reason for the blockage of elongation fork movement in those regions. Elucidation of the function of Mcm10 awaits identification of the DNA sequences where the replication fork pauses and the gene products associated with the pause sites in the *mcm10* mutant.

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