MOLECULAR AND CELLULAR BIOLOGY, Sept. 1996, p. 5081–5090 Vol. 16, No. 9
0270-7306/96/$04.00+0

Physical Interactions among Mcm Proteins and Effects of Mcm Dosage on DNA Replication in *Saccharomyces cerevisiae*

MING LEI, YASUO KAWASAKI, AND BIK K. TYE*

Section of Biochemistry, Molecular and Cell Biology, Cornell University, Ithaca, New York 14853

Received 21 February 1996/Returned for modification 22 April 1996/Accepted 25 June 1996

Mcm2, Mcm3, and Mcm5/Cdc46 are conserved proteins essential for the initiation of DNA synthesis at replication origins in *Saccharomyces cerevisiae*. The accumulation of these proteins in the nucleus before the onset of DNA synthesis suggests that they play a role in restricting DNA synthesis to once per cell cycle. In this work, we show that Mcm2, Mcm3, and Mcm5 self-interact and interact with one another to form complexes. Mcm2 and Mcm3 are abundant proteins, present in approximately 4×10^4 and 2×10^5 copies per cell, respectively. Reducing the dosage of Mcm2 by half results in diminished usage of specific replication origins. These results together suggest that a significant molar excess of Mcm proteins relative to replication origins is required for the proper initiation of all replication origins.

Eukaryotic chromosomes are replicated exactly once in each cell cycle. Replication of these large chromosomes is achieved by the initiation of DNA synthesis at multiple origins. Initiation of DNA synthesis at these replication origins is under both cell cycle and developmental regulation (18). In each cell cycle, DNA synthesis can occur only during S phase, and initiation of DNA synthesis at the large number of replication origins follows a strict temporal order (15, 37, 41). In developing organisms such as *Drosophila melanogaster*, depending on the stage of development, cell divisions may vary significantly in duration. Under these vastly different circumstances, the rate of DNA synthesis is regulated by the density of initiation events on each chromosome (4). The precise mechanism that ensures that each chromosome is replicated only once per cell cycle is unknown. However, any model that attempts to explain the regulation of the initiation of DNA synthesis in eukaryotes needs to address two important features, i.e., selective origin usage (31) and restriction of DNA synthesis to once per cell cycle (3, 36).

The Mcm2-3-5 proteins are a family of conserved proteins essential for the initiation of DNA synthesis at replication origins in *Saccharomyces cerevisiae*. The properties of these proteins suggest that they play roles in determining origin usage (31, 46) and in restricting DNA synthesis to once per cell cycle (19, 47). These proteins, originally identified in *S. cerevisiae*, are ubiquitous in eukaryotes (11, 23, 44). There are currently six known members in the Mcm2-3-5 protein family in *S. cerevisiae* (12, 17, 20, 22a, 45, 46). Mcm2, Mcm3, and Mcm5/Cdc46 are the best studied among the known members. Conditional mutants defective in *MCM2*, *MCM3*, or *MCM5*/*CDC46* have been described previously (17, 20, 46). Under semipermissive conditions, the usage of all replication origins tested is diminished in the *mcm* mutants (31, 38a), suggesting that each of the Mcm proteins is required for the initiation at most if not all replication origins. Under permissive conditions, these *mcm* mutations dramatically reduced the usage of selected replication origins. This effect could be seen both for autonomously replicating sequences (ARSs) on plasmids (9, 46) and for the same ARSs in their native chromosomal locations (47). This origin-specific minichromosome maintenance defect (Mcm^-) of the *mcm* mutants suggests that Mcm proteins play a regulatory role directed at the initiation of DNA synthesis at individual replication origins that are inherently different.

Mcm proteins are synchronously localized in the nucleus between late M phase and the beginning of S phase (12, 19, 47). A fraction of the nuclear Mcm proteins appeared to be tightly associated with chromatin (47). The accumulation of the Mcm proteins in the nucleus before the onset of S phase and their abrupt disappearance from the nucleus at the beginning of S phase suggest that they play a crucial role in restricting DNA replication to once per cell cycle (44).

Recently, the essential roles of Mcm homologs in DNA replication have been confirmed both in vitro and in vivo in multicellular organisms. Using in vitro-assembled nuclei in *Xenopus* egg extracts, three groups independently showed that XMcm3 is an essential component of the DNA replication activity which is restricted to one round of synthesis (10, 27, 30). Immunodepletion of XMcm3 from extracts resulted in a loss of DNA replication activity. Replenishing the extracts with the immunoprecipitate restored activity. Interestingly, the immunoprecipitate also sedimented two other members of the Mcm protein family, suggesting that this essential component for DNA replication is in fact a complex of Mcm proteins. Insertion mutations that inactivated *MCM* genes have been identified by gene trap mutagenesis in *Arabidopsis* (39) and *Drosophila* (43) species. In both cases, the *MCM* genes were shown to be expressed in proliferating cells and essential for early development. Other phenotypes of these mutants, such as prolongation of S phase in proliferating cells, are consistent with a role for the plant and insect *MCM* genes in DNA replication.

The six members of the yeast Mcm protein family, ranging in size from 775 to 1,017 amino acids, share three conserved regions (12, 22a, 44, 45). The largest conserved region, domain II (see Fig. 1), contains the putative ATPase consensus motif (25). Despite their structural and functional similarities, each of these proteins is essential for viability (17, 20, 46), suggesting that they act independently in a common pathway or interact with each other to carry out an essential function. The possibility that these proteins function as a complex in *S. cerevisiae* was suggested by the specificity of the different *mcm* mutations for the same ARSs in their minichromosome maintenance defect (31). This idea is reinforced by allele-specific suppressor mutations identified within members of this gene family (20).

* Corresponding author. Mailing address: 325 Biotechnology Bldg., Cornell University, Ithaca, NY 14853-2703.

TABLE 1. Plasmids and yeast strains used in this study

Plasmid or strain	Description or genotype	Source or reference
Plasmids		
BTM.116	2μm *TRP1 lexA*(1-202)	S. Fields
BTM.MCM2(1-889)	2μm *TRP1 lexA-MCM2*(1-889)	This study
BTM.MCM2(1-705)	2μm *TRP1 lexA-MCM2*(1-707)	This study
BTM.MCM2(499-705)	2μm *TRP1 lexA-MCM2*(499-705)	This study
BTM.MCM3(1-917)	2μm *TRP1 lexA-MCM3*(1-917)	This study
BTM.MCM3(1-781)	2μm *TRP1 lexA-MCM3*(1-781)	This study
BTM.MCM3(106-971)	2μm *TRP1 lexA-MCM3*(106-971)	This study
BTM.MCM3(365-568)	2μm *TRP1 lexA-MCM3*(365-568)	This study
BTM.MCM5(1-775)	2μm *TRP1 lexA-MCM5*(1-775)	This study
BTM.MCM5(1-446)	2μm *TRP1 lexA-MCM3*(1-446)	This study
BTM.MCM5(1-309)	2μm *TRP1 lexA-MCM3*(1-309)	This study
BTM.MCM5(481-775)	2μm *TRP1 lexA-MCM3*(481-775)	This study
GAD.2F	2μm *LEU2 GAL4*(768-881)	S. Fields
GAD.MCM2(1-889)	2μm *LEU2 GAL4*(768-881)-*MCM2*(1-889)	This study
GAD.MCM3(1-971)	2μm *LEU2 GAL4*(768-881)-*MCM3*(1-971)	This study
GAD.MCM5(1-775)	2μm *LEU2 GAL4*(768-881)-*MCM5*(1-775)	This study
SH18-34	2μm *URA3 GAL1* (*lexAop*)-*lacZ*	S. Hanes and R. Brent
EG(KT)	2μm *URA3 GAL1*-GST	D. Mitchell
EG(KT)MCM2	2μm *URA3 GAL1*-GST-*MCM2*	This study
EG(KT)MCM3	2μm *URA3 GAL1*-GST-*MCM3*	This study
YCp101	*LEU2* ARS1 *CEN5*	This laboratory
YES3	*URA3* ARS1 *CEN5*	This laboratory
YCpH2B	*URA3 LEU2* ARSH2B *CEN5*	This laboratory
YCpHO	*URA3 LEU2* ARSHO *CEN5*	This laboratory
YCp121	*LEU2* ARS121 *CEN5*	This laboratory
Strains		
EGY40	α *ura3 trp1 leu2 his3*	E. Golemis and R. Brent
8534-8c	α *ura3-52 leu2-3,112 his4Δ34*	G. Fink
BJ2168	**a** *pep4-3 prc1-407 prb-1122 ura3-52 trp1 leu2*	E. Jones
CB001	**a** *leu2 ura3 trp1 prb pep4*::*URA3*	A. Sugino
8534-10A	**a** *ura3-52 leu2-3,112 his4Δ34*	17
RY71A	α *ura3-52 leu2-3,112 his3Δ200 lys2-801Δtrp1 ade2-101*	
8534-10A mcm2::Tn3	**a** *ura3-52 leu2-3,112 his4Δ34 mcm2*::Tn*3*(*URA3*)	46
RY71A	α *ura3-52 leu2-3,112 his3Δ200 lys2-801Δtrp1 ade2-101*	
8534-10A mcm3::Tn3	**a** *ura3-52 leu2-3,112 his4Δ34 mcm3*::Tn*3*(*LEU2*)	17
RY71A	α *ura3-52 leu2-3,112 his3Δ200 lys2-801Δtrp1 ade2-101*	

In this paper, we used four independent methods to investigate the physical interactions between Mcm2, Mcm3, and Mcm5/Cdc46 in *S. cerevisiae.*

MATERIALS AND METHODS

Plasmids and strains. Plasmids and strains used in this study are shown in Table 1. Plasmids were constructed by standard techniques. When PCR-amplified DNA fragments were used, the restriction sites generated at both ends of the PCR fragment were immediately adjacent to the sequence that was amplified. For pBTM116.MCM2(1-889), the coding sequence for the N terminus of Mcm2(1-514) was amplified by PCR as a *Bam*HI-*Pst*I fragment and cloned into pBTM.116 at the *Bam*HI-*Pst*I sites and the coding sequence for the C terminus of Mcm2(515-889) was isolated from PUC8.MCM2 (45a) as a *Pst*I fragment and cloned into pBTM116.MCM2(1-514) at the *Pst*I site. For pBTM116.MCM2(1-705), the coding sequence for this portion of *MCM2* was isolated as a *Bam*HI-*Bgl*II fragment from pBTM116.MCM2(1-889) and cloned into the vector at the *Bam*HI site. For pBTM116.MCM2(499-705), the coding sequence for this portion of *MCM2* was amplified by PCR as a *Bgl*II fragment and cloned into the vector at the *Bam*HI site. For pBTM116.MCM3(1-971), the entire coding region of the *MCM3* gene was isolated as a *Stu*I-*Sal*I fragment from YIP5.MCM3 (17) and cloned into the vector at the *Bam*HI (blunt ended by T4 DNA polymerase)-*Sal*I site. For pBTM116.MCM3(1-781), the coding sequence for this portion of *MCM3* was isolated as a *Bam*HI fragment from pBTM116.MCM3(1-971) and cloned into the vector at the *Bam*HI site. For pBTM116.MCM3(106-971), the *Eco*RI fragment was excised from pBTM116.MCM3(1-971) and the remainder of the plasmid was religated. For pBTM116.MCM3(365-568), the coding sequence for this portion of *MCM3* was amplified by PCR as a *Bam*HI fragment and cloned into the vector at the *Bam*HI sites. For pBTM116.MCM5(1-775), the entire coding region of the *MCM5* gene was amplified by PCR as a *Bgl*II fragment and cloned into the vector at the *Bam*HI site. For pBTM116.MCM5(1-446), the coding sequence for this portion of *MCM5* was amplified by PCR as a *Sma*I-*Pst*I fragment and cloned into the vector at the *Sma*I-*Pst*I sites. For pBTM116.MCM5(1-309), the 1.1-kb *Bam*HI fragment was excised from pBTM116.MCM5(1-775) and the remainder of the plasmid was religated; this construct will produce an additional Arg following amino acid 775. For pBTM116.MCM5(481-775), the *Eco*RI fragment was excised from pBTM116.MCM5(1-775) and the remainder of the plasmid was religated. For pGAD2F.MCM2(1-889), the entire coding region of the *MCM2* gene was amplified by PCR as a *Bam*HI fragment and cloned into pGAD2F (16) at the *Bam*HI site. For pGAD2F.MCM3(1-971), the coding sequence for the C terminus of Mcm3(782-971) was amplified by PCR as a *Bam*HI-*Bgl*II fragment and cloned into the vector at the *Bam*HI site to yield pGAD2F.MCM3B3′; then the coding region for the N terminus of Mcm3(1-781) was isolated from pBTM116.MCM3(1-971) as a *Bam*HI fragment and cloned into pGAD2F.MCM3B3′ at the *Bam*HI site. For pGAD2F.MCM5(1-775), the entire coding region of the *MCM5* gene was amplified by PCR as a *Bgl*II fragment and cloned into the vector at the *Bam*HI site. For pEG(KT)-MCM2, the coding sequence for the N terminus of Mcm2(1-319) was amplified by PCR as an *Xba*I-*Mlu*I fragment and the coding sequence for the C terminus of Mcm2(320-889) was isolated as an *Mlu*I-*Sal*I fragment from pM46-33 (46). These two fragments were ligated and cloned into pEG(KT) at the *Xba*I-*Sal*I site. For pEG(KT)-MCM3, coding sequence for the N terminus of Mcm3(1-106) was PCR amplified as an *Xba*I-*Eco*RI fragment, the coding sequence for the C terminus of Mcm3(107-971) was isolated as an *Eco*RI-*Mlu*I fragment from R61-1 (17), and these two fragments were ligated and cloned into the vector at the *Xba*I-*Sal*I (blunt-ended) site.

Immunoblotting. Immunoblotting was performed using standard techniques. The primary antibodies used were polyclonal rabbit antisera. Secondary goat anti-rabbit horseradish peroxidase-conjugated antibodies were used for chemiluminescence detection.

Two-hybrid analysis. A DNA binding domain plasmid, an activation domain plasmid, and the reporter plasmid, pSH18-34 (kindly provided by S. Homes and R. Brent), were introduced into the yeast strain EGY40 by transformation. For

the colony color assay, transformants were patched on X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) plates and the color was scored after a 3-day incubation of the plates at 30°C. For the β-galactosidase activity assay (32), transformants were grown to saturation in liquid selective medium at 30°C and then diluted 1:50 using the same medium and grown to an optical density at 600 nm (OD_{600}) of ~1. One milliliter of the culture from each sample was used for the assay.

Coimmunoprecipitation of LexA-Mcm fusion proteins with Mcm proteins. Yeast cells (EGY40 transformed with BTM116, BTM.MCM2, BTM.MCM3, or BTM.MCM5) were harvested from 50-ml cultures at an OD_{600} of ~1 and resuspended in 600 μl of ice-cold buffer A (Na_2HPO_4-NaH_2PO_4 [pH 7.0], 50 mM; NaCl, 100 mM; glycerol, 10%; phenylmethylsulfonyl fluoride, 1 mM; leupeptin, 0.5 μg/ml; pepstatin, 0.7 μg/ml). The cells were then homogenized by glass beads, and 450 μl of soluble proteins was recovered. Five microliters of anti-LexA serum was added to 400 μl of the soluble protein extract, and the mixture was incubated on ice for 1.5 h. Protein A–Sepharose CL-4B (Sigma; 100 μl) equilibrated in buffer A at 100 mg/ml was added, and the mixture was incubated at 4°C with gentle shaking for 1.5 h. The mixture was then spun at 4°C for 20 s in a bench-top centrifuge. The pellet was washed three times by resuspending in 500 μl of ice-cold buffer A. The immunoprecipitate was finally resuspended in 100 μl of buffer A and boiled to release proteins from agarose beads. Then 10 μl each of the soluble proteins and the immunoprecipitate was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), blotted onto Immobilon-P filters (Millipore), and probed with anti-LexA antisera or antibodies specific to Mcm2, Mcm3, or actin. Ten percent gels were used for the analysis of LexA and actin. Six percent gels were used for the analysis of Mcm2, Mcm3, and LexA-Mcm fusion proteins.

GST fusion protein purification and affinity column chromatography. Glutathione *S*-transferase (GST)–Mcm2 or GST-Mcm3 was overexpressed in yeast strains carrying pEG(KT)MCM2 or pEG(KT)MCM3. Harvested yeast cells were homogenized by glass beads in buffer B (5% glycerol, 50 mM Tris-HCl [pH 7.5], 0.1% Triton X-100, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride) containing 0.5 M NaCl. The soluble protein extracts were diluted 1:4 with buffer B to a final NaCl concentration of 0.1 M and then incubated with glutathione-Sepharose 4B (Pharmacia) in suspension at 4°C for 2 h. The resin was washed extensively with buffer B containing 0.1 M NaCl. For the purification of Mcm proteins, GST fusion proteins were released with buffer B–10 mM glutathione. About 100 μg of each of the GST fusion proteins (>90% pure) was obtained from 1 liter of culture.

For affinity column chromatography, the buffer B-washed resin was packed in 0.5-ml columns. Yeast protein extract prepared from CB001 was applied to these columns. The columns were washed with 10 ml of buffer B–0.1 M NaCl, and 500-μl fractions were collected. The last two fractions of this elution were analyzed (see Fig. 3, lanes 2 and 3). Ten milliliters of buffer B–0.5 M NaCl was used to elute proteins from the column, and 500-μl fractions were collected. The first two fractions of this elution were analyzed (see Fig. 3, lanes 4 and 5). GST-Mcm2 or GST-Mcm3 was eluted in buffer B–10 mM glutathione.

Glycerol gradient sedimentation and phosphatase treatment of peak fractions. Soluble proteins from BJ2168 were extracted as described for coimmunoprecipitation experiments. Protein extracts (250 μl) were loaded on top of a 10.5-ml 20 to 60% glycerol gradient in buffer A (in Beckman tube 331372). The gradient was centrifuged in an SW41Ti rotor at 40,000 rpm for 40 h at 4°C and then collected from the top into 43 fractions of 250 μl each. The pellet on the bottom of the tube was resuspended in 250 μl of buffer A. Ten microliters of each sample was mixed with 2× sample buffer and analyzed by SDS-PAGE in an 8% gel. Protein size markers were analyzed in parallel in a separate tube. Sedimentation coefficients were determined as follows: $\Delta(\ln r_b)/\omega^2\Delta t$, where r_b is the distance from the midpoints of the protein bands (peaks) to the axis of rotation (in centimeters); ω is the angular velocity, defined as $2\pi \times$ revolutions per second; and Δt is the duration of centrifugation (in seconds). For the phosphatase treatment, the pooled fractions were dialyzed against buffer A. Samples (10 μl) were treated with 100 U of lambda protein phosphatase (NEB) at 30°C for 30 min.

Quantitation of cellular concentrations of Mcm2 and Mcm3. Yeast strains 8534-8C and CB001 were grown at 30°C in yeast extract-peptone-dextrose (YEPD) to early log phase (OD_{600} = 1). Cell densities in the cultures were determined by counting with the hemocytometer. Known numbers of cells were harvested and disrupted by glass beads in buffer B containing 0.5 M NaCl and 1% SDS until more than 90% cells were lysed. Soluble proteins from each sample were recovered. Purified GST-Mcm2 and GST-Mcm3 and the protein extracts were analyzed by SDS–6% PAGE, blotted onto Immobilon-P filters, and probed with Mcm2- or Mcm3-specific antibodies.

Quantitation of the relative concentrations of Mcm2 and Mcm3 in diploid strains. Wild-type and heterozygous mcm/null diploid strains growing in YEPD medium at 30°C were harvested at early log phase (OD_{600} = 1). Soluble cellular proteins were extracted as described above from the same number of cells of each of these strains. The protein extracts were analyzed by SDS-PAGE in 8% gels. Two identical protein blots were prepared. One was probed with Mcm2-specific antibodies, and the other was probed with Mcm3-specific antibodies (46). Both filters were then probed with β-tubulin-specific antibodies. The intensities of Mcm2, Mcm3, and β-tubulin cross-reacting signals were quantitated by densitometric analysis. The relative amounts of Mcm2 and Mcm3 in each strain were normalized against that of β-tubulin in the same strain.

Mitotic plasmid loss rates. The mitotic plasmid loss rates were determined by a modified version of the procedure described by Gibson et al. (17). Yeast transformants were streaked onto selective plates to obtain fresh and well-separated colonies. A single colony from the plate was transferred to 1 ml of YEPD medium in a 1.5-ml microcentrifuge tube and thoroughly resuspended by vortexing for 20 s. Dilutions of this resuspension were plated onto YEPD agar plates. After approximately 14 generations of growth at 30°C, all cells in a single colony were transferred from the YEPD plate to 1 ml of YEPD liquid medium in a 1.5-ml microcentrifuge tube by using the sharp end of a sterilized Pasteur pipette. The pipette was rinsed several times to make sure that no yeast cells were retained in the pipette. The transferred colony was thoroughly resuspended in the medium by vortexing for 20 s. Dilutions of this resuspension were plated onto YEPD and selective plates. The fraction of cells containing the plasmid (F) was the number of colonies on a selective plate divided by the number of colonies on a YEPD plate. The plasmid loss rate per cell division was determined by $1 - F^{1/N}$, where N is the number of generations. For each transformant, at least three colonies from the YEPD plate were analyzed independently. Details of this procedure will be published elsewhere.

RESULTS

Mcm2, Mcm3, and Mcm5 interactions by two-hybrid analysis. To investigate the interactions between Mcm2, Mcm3, and Mcm5, we first used the two-hybrid system (16). Plasmids encoding the Mcm2, -3, or -5 protein fused to LexA or to the Gal4 activation domain were constructed (Fig. 1). Each of the full-length Mcm2, Mcm3, and Mcm5 fusion proteins functionally complemented the null or conditional alleles of the corresponding *MCM* genes (data not shown). In contrast, none of the truncated fusion proteins complemented the null or conditional alleles, although stable fusion proteins were produced in *S. cerevisiae* in each case (data not shown). Several inferences could be made from the two-hybrid studies (Fig. 1). (i) All pairwise (homo or hetero) combinations of full-length Mcm proteins interacted, but to different extents. (ii) The pair that had the strongest interaction was Mcm3 and Mcm5. C-terminal deletions in Mcm3 [BTM.MCM3(1-781)] strengthened this interaction, suggesting that conformational changes modulated their interactions. Differences in β-galactosidase activities were consistently observed between certain pairs in reverse combinations (e.g., Mcm3 versus Mcm5 or Mcm3 versus Mcm2), presumably because of differences in presentation of the interacting surfaces in the fusion proteins. (iii) The conserved domain II alone was not sufficient to foster interactions between Mcm2, Mcm3, and Mcm5 [e.g., BTM.MCM2 (409-705) and BTM.MCM3(365-568)]. These results suggest that each Mcm protein has the capacity to interact with multiple members of the family and that contact points between specific pairs of Mcm proteins are nonequivalent. However, because extensive deletions invariably gave negative results in this experiment (data not shown), we were unable to define the domain of interaction between each of the pairs to a small region.

Coimmunoprecipitation of Mcm2, Mcm3, and Mcm5. If Mcm2, Mcm3, and Mcm5 interact with one another as suggested by the two-hybrid studies, immunoprecipitation of one of the proteins should sediment a complex containing the others. Soluble cellular proteins extracted from the yeast strain EGY40 expressing either LexA or LexA-Mcm fusion proteins were subjected to immunoprecipitation using anti-LexA antiserum (Fig. 2). The presence of Mcm2 or Mcm3 in the precipitates was examined with affinity-purified antibodies specific to Mcm2 or Mcm3 (47). As a control, the presence of actin in the precipitates was also examined. Under the conditions that we used, when LexA was immunoprecipitated, neither Mcm2, Mcm3, nor actin could be detected in the immunoprecipitate. When LexA-Mcm2 was immunoprecipitated, Mcm3 was coprecipitated but actin was not. When LexA-Mcm3 or LexA-

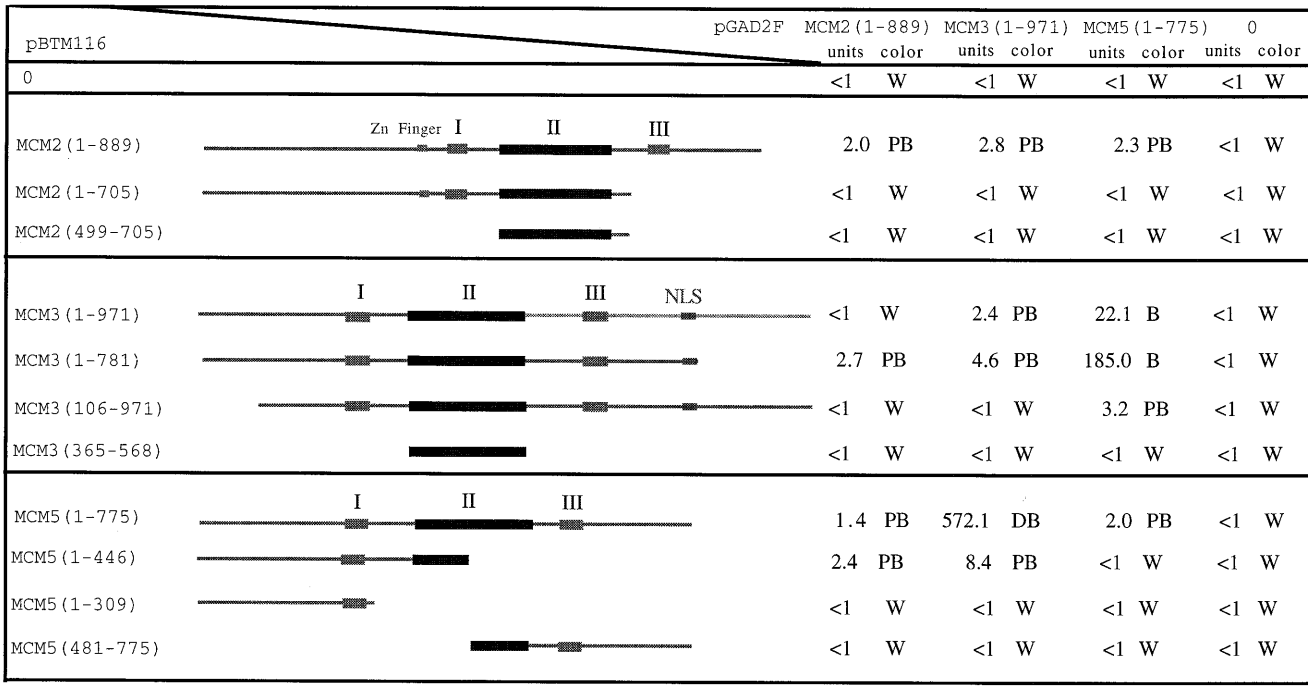

FIG. 1. Schematic representation of the plasmids used in the two-hybrid analysis and the physical interactions between Mcm2, Mcm3, and Mcm5/Cdc46 measured by the expression of the *lacZ* reporter gene. The horizontal line represents the coding region of *MCM2*, *MCM3*, and *MCM5/CDC46*. The three conserved domains (I, II, and III) and the nuclear localization sequence (NLS) in Mcm3 are represented by boxes as labelled. Numbers in parentheses indicate the portion of each Mcm protein in the fusion. Yeast transformants of EGY40 carrying each pair of the plasmids and the *lacZ* reporter gene were assayed for β-galactosidase activity both by colony color on X-Gal selective plates and in cell extracts using *ortho*-nitrophenyl-β-D-galactopyranoside. units, units of β-galactosidase activity (each entry represents the average value from two independent transformants); color, intensity of X-Gal staining (W, white; B, blue; PB, pale blue; DB, dark blue).

Mcm5 was immunoprecipitated, both Mcm2 and Mcm3 were coprecipitated but actin was not. These results further indicate that the Mcm2, Mcm3, and Mcm5 proteins can physically associate with each other. The additional bands detected by LexA and Mcm2 antibodies (third column, first and second rows) are probably due to protein degradation. Although Mcm2 self-interaction was detected in the two-hybrid system, we were unable to detect coprecipitation of Mcm2 with LexA-Mcm2.

Retention of Mcm2 and Mcm3 on GST-Mcm2 affinity columns. The self-interaction of Mcm2 and its interaction with Mcm3 were investigated further by GST-Mcm2 affinity chromatography. The GST-Mcm2 fusion protein, which functionally complemented an *mcm2* null mutation (data not shown), was bound to glutathione-Sepharose resin in a column. Yeast protein extracts were applied to the column, and the column was extensively washed with buffer containing 0.1 M NaCl (Fig. 3A and B, lanes 2 and 3). Mcm2 (Fig. 3A) and Mcm3 (Fig. 3B) retained in the column were completely dissociated by buffer containing 0.5 M NaCl (lanes 4 and 5). With antisera specific to actin (Fig. 3C) and DNA polymerase α (data not shown), the retention of these proteins in this column was also examined. Under the same conditions, neither protein was retained. These results indicate that both Mcm2 and Mcm3 specifically bind GST-Mcm2. Larger amounts of Mcm3 than Mcm2 were consistently retained by the GST-Mcm2 column. This observation, in corroboration with the results from coimmunoprecipitation experiments and two-hybrid analysis, may reflect the stoichiometry and/or differences in affinity of Mcm2 and Mcm3 in complexes formed on the column.

Affinity chromatography using GST-Mcm3 was also carried out under similar conditions. However, neither Mcm2 nor Mcm3 was retained by this column even though GST-Mcm3 complemented the *mcm3-1* mutation (data not shown). Perhaps the majority of GST-Mcm3, when overproduced in *S. cerevisiae*, does not have the appropriate modification for complex formation. This negative result, however, served as an additional control for the specific binding of Mcm2 and Mcm3 to the GST-Mcm2 affinity column. Retention of Mcm5 by affinity chromatography was not pursued because of the lack of Mcm5-monospecific antibodies.

The Mcm proteins form two size classes of complexes. Having shown that the Mcm proteins can interact, we used glycerol gradient sedimentation to investigate if these interactions are limited to dimeric complexes or if they also promote the formation of larger complexes. Soluble cellular proteins were subjected to centrifugation in a 20 to 60% glycerol gradient. The fractions collected were analyzed for the presence of Mcm2 and Mcm3 (Fig. 4A and B). Under the conditions that we used, Mcm2 was detected in two separate peaks, fractions 9 to 11 and 23 to 25 (Fig. 4A). Mcm3 was detected in two broad peaks, fractions 9 to 15 and 23 to 29 (Fig. 4B), that overlapped with the Mcm2 peaks. The first overlapping peak (peak I) had a sedimentation coefficient of about 0.7S, as expected for a globular protein of about 150 to 200 kDa in size. The second overlapping peak (peak II) had a sedimentation coefficient of about 1.4S to 1.5S, as expected for a globular protein of about 443 to 669 kDa in size. Mcm3 detected in fractions at or close to the bottom of the gradient appeared to be denatured protein aggregates. DNA did not appear to be a component of these complexes. Treating the protein extract with 200 U of DNase per ml (47) did not affect the distribution of Mcm2 or Mcm3 in the gradient (data not shown). Although Mcm2 and Mcm3 were the only two proteins probed in these complexes,

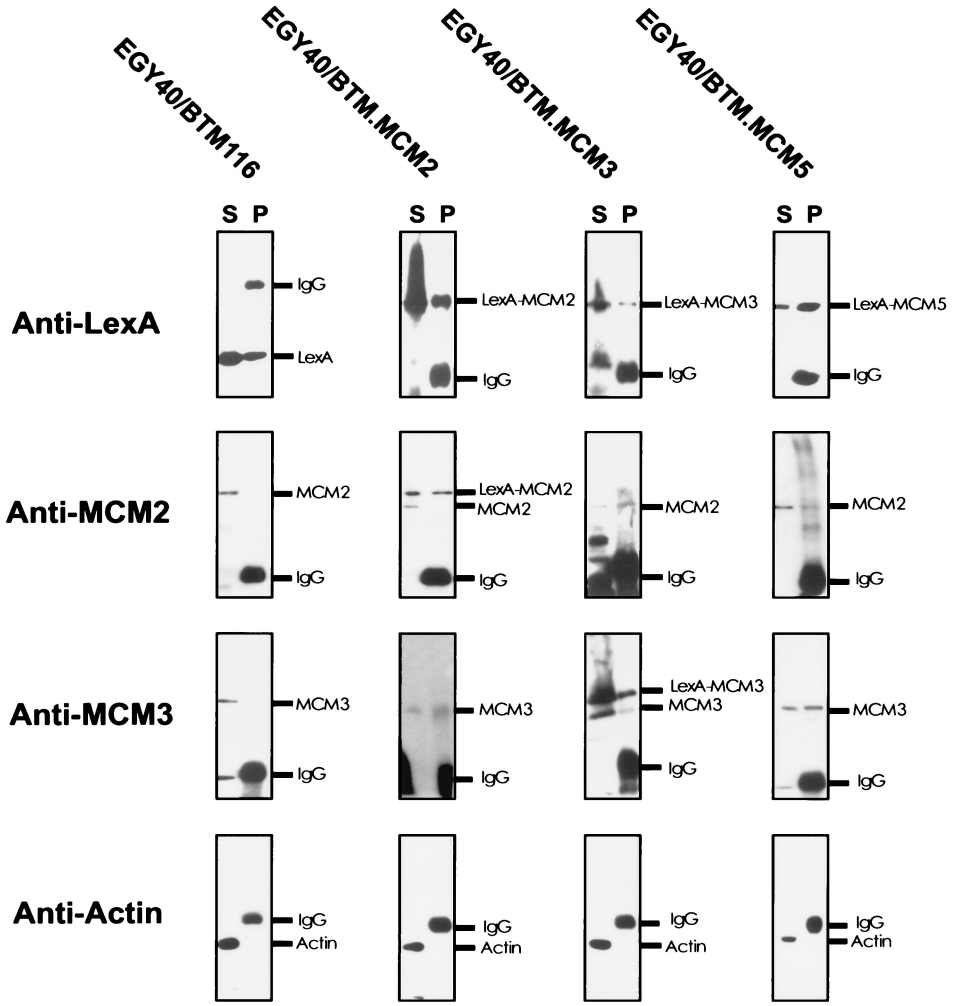

FIG. 2. Coimmunoprecipitation of Mcm2, Mcm3, and Mcm5/Cdc46. Proteins extracted from yeast strain EGY40 expressing LexA, LexA-Mcm2, LexA-Mcm3, or LexA-Mcm5 fusion proteins were precipitated by anti-LexA serum. The protein extracts and the precipitates were analyzed for the presence of LexA or LexA-Mcm fusion proteins (top row), Mcm2 (second row), Mcm3 (third row), or actin (bottom row). Lanes S, soluble protein extract; lanes P, immunoprecipitate. IgG, immunoglobulin G.

the high molecular mass suggested the presence of other proteins.

We noticed that Mcm3 from peak I and peak II had different mobilities in SDS-PAGE (Fig. 4B, lanes 9 to 15 and 23 to 29, and 4C, lanes 5 and 6). This difference was not observed in Mcm2 (Fig. 4C, lanes 1 to 4). To investigate if the dissimilar mobilities of the Mcm3 in the two peaks are due to differences in the phosphorylation states of the Mcm3 protein, we treated proteins from the peak fractions with lambda protein phosphatase. This treatment did not affect the mobility of Mcm3 from peak II (Fig. 4C, lanes 6 and 8). However, it changed the mobility of a fraction of Mcm3 from peak I to that of Mcm3 from peak II (Fig. 4C, lanes 5 and 7), suggesting that Mcm3 is hyperphosphorylated in the smaller complexes and hypophosphorylated in larger complexes.

Mcm2 and Mcm3 are abundant proteins, yet Mcm2 is limiting for DNA replication. Depending on the association-dissociation constants for multimolecular interactions, the assembly of multimeric complexes may require high subunit concentrations. Our finding that Mcm2 and Mcm3 are associated with large complexes led us to measure the intracellular concentrations of Mcm2 and Mcm3 in logarithmically growing yeast cells. Soluble proteins, which accounted for more than 90% of the Mcm proteins in yeast cells (48), were extracted from known numbers of cells and calibrated against known amounts of the purified GST-Mcm2 (Fig. 5A) and GST-Mcm3 proteins (Fig. 5B). This calculation gave an estimation of 6.6 fg, or 4×10^4 Mcm2 molecules, and 36 fg, or 2×10^5 Mcm3 molecules, per cell. Thus, the molar ratio of the cellular concentrations of Mcm3 to Mcm2 is approximately 5:1.

Replication initiation complexes are expected to be limiting in concentration to ensure that no excess complexes are available for reinitiation within the same cell cycle (3). The haploid yeast genome contains between 200 and 400 replication origins (8, 34). We showed that the intracellular concentrations of Mcm2 and Mcm3 are about 100 and 500 times that of their putative targets, respectively. To investigate if abundance equates with excesses of these proteins, we examined the effect of *MCM2* and *MCM3* gene dosage on the stability of minichromosomes. Isogenic diploids that contained a single or two copies of the functional *MCM2* or *MCM3* gene were used in this experiment.

To verify that the intracellular concentrations of Mcm2 and Mcm3 are proportional to the gene dose of *MCM2* and *MCM3*,

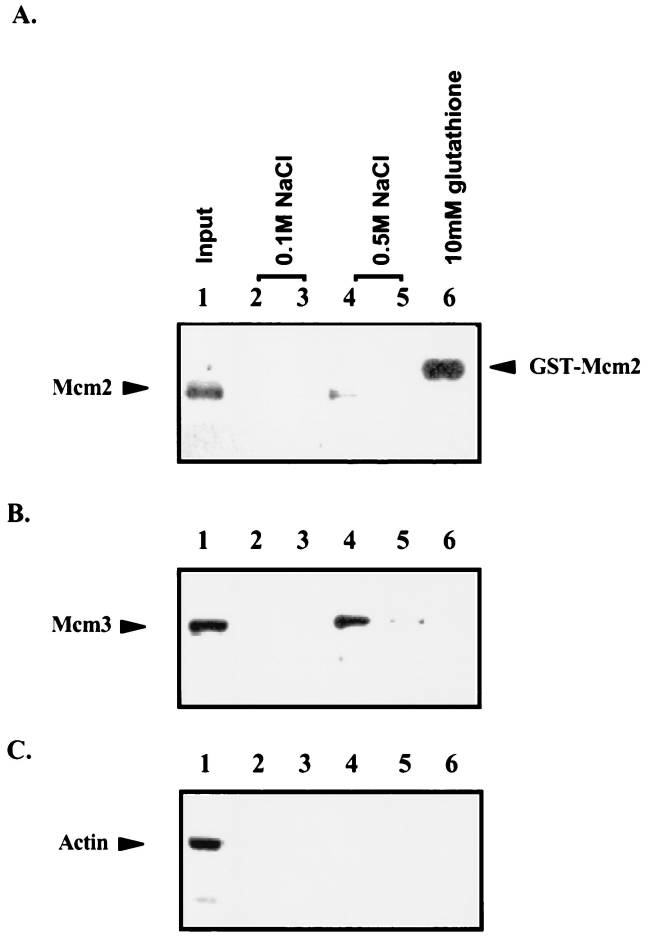

FIG. 3. GST-Mcm2 affinity column chromatography. Lanes 1, input soluble yeast proteins; lanes 2 and 3, 0.1 M NaCl eluate; lanes 4 and 5, 0.5 M NaCl eluate; lanes 6, 10 mM glutathione eluate. Western blots (immunoblots) were probed with antibodies against Mcm2 (A), Mcm3 (B), and actin (C).

soluble cellular proteins were extracted from a wild-type yeast strain and diploids heterozygous for a null allele of the *MCM2* or *MCM3* gene. These protein extracts were analyzed by SDS-PAGE followed by immunoblotting using antibodies specific to Mcm2 (Fig. 6A) or Mcm3 (Fig. 6B) and β-tubulin. The heterozygous *MCM2*/*mcm2*::Tn*3* diploid contains about 50% of the Mcm2 protein present in the wild-type diploid and the heterozygous *MCM3*/*mcm3*::Tn*3* diploid (Fig. 6A). Similarly, the heterozygous *MCM3*/*mcm3*::Tn*3* diploid contains about 50% of the Mcm3 protein present in the wild-type diploid and the heterozygous *MCM2*/*mcm2*::Tn*3* diploid (Fig. 6B). These results confirmed that the intracellular concentrations of the Mcm2 and Mcm3 proteins are proportional to the gene dose of *MCM2* and *MCM3* in each of the diploid strains. To examine if half the gene dose of *MCM2* or *MCM3* affected the normal growth of diploid strains, growth rates of each of the heterozygous diploids and their isogenic wild-type diploid were compared. We detected no difference in the rates of cell division of these strains in YEPD medium at 30°C (data not shown).

To investigate the effect of reducing the intracellular concentrations of Mcm2 and Mcm3 by 50% on the initiation of DNA synthesis at replication origins, we examined the stability of minichromosomes in these strains. The loss rates of several minichromosomes, each carrying a different ARS, ARS1, ARSH2B, ARSHO, or ARS121, were measured at 30°C (Table 2). The heterozygous *MCM3*/*mcm3*::Tn*3* strain had no effect on the stability of the three minichromosomes (pYES3, $YCpH_2B$, and YCpHO) examined, regardless of the ARS present on the minichromosome. In contrast, the *MCM2*/*mcm2*::Tn*3* diploid exerted dramatic effects on the stability of minichromosomes carrying ARS1 and ARSHO, a small effect on ARS121, but no effect on ARSH2B. The reduced stability of minichromosomes carrying ARS1 and ARSHO in the *MCM2*/*mcm2*::Tn*3* heterozygous diploid is not likely due to growth defects in the strain, since there was no measureable difference in growth rates between the heterozygous and the wild-type diploids. Because the insertion site of the Tn*3* transposon in the *mcm2*::Tn*3* allele is immediately 5′ proximal to the translational start codon of the *MCM2* open reading frame (46), no truncated Mcm2 protein was made from this allele. Therefore, the reduced stability could not be due to truncated nonfunctional Mcm2 interfering with the assembly of functional Mcm complexes but could be due to a reduced concentration of Mcm2 in the diploid cells. The fact that reduced activity was observed at some but not all ARSs suggests that this effect is due to defects in the initiation at origins rather than defects in later steps of DNA replication or chromosome segregation. These results indicate that Mcm2, but not Mcm3, is limiting for the initiation of DNA synthesis. However, at half the normal concentration, Mcm2 is limiting for the initiation at only some but not all ARSs.

DISCUSSION

DNA replication initiation in *S. cerevisiae*. The haploid genome of 16 chromosomes of *S. cerevisiae* is estimated to contain between 200 and 400 replication origins on the basis of the frequency of occurrences of ARSs in a yeast genomic library (8). ARSs are largely divergent sequences, all of which contain an 11-bp ARS consensus sequence (5). Initiation of DNA synthesis occurs at defined DNA sequences on chromosomes corresponding to ARSs (21). However, not all ARSs are active as replication origins in the chromosome (14). An origin recognition complex of six protein subunits constitutively and specifically binds the ARS consensus sequence (2, 13) of active as well as silent replication origins (29). Initiation events are likely to be mediated through interactions between regulatory proteins and the origin recognition complex. The Mcm proteins have been suggested to play a key role in the relay of initiation signals at the beginning of S phase to origin recognition complex-bound replication origins that are to be activated (28).

Mcm complexes. We have used four methods to show that members of the Mcm family of yeast replication proteins, Mcm2, Mcm3, and Mcm5, self-interact and interact with one another. Two-hybrid study, coimmunoprecipitation analysis, and affinity chromatography allowed detection of interactions between specific pairs of Mcm proteins. Sizing of native Mcm complexes by glycerol gradient sedimentation suggests that two distinct classes of complexes, consistent with dimers and multimers of Mcm proteins, are formed. Our study is consistent with those of amphibian (10, 27, 30) and mammalian (6, 33) cells in which homologs of Mcm proteins copurify or coprecipitate in serological reactions. The significance of the stronger interaction observed between Mcm3 and Mcm5 is unclear. Ordered assembly of large complexes often involves nucleating subunits that have a higher affinity for each other than for subsequent subunits (1, 26). It is also possible that the high affinity of Mcm3 for Mcm5 regulates the effective concentrations of Mcm3 and Mcm5 in the active pool of Mcm proteins for complex assembly. Finally, we cannot rule out the possibil-

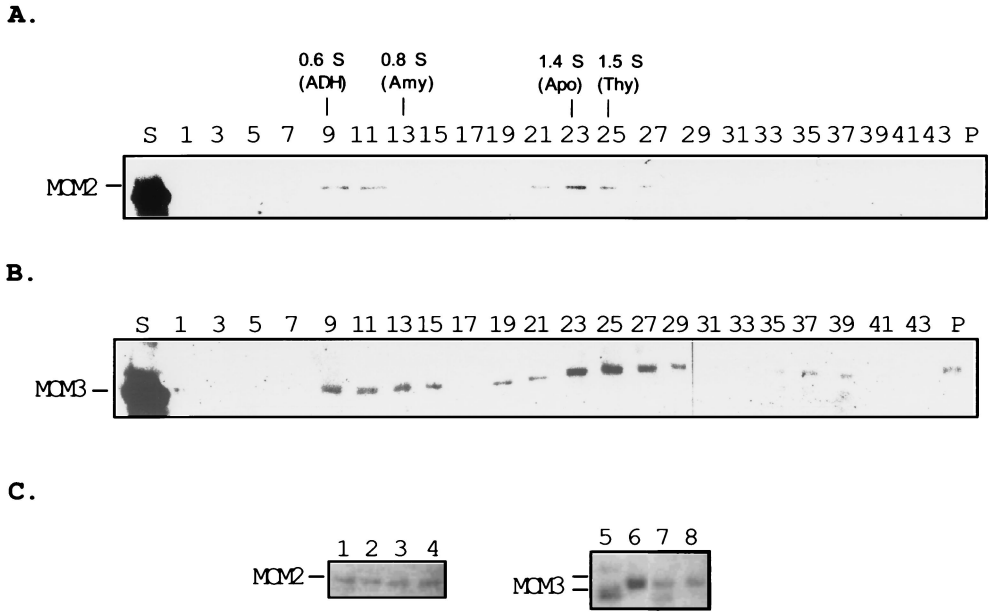

FIG. 4. Cosedimentation of Mcm2 and Mcm3 in glycerol gradient centrifugation. Input soluble proteins (lanes S), every other fraction, and resuspended pellet (lanes P) were analyzed. Fraction 1 corresponds to the top fraction, and fraction 43 corresponds to the bottom fraction of the gradient. (A and B) Distributions of Mcm2 and Mcm3 in the fractions analyzed by immunoblotting using Mcm2- or Mcm3-specific antibodies, respectively. Markers: ADH, alcohol dehydrogenase (150,000 Da); Amy, β-amylase (200,000 Da); Apo, apoferritin (443,000 Da); and Thy, thyroglobulin (669,000 Da). (C) Phosphoisoforms of Mcm2 and Mcm3 proteins associated with small and large complexes. Pooled fractions from peak I (fractions 9 to 11) and peak II (fractions 23 to 26) were analyzed by SDS-PAGE followed by immunoblotting using antibodies specific to Mcm2 (lanes 1 to 4) or Mcm3 (lanes 5 to 8). Lanes 1 and 5, peak I; lanes 2 and 6, peak II; lanes 3 and 7, peak I treated with phosphatase; lanes 4 and 8, peak II treated with phosphatase.

ity that Mcm3 and Mcm5 form complexes that serve specific functions not carried out by other complexes.

We are unable to distinguish whether the Mcm proteins interact to form a single multimeric complex or multiple distinct multimeric complexes, each differing in composition and/or stoichiometry of the subunits involved. The observation that Mcm2 and Mcm3 sedimented in glycerol gradients as large complexes that have overlapping but noncoincidental peaks leaves room for speculation (Fig. 4). There is genetic evidence for the Mcm proteins carrying out essential functions as heteromeric complexes in vivo. The allele-specific suppression of the cold sensitivity of *cdc54* by a heat-sensitive allele of *cdc46* (20) is a classic genetic demonstration of a restoration of function by conformational changes of interacting proteins (22). The observation that *mcm2* (31), *mcm3* (17, 46), and *cdc46* (9) mutants exert their most dramatic effects on the activity of a similar subset of ARSs suggests that they act in unison, most likely as subunits of a complex at these replication origins.

Although only the presence of Mcm2 and Mcm3 was exam-

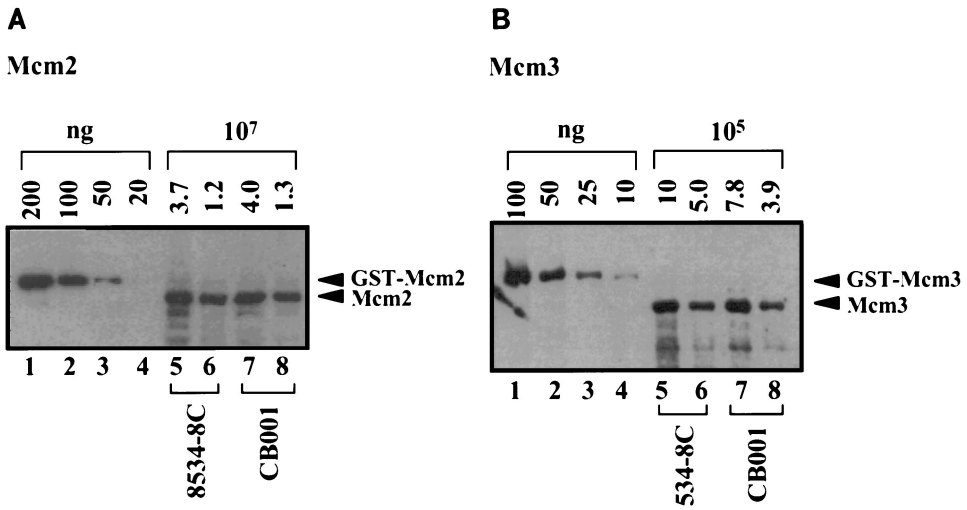

FIG. 5. Quantitation of soluble Mcm2 (A) and Mcm3 (B) in yeast cells. Lanes 1 to 4, purified GST fusion proteins. The amount of protein used is shown above each lane. Lanes 5 and 6, lysate from strain 8534-8C; lanes 7 and 8, lysate from strain CB001. The number of yeast cells from which the protein extract was prepared is indicated above each lane. Immunoblots were probed by Mcm2-specific (A) or Mcm3-specific (B) antibodies.

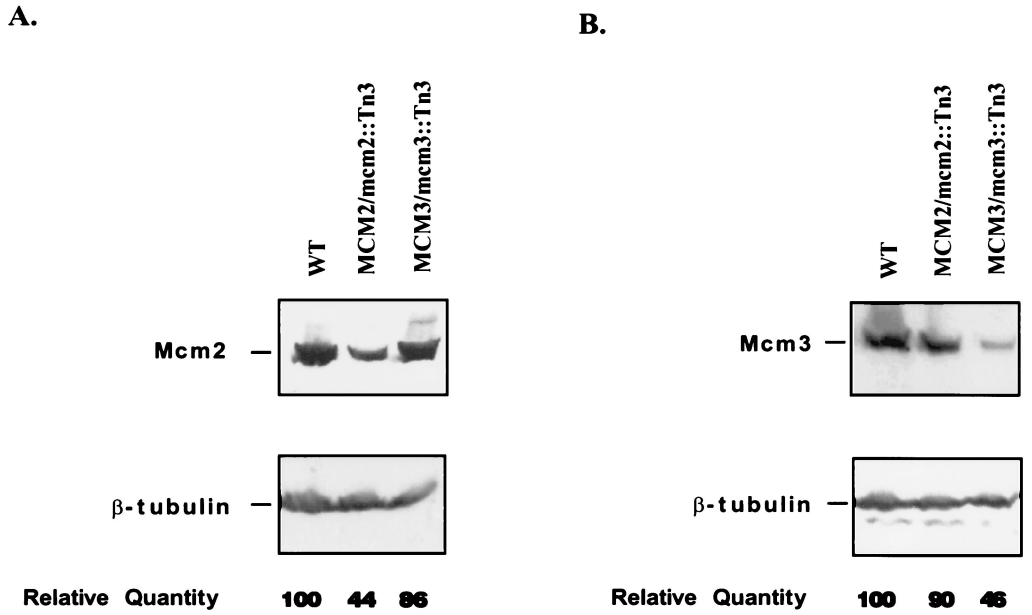

FIG. 6. Quantitation of Mcm2 and Mcm3 in the diploid strains. (A) Immunoblots of soluble proteins extracted from diploid strains 8534-10A/RY71A (wild type [WT]), 8534-10A/RY71A mcm2::Tn3 (*MCM2*/*mcm2*::Tn*3*), and 8534-10A/RY71A mcm3::Tn3 (*MCM3*/*mcm3*::Tn*3*). The blot was probed by Mcm2- and β-tubulin-specific antibodies. The intensity of the Mcm2 signal in each lane is normalized against that of β-tubulin in the same lane. The amount of Mcm2 in the wild-type diploid is given a value of 100. The amounts of Mcm2 in the heterozygous diploids relative to that of the wild-type diploid are indicated below each lane. (B) An identical blot (as shown in panel A) was probed with Mcm3- and β-tubulin-specific antibodies. The amount of Mcm3 in the wild-type diploid is given a value of 100. The amounts of Mcm3 in the heterozygous diploids relative to that in the wild-type diploid are indicated below each lane.

ined in the larger complexes, the size of these large complexes does not exclude the presence of additional subunits. Other members of the Mcm2-3-5 protein family, Mcm5/Cdc46, Cdc47, and Cdc54, are likely components of this large complex. Interactions between Mcm3, Mcm5/Cdc46, and Cdc47 have been observed in *S. cerevisiae* by two-hybrid analysis (27a). Coimmunoprecipitation of homologs of Mcm2, Cdc47, and Cdc54 in HeLa cell extracts has been reported (33).

Recent studies indicated that the Mcm proteins and their mammalian homologs undergo phosphomodifications (24, 33, 42) in a cell cycle-dependent manner (48). Sequence analysis indicates that the yeast Mcm3 protein contains multiple Cdc28/Cdc2 kinase consensus phosphorylation sites (44). Our finding that Mcm3 is hypophosphorylated in multimeric complexes suggests that phosphomodifications regulate the assembly of these complexes.

Dosage effect of Mcm2 on DNA replication. We showed that Mcm2 and Mcm3 are present in 4×10^4 and 2×10^5 copies per cell, respectively. The abundance of the yeast Mcm proteins is in accordance with that reported for the human Mcm3 protein, which was estimated to be over 10^6 molecules per cell (6). The abundance of the Mcm proteins suggests that their target sites are not limited to replication origins. A role for the Mcm proteins in the decondensation of chromatin (44) or the marking of unreplicated chromatin (42) has been suggested because of their association with chromatin at G_1 phase and their dissociation from chromatin during S phase.

The abundance of cellular Mcm2 and Mcm3 prompted us to investigate if the intracellular concentrations of the Mcm proteins have a regulatory role in the initiation of DNA synthesis. We found that Mcm2, but not Mcm3, is limiting for DNA replication. A plausible interpretation is that Mcm2 is the limiting subunit for the assembly of functional replication initiation complexes. This interpretation is consistent with the 5:1 molar ratio in cellular concentrations of Mcm3 to Mcm2. Furthermore, it explains our earlier finding that overproduction of Mcm2 partially suppressed the *mcm3-1* mutation while overproduction of Mcm3 exacerbated the *mcm2-1* mutation (46) and resulted in an Mcm^- defect even in the wild-type strain (17). In this scenario, if Mcm2 is limiting and Mcm3 is compromised such that partially active or less complexes are formed, an increase in the Mcm2 concentration will favor the formation of more complexes that will compensate for the Mcm3 defect. Conversely, if Mcm2 is defective, overproducing Mcm3, which is already in excess, would create a further stoichiometric imbalance that would favor the assembly of partial complexes but not that of active complexes.

The most revealing information on the mechanism that reg-

TABLE 2. Loss rates of plasmids carrying different ARSs in the diploid strains

Strain description	Loss rate/cell division (%[a]) of plasmid:				
	YCp101 (ARS1)	YES3 (ARS1)	YCpH_2B (ARSH_2B)	YCpHO (ARSHO)	YCp121 (ARS121)
Wild type	2.6 ± 1.1	1.2 ± 0.2	2.8 ± 0.1	2.8 ± 0.1	0.5 ± 0.1
MCM2/*mcm2*::Tn*3*	16.8 ± 1.4	—	2.8 ± 1.4	10.7 ± 0.4	1.0 ± 0.1
MCM3/*mcm3*::Tn*3*	—	1.2 ± 0.4	2.8 ± 0.6	1.7 ± 1.7	—

[a] Values represent the average from three measurements ± the standard error. —, not determined because of incompatible markers in the plasmid and diploid strain.

ulates origin usage came from the discriminating effects of limiting Mcm2 on the activity of different ARSs. A plausible model for the regulation of origin usage comes into focus. If each complex were to regulate the initiation of a large number of replication origins (31), then origin usage could be determined by the binding constant inherent to each replication origin for that complex. Thus, increasing the effective concentration of that initiation complex, either by increasing subunit concentrations or by altering subunit conformations, may result in the activation of a larger set of replication origins. Conversely, decreasing the effective concentrations of the same complex may result in the activation of only a subset of replication origins. Relevant to this idea is the observation that in developing eukaryotes, Mcm proteins are more abundant in tissues undergoing rapid cell divisions (39, 40, 43). The observation that limiting Mcm2 had no effect on the growth rate of yeast cells suggests that yeast cells tolerate flexibility in the number of replication origins utilized without consequences in the timing of the complete replication of chromosomes. Since the rate of replication fork movement is 2.4 to 6.3 kb/min (35, 38) and the length of S phase is 25 to 40 min at 30°C, each yeast chromosome would require no more than a couple of initiation events for its complete replication within S phase (see reference 7 for a review). Perhaps the larger than necessary number of replication origins on chromosomes serves to engage all available replication initiation complexes to ensure that no active complex remains unconsumed after each round of initiation.

ACKNOWLEDGMENTS

We thank Mariana Wolfner and Jeff Roberts for critical reading of the manuscript. We gratefully acknowledge gifts of anti-LexA antibodies from Jeff Roberts, antiactin antibodies from Tony Bretscher, and anti-β-tubulin antibodies from Tim Huffaker.

This work was supported by NIH grant GM34190 to B.K.T. Y.K. was supported by a fellowship from the Human Frontier Science Program.

REFERENCES

1. **Alfano, C., and R. McMacken.** 1989. Ordered assembly of nucleoprotein structures at the bacteriophage lambda replication origin during the initiation of DNA replication. J. Biol. Chem. **264:**10699–10708.
2. **Bell, S., and B. Stillman.** 1992. ATP-dependent recognition of eukaryotic origins of DNA replication by a multiprotein complex. Nature (London) **357:** 128–134.
3. **Blow, J. J., and R. A. Laskey.** 1988. A role for the nuclear envelope in controlling DNA replication within the cell cycle. Nature (London) **332:**546–548.
4. **Blumenthal, A., H. J. Kriegstein, and D. S. Hogness.** 1973. The units of DNA replication in *Drosophila melanogaster* chromosomes. Cold Spring Harbor Symp. Quant. Biol. **38:**205–223.
5. **Broach, J., Y. Li, J. Feldman, M. Jayaram, J. Abraham, K. Nasmyth, and J. Hicks.** 1983. Localization and sequence analysis of yeast origins of DNA replication. Cold Spring Harbor Symp. Quant. Biol. **47:**1165–1173.
6. **Burkhart, R., D. Schulte, C. Musahl, F. Göhring, and R. Knippers.** 1995. Interactions of human nuclear proteins P1Mcm3 and P1Cdc46. Eur. J. Biochem. **228:**431–438.
7. **Campbell, J. L., and C. S. Newlon.** 1991. Chromosomal DNA replication, p. 41–146. *In* J. Broach (ed.), The molecular and cellular biology of the yeast Saccharomyces: genome dynamics, protein synthesis, and energetics, vol. 1. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
8. **Chan, C. S., and B. K. Tye.** 1980. Autonomously replicating sequences in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **77:**6329–6333.
9. **Chen, Y., K. M. Hennessy, D. Botstein, and B. K. Tye.** 1992. CDC46/MCM5, a yeast protein whose subcellular localization is cell-cycle regulated, is involved in DNA replication at ARSs. Proc. Natl. Acad. Sci. USA **89:**10459–10463.
10. **Chong, J., H. M. Mahbubani, C. Y. Khoo, and J. J. Blow.** 1995. Purification of an MCM-containing complex as a component of the DNA replication licensing system. Nature (London) **375:**418–421.
11. **Chong, J. P., P. Thommes, and J. J. Blow.** 1996. The role of MCM/P1 proteins in the licensing of DNA replication. Trends Biochem. Sci. **21:**102–106.
12. **Dalton, S., and L. Whitebread.** 1995. Cell cycle-regulated nuclear import and export of Cdc47, a protein essential for initiation of DNA replication in budding yeast. Proc. Natl. Acad. Sci. USA **92:**2514–2518.
13. **Diffley, J. F. X., and J. H. Cocker.** 1992. Protein-DNA interactions at a yeast replication origin. Nature (London) **357:**169–172.
14. **Dubey, D. D., L. R. Davis, S. A. Greenfeder, L. Y. Ong, J. Zhu, J. R. Broach, C. S. Newlon, and J. A. Huberman.** 1991. Evidence suggesting that the *ARS* elements associated with silencers of the yeast mating-type locus *HML* do not function as chromosomal DNA replication origins. Mol. Cell. Biol. **11:**5346–5355.
15. **Ferguson, B. M., B. J. Brewer, A. E. Reynolds, and W. L. Fangman.** 1991. A yeast replication origin is activated late in S phase. Cell **65:**507–515.
16. **Fields, S., and O. K. Song.** 1989. A novel genetic system to detect protein-protein interaction. Proc. Natl. Acad. Sci. USA **340:**245–246.
17. **Gibson, S. I., R. T. Surosky, and B.-K. Tye.** 1990. The phenotype of the minichromosome mutant *mcm3* is characteristic of mutants defective in DNA replication. Mol. Cell. Biol. **10:**5707–5720.
18. **Hand, R.** 1978. Eucaryotic DNA: organization of the genome for replication. Cell **15:**317–325.
19. **Hennessy, K. M., C. D. Clark, and D. Botstein.** 1990. Subcellular localization of yeast CDC46 varies with the cell cycle. Genes Dev. **4:**2252–2263.
20. **Hennessy, K. M., A. Lee, E. Chen, and D. Botstein.** 1991. A group of interacting yeast DNA replication genes. Genes Dev. **5:**958–969.
21. **Huberman, J. A., J. G. Zhu, L. R. Davis, and C. S. Newlon.** 1988. Close association of a DNA replication origin and an ARS element on chromosome III of the yeast, *Saccharomyces cerevisiae*. Nucleic Acids Res. **16:**6373–6384.
22. **Jarvik, J., and D. Botstein.** 1975. Conditional-lethal mutations that suppress genetic defects in morphogenesis by altering structural proteins. Proc. Natl. Acad. Sci. USA **72:**2738–2742.

22a. **Kawasaki, Y., and B. Tye.** Unpublished results.

23. **Kearsey, S. E., D. Maiorano, E. C. Holmes, and I. Todorov.** 1996. The role of MCM proteins in the cell cycle control of genome duplication. Bioessays **18:**183–189.
24. **Kimura, H., N. Nazaki, and K. Sugimoto.** 1994. DNA polymerase α associated protein P1, a murine homolog of yeast Mcm3, changes its intranuclear distribution during the DNA synthetic period. EMBO J. **13:**4311–4320.
25. **Koonin, E. V.** 1993. A common set of conserved motifs in a vast variety of putative nucleic acid-dependent ATPases including MCM proteins involved in the initiation of eukaryotic DNA replication. Nucleic Acids Res. **21:**2541–2547.
26. **Kornberg, R. D., and A. Klug.** 1981. The nucleosome. Sci. Am. **244:**52–64.
27. **Kubota, Y., S. Mimura, S.-I. Nishimoto, H. Takisawa, and H. Nojima.** 1995. Identification of the yeast MCM3-related protein as a component of Xenopus DNA replication licensing factor. Cell **81:**601–610.

27a. **Lei, M., M. Osman, and B. Tye.** Unpublished results.

28. **Li, J., and I. Herskowitz.** 1993. Isolation of *ORC6*, a component of the yeast origin recognition complex by a one-hybrid system. Science **262:**1870–1874.
29. **Loo, S., C. A. Fox, J. Rine, R. Kobayashi, B. Stillman, and S. Bell.** 1995. The origin recognition complex in silencing, cell cycle progression, and DNA replication. Mol. Biol. Cell **6:**741–756.
30. **Madine, M. A., C.-Y. Khoo, A. D. Mills, and R. A. Laskey.** 1995. MCM3 complex required for cell cycle regulation of DNA replication in vertebrate cells. Nature (London) **375:**421–424.
31. **Maine, G. T., P. Sinha, and B.-K. Tye.** 1984. Mutants of *S. cerevisiae* defective in the maintenance of minichromosomes. Genetics **106:**365–385.
32. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
33. **Musahl, C., D. Schulte, R. Burkhart, and R. Knippers.** 1995. A human homologue of the yeast replication protein Cdc21 interactions with other Mcm proteins. Eur. J. Biochem. **230:**1096–1101.
34. **Newlon, C. S., and W. G. Burke.** 1980. Replication of small chromosomal DNAs in yeast, p. 399–409. *In* B. Alberts (ed.), Mechanistic studies of DNA replication and recombination, vol. 19. Academic Press, Inc., New York.
35. **Petes, T. D., and D. H. Williamson.** 1975. Fiber autoradiography of replicating yeast DNA. Exp. Cell Res. **95:**103–111.
36. **Rao, P. N., and R. T. Johnson.** 1970. Mammalian cell fusion: studies on the regulation of DNA synthesis and mitosis. Nature (London) **225:**159–164.
37. **Reynolds, A. E., R. M. McCarroll, C. S. Newlon, and W. L. Fangman.** 1989. Time of replication of ARS elements along yeast chromosome III. Mol. Cell. Biol. **9:**4488–4494.
38. **Rivin, C. J., and W. L. Fangman.** 1980. Replication fork rate and origin activation during the S phase of Saccharomyces cerevisiae. J. Cell Biol. **85:**108–115.

38a. **Shirahige, K., and H. Yoshikawa.** Unpublished results.

39. **Springer, P. S., W. R. McCombe, V. Sundaresan, and R. A. Martienssen.** 1995. Gene trap tagging of *PROLIFERA*, an essential *MCM2-3-5*-like gene in *Arabidopsis*. Science **268:**877–880.
40. **Starborg, M., and C. Höög.** 1995. The murine replication protein P1 is differentially expressed during spermatogenesis. Eur. J. Cell Biol. **68:**206–210.
41. **Taylor, J. H.** 1960. Asynchronous duplication of chromosomes in culture

cells of Chinese hamster. J. Biophys. Biochem. Cytol. **7:**455–463.
42. **Todorov, I. T., A. Attaran, and S. E. Kearsey.** 1995. BM28, a human member of the MCM2-3-5 family, is displaced from chromatin during DNA replication. J. Cell Biol. **129:**1433–1445.
43. **Treisman, J. E., P. J. Follette, P. H. O'Farrell, and G. M. Rubin.** 1995. Cell proliferation and DNA replication defects in a *Drosophila MCM2* mutant. Genes Dev. **9:**1709–1715.
44. **Tye, B. K.** 1994. The Mcm2-3-5 proteins: are they replication licensing factors? Trends Cell Biol. **4:**160–166.
45. **Whitebread, L. A., and S. Dalton.** 1995. Cdc54 belongs to the Cdc46/Mcm3 family of proteins which are essential for initiation of eukaryotic DNA replication. Gene **155:**113–117.
45a. **Yan, H.** Unpublished results.
46. **Yan, H., S. Gibson, and B. K. Tye.** 1991. Mcm2 and Mcm3, two proteins important for ARS activity, are related in structure and function. Genes Dev. **5:**944–957.
47. **Yan, H., A. M. Merchant, and B.-K. Tye.** 1993. Cell cycle-regulated nuclear localization of MCM2 and MCM3, which are required for the initiation of DNA synthesis at chromosomal replication origins in yeast. Genes Dev. **7:**2149–2160.
48. **Young, M., and B. Tye.** Unpublished results.