Mcm10 and the MCM2–7 complex interact to initiate DNA synthesis and to release replication factors from origins

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MCM2–7, a complex of six subunits, is an essential component of the prereplication chromatin that is assembled at Saccharomyces cerevisiae replication origins during G1 phase. To elucidate the action of MCM2–7 during the transition from initiation to elongation replication, we have focused our studies on Mcm10, a replication initiation protein that physically interacts with members of the MCM2–7 complex. We show that Mcm10 is a chromatin-associated protein that mediates the association of the MCM2–7 complex with replication origins. Furthermore, diminished interaction between Mcm10 and Mcm7, a subunit of the MCM2–7 complex, by a mutation in either Mcm10 or Mcm7 inhibits replication initiation. Surprisingly, a double mutant containing both the mcm10-1 and mcm7-1 (cdc47-1) alleles restores interaction between Mcm10 and Mcm7 and corrects all of the defects exhibited by each of the single mutants, including the stalling of replication forks at replication origins typically seen in mcm10-1 cells. This mutual compensation of defects between two independently isolated mutations is allele specific. These results suggest that Mcm10, like Mcm7, is a critical component of the prereplication chromatin and that interaction between Mcm10 and Mcm7 is required for proper replication initiation and prompt release of origin-bound factors.

[Key Words: MCM2–7 complex; Mcm10; Cdc45; DNA synthesis; replication initiation]

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Fidelity of genome duplication is essential for the procreation of life. Eukaryote genomes are organized into multiple chromosomes and initiation of DNA synthesis starts from not one but numerous sites on each chromosome. To ensure fidelity, these multiple initiation events must be coordinated such that on completion, the entire genome is duplicated exactly once. The strategy for this regulated process is beginning to emerge from the collective works of a number of laboratories. Key to this strategy is the periodic recruitment and discharge of a hexameric complex, MCM2–7, to forge a cycle of activity and inactivity at replication origins [Diffley 1996; Tye 1999]. Although the concept of the temporal separation of an active and an inactive chromatin state at replication origins is relatively simple, the number of protein factors involved in this process suggests a complex and intricate scheme. The origin recognition complex (ORC) is a complex of six subunits that constitutively binds origin DNA and “bookmarks” the sequences [Bell and Stillman 1992]. Recruitment of the MCM complex to replication origins is facilitated by the short-lived replication initiation factor Cdc6 (Coleman et al. 1996; Donovan et al. 1997). At the cue of the cell cycle-dependent kinases Cdc7-Dbf4 and Cdc28-Clb, a sequence of events involving participation of Cdc45 activates the transition of replication initiation to replication elongation [Lei et al. 1997; Zou et al. 1997; Zou and Stillman 1998]. During this transitional process, the inactive MCM2–7 complex is believed to transform into an active helicase that first melts the origin DNA and then processively unwinds the growing fork [Aparicio et al. 1997; Kelman et al. 1999; You et al. 1999; Chong et al. 2000]. Replication origins that are no longer occupied by the MCM complex are inactive and this period of inactivity extends into the next G1 phase when a new cycle of activity begins again with the recruitment of the MCM2–7 complex.

Although there is strong evidence that the MCM2–7 complex plays a key role both in initiation and in elongation replication, the mechanistic detail of the different...
roles that it plays is unknown. In particular, the relationship between MCM2–7 and the accessory factors that facilitate its activities requires further investigation. For example, the recruitment of the MCM2–7 complex to replication origins is dependent on ORC (Santocanale and Diffley 1996) and Cdc6 [Liang and Stillman 1997; Tanaka et al. 1997], yet there is little evidence for physical interactions between MCM2–7 with either of these proteins. On the other hand, the MCM2–7 complex is known to interact with two replication initiation factors, Cdc45 [Hopwood and Dalton 1996] and Mcm10 [Merchant et al. 1997]. However, the context and the functional significance of these interactions have yet to be explored. To understand better the roles of the MCM2–7 complex in DNA replication, we focus our studies on Mcm10 and its functional relationship with subunits of the MCM2–7 complex.

Originally, Mcm10 was identified in the same screen in which several members of the MCM2–7 family were first isolated [Maine et al. 1984], although Mcm10 shows no structural similarity to members of this family. Physical interactions between Mcm10 and several members of the MCM2–7 family in a two-hybrid assay suggest a functional relationship between these proteins through contacts [Merchant et al. 1997]. Phenotypes of the mcm10 mutants suggest that Mcm10 may have multiple roles in regulating DNA replication. First, the mcm10-1 mutant is defective in the initiation of DNA synthesis. This defect is illustrated by the instability of minichromosomes even at the permissive temperature of 30°C, and by the reduced frequency of replication initiation in two-dimensional gel analysis. Second, a pausing phenotype shown by two-dimensional DNA gel analysis indicates stalling of the replication machinery as origin regions are passively replicated. This accumulation of paused replication forks suggests a second role for Mcm10, perhaps in the removal of some tightly bound protein factors from the origin that are blocking the replication machinery, or perhaps in the elongation step itself.

In this paper we discuss new evidence for the roles of Mcm10 in the multistep replication initiation process. First, we show that Mcm10 is a chromatin-associated protein that is also localized at replication origins. Removal of Mcm10 from chromatin results in the dissociation of Mcm2 from chromatin. The suggestion that Mcm10 mediates the association of the MCM2–7 complex with replication origins is supported by the genetic interaction of Mcm10 with Mcm7, a subunit of the MCM2–7 complex. Two independently isolated mutants, mcm10-1 and mcm7-1, were studied. Individual mutants of Mcm10 or Mcm7 are defective in DNA replication and show diminished interactions between the two proteins. However, we find that the combined effects of lesions in both Mcm7 and Mcm10 result in the mutual suppression of all defects exhibited by the individual mutants. We show evidence that the mutual suppression is due to enhanced physical interaction between the mutant Mcm10-1 and Mcm7-1 proteins. The necessity of physical contact between the Mcm10 and Mcm7 proteins for their functions supports the notion that Mcm10 is an integral part of the prereplication chromatin and may play a role in the subsequent release of the MCM2–7 complex from replication origins during replication initiation.

Results

Elongation forks pause at functional replication origins in a mcm10 mutant

Lesions in Mcm10 not only reduce significantly the frequency of initiation events at replication origins but also induce pausing of elongation forks near these sites [Fig. 1, cf. A and B]. The diagram in Figure 1B depicts the accumulation of two species of replication intermediates that result from the pausing of elongation forks from opposite directions at a single site at or near a replication origin typically seen in a mcm10 mutant. The interaction between Mcm10 and subunits of MCM2–7 suggests that the pausing of elongation forks in the mcm10-1 mutant may be related directly to the chromatin structure at replication origins. To investigate this possibility, we introduced mutations into the ORI1 locus and examined the effects of these mutations on the mcm10-1 mutant phenotypes [Fig. 1C]. Linker substitution analysis by Marahrens and Stillman [1992] identified several functionally important sequence modules termed A, B1, B2, and B3 elements in ORI1. The origin activity requires the integrity of the A element and at least two of the

![Figure 1](https://genesdev.cshlp.org/)

**A ORI1 in MCM10**

**B ORI1 in mcm10-1**

**C ori1 in mcm10-1**

Figure 1. Two-dimensional DNA gel analysis of replicative intermediates in the ORI1 region in the mcm10-1 mutant. (A) ORI1 in a wild-type strain. (B) ORI1 in the mcm10-1 mutant strain. The cartoon is an interpretation of the result showing that the intense spots are due to accumulations of specific species of replicative intermediates. (C) The ori1 alleles in the mcm10-1 mutants: (a) ori1-a, (b) ori1-b2b3, (c) ori1-b1, (d) ori1-b3. Pause signals are indicated by open triangles. All ori1 alleles were constructed in isogenic mcm10-1 background.
three B elements. Mutations in any one of the three B elements compromise but do not abolish origin activity. We constructed isogenic mcm10-1 strains with previously described linker-substitution mutations in A, B1, B2 + B3, or B3 elements of ORI1 (Marahrens and Stillman 1992).

Effects of these mutations on mcm10-1 mutant phenotypes were then examined by two-dimensional DNA gel analysis (Brewer and Fangman 1987). Two intense spots or “pause signals” (open triangles) on the Y arc result from replication intermediates generated by elongation forks from opposite directions pausing at ORI1 (Fig. 1B). When a mutation that abolishes origin activity was introduced into the A element of ORI1, this locus [ori1-a] was no longer functional as a replication origin as indicated by the absence of an initiation bubble (Fig. 1C, a). Also missing in this ori1-a strain were the pause signals on the Y arc (Fig. 1C, a). When mutations that abolish origin activity were introduced into both B2 and B3 elements of ORI1 [ori1–b2b3], phenotypes similar to those in the ori1-a strain were observed: no initiation bubble and no pause signals on the Y arc (Fig. 1C, b). These results suggest that pausing of elongation forks at replication origins requires the integrity of cis-acting elements that are essential for the assembly of prereplication complex (pre-RC) at replication origins.

Mutations in B1 or B3 elements, which impair but do not abrogate origin activity (Marahrens and Stillman 1992), were also introduced into ORI1 in the mcm10-1 strain. In these mutant strains [ori1–b1 and ori1–b3], the intensity of the bubble arcs was detectable but considerably reduced, compared to ORI1 (Fig. 1B) in the same mcm10-1 background, consistent with defects that compromise rather than abolish replication initiation (Fig. 1C, c,d). However, pausing of elongation forks was still observed in the Y arc. These results suggest that replication origins that are compromised in initiation efficiency yet competent to initiate DNA synthesis can also impede the migration of elongation forks in the mcm10-1 mutant. The requirement for a competent origin to induce fork pausing in the mcm10 mutant suggests an intimate relationship between Mcm10 and the chromatin structure at the origin.

All cellular Mcm10 proteins are constitutively associated with chromatin

To gain insights into the functions of Mcm10, we examined the subcellular location of the protein. Whole cell extracts from G1-, S-, or M-phase cells were fractionated into a soluble fraction that includes both cytosol and nucleosol, and a chromatin-enriched pellet fraction (Donovan et al. 1997; Liang and Stillman 1997). The distribution of Mcm10 in these fractions was examined by immunoblot analysis (Fig. 2A). Mcm10 was not detected in the soluble fractions and was found only in the pellets or chromatin fractions of cells at G1, S, or M phases of the cell cycle (Fig. 2A, top). In contrast, actin, which is found only in the cytoplasm, was detected only in the soluble fraction (Fig. 2A, middle). The subcellular distri-

![Figure 2.](image-url)
Yeast cells were first synchronized at G1 phase and examined in a time course experiment (Fig. 2B). Yeast cells were first synchronized at G1 phase with α factor and then released to fresh medium. Aliquots of cells were harvested at 10-min intervals until the completion of one cell cycle. Chromatin fractions were prepared from these cells and analyzed for the presence of Mcm2 and Mcm10. Mcm2, a subunit of the MCM2–7 complex, associated with chromatin periodically from early G1 phase to the beginning of S phase (Fig. 2B, bottom) as previously shown (Liang and Stillman 1997; Young and Tye 1997). In contrast, Mcm10 associated with chromatin throughout the cell cycle at a constant level (Fig. 2B, top). These results suggest that Mcm10 is constitutively chromatin bound.

The interaction between Mcm10 and the MCM2–7 proteins as well as the localization of Mcm10 at ORI1 suggests that Mcm10 is likely a component of the pre-RC. To investigate whether Mcm10 plays a role in the formation and maintenance of the pre-RC, we examined the chromatin association of Mcm2 and Orc3, a subunit of ORC, when Mcm10 is removed from chromatin. This experiment was performed using temperature-sensitive mcm10 mutant cells synchronized in G1 phase, because MCM2–7 proteins are associated with chromatin only during G1 phase. mcm10 mutant cells were arrested at the G1 phase with α factor at 30°C and then shifted to 37°C in the presence of additional α factor. Cells were harvested at 1-hr intervals up to 5 hr after the temperature shift and fractionated as described above. The chromatin-enriched pellet fractions were analyzed for the presence of Mcm10, Orc3, Mcm2, and histone H2B. Two temperature-sensitive mcm10 mutants, mcm10-1 (Merchant et al. 1997), and mcm10-43 (Solomon et al. 1992) were examined. These two mutant alleles showed similar phenotypes in two-dimensional DNA gel analysis [M. Lei, unpubl.]. When cells were shifted to the restrictive temperature, the binding of the Mcm10-1 protein to chromatin was not affected [data not shown]. However, Mcm10-43 is released gradually from the chromatin fraction [Fig. 3A, a] into the soluble fraction [Fig. 3A, b] during this time course. The removal of Mcm10 had no effect on the chromatin association of Orc3. Orc3 remained associated with chromatin throughout the time course [Fig. 3A, c]. However, as Mcm10 was removed from chromatin, Mcm2 was also released from chromatin [Fig. 3A, d]. Both Mcm10 and Mcm2 remained associated with chromatin 5 hr after shift to 37°C in a wild-type strain [Fig. 3C, a,b]. As a control, the chromatin binding of histone H2B remained constant throughout the time course [Fig. 3A, e]. Thus, the chromatin association of Mcm2, but not that of Orc3, depends on the stable association of Mcm10 with chromatin.

To investigate whether the binding of Mcm10 to chromatin depends on the association of ORC with chromatin, we carried out a similar temperature shift experiment using the temperature-sensitive orc2-1 mutant and examined the chromatin association of MCM10 and Mcm2, when Orc3 is removed from chromatin. The orc2-1 mutation severely affects the formation of the ORC complex and its chromatin binding (Bell et al. 1993). Consistent with this observation, we found that the chromatin association of Orc3 is significantly compromised in this mutant. Subunits of ORC, which are found only in the chromatin fraction in wild-type cells [Fig. 3C, c] (Liang and Stillman 1997), were detected in the soluble fraction in the orc2-1 mutant even at a permissive temperature [Fig. 3B, b]. Shifting to the restric-
that during the G1 phase, the binding of MCM2–7 to like Orc3 (Fig. 3B, d). Taken together, our results suggest Mcm2 is dissociated completely from chromatin much impact on the chromatin binding of Mcm2. At 3 hr, pendent on that of Orc3. Orc2-1 also had a profound Thus, the chromatin association of Mcm10 was not de- ter shifting to the restrictive temperature (Fig. 3B, c,e). H2B, remained associated with chromatin up to 5 hr af- matin in the mutant (Fig. 3B, a). The binding of Mcm10 bound to chromatin. (Fig. 3B, b). Cells were treated with with antibodies specific for Mcm10, Orc3, Mcm2, or histone H2B as indicated. (B) Chromatin binding of Orc3, Mcm10, Mcm2, and histone H2B during G1 phase in MW23 cells (a ura3-53 can1-11 mcm10-43) (Solomon et al. 1992). Cells were treated with a factor (10 µg/ml) for 3 hr at 30°C and again with the same amount before being shifted to 37°C for 0–5 hr [lanes 1–5]. Pellet [P] and soluble fractions [S] were prepared as described in Fig. 2, and analyzed in SDS-PAGE. Western blots were probed with antibodies specific for Mcm10, Orc3, Mcm2, or histone H2B as indicated. The anti-Orc3 antibody was a gift from Bruce Stillman (Cold Spring Harbor Laboratory) and the anti-H2B antibody was indicated. The anti-Orc3 antibody was a gift from Bruce Stillman (Cold Spring Harbor Laboratory) and the anti-H2B antibody was a gift from Mike Grunstein (UCLA). (C) Mcm10, Mcm2, and Orc2 are associated with chromatin in the presence of a factor after 5 hr of incubation at 37°C in the W303/Bar1 strain.

tive temperature completely removed Orc3 from chromatin in the mutant (Fig. 3B, a). The binding of Mcm10 to chromatin was not affected. Mcm10, like histone H2B, remained associated with chromatin up to 5 hr after shifting to the restrictive temperature (Fig. 3B, c,e). Thus, the chromatin association of Mcm10 was not dependent on that of Orc3. Orc2-1 also had a profound impact on the chromatin binding of Mcm2. At 3 hr, Mcm2 is dissociated completely from chromatin much like Orc3 (Fig. 3B, d). Taken together, our results suggest that during the G1 phase, the binding of MCM2–7 to chromatin is dependent on the binding of both ORC and Mcm10; the binding of Mcm10 is not dependent on, nor required for the binding of ORC to chromatin, and vice versa.

The heat sensitivity of mcm10-1 is suppressed by an independently isolated mutant allele of the replication initiation factor MCM7

To elucidate the role played by Mcm10 in replication initiation, genetic interactions were examined by crossing mcm10-1 with known mutants of the MCM2–7 family. Known mutants in MCM2–7 were initially identified by one of two strategies. Some mutants were identified by their mcm (minichromosome maintenance) defects (Maine et al. 1984), whereas others were identified by their cdc (cell division cycle) defects (Moir et al. 1982) but were later assigned a mcm notation (Table 1; Chong et al. 1995; Kearsey et al. 1995). Throughout this paper, we will use the mcm nomenclature for all mutants of the MCM2–7 family in the interest of clarity.

The results of sporulation and dissection are shown in Table 1. To determine the phenotypes of the double mutants, we examined the growth pattern of the tetradtype (TT) tetrads. Because MCM10 is not linked to any of the MCM2–7 genes, statistically, four of every six tetrads are the tetratype. A TT tetrad consists of a wild-type spore, one spore for each parental mutant, and one spore for the double mutant. Because the phenotypes of all parental mutants are known, the phenotype of the double mutant spore can be easily discerned. Most crosses between parents with unlinked heat-sensitive mutant alleles gave a 3:1 heat-sensitive: heat-resistant TT pattern of growth, indicating that the double mutant was heat sensitive like the parental strains. However, in two of the crosses, mcm10-1 × mcm7-1 (cdc47-1) and mcm10-1 × mcm5-461 (cdc46-1), the TT tetrads had two spores that grew well at 37°C, indicating that the double mutant does not have the heat-sensitive phenotype of the parents (Fig. 4A).

To confirm that the inferred double mutant did con- Table 1. Double mutant phenotypes of mcm10-1 with alleles of mcm2–mcm7 or cdc45

| Mutant | Phenotype² | 30°C | 37°C | 14°C |
|--------|------------|------|------|------|
| mcm2-1 | ts          | ++   | −    | +    |
| mcm3-1 | ts          | ++   | −    | ++   |
| mcm4-1 (cdc45) | cs | ++ | − | − |
| mcm5-461 (cdc46) | ts | ++ | + | ++ |
| mcm5-462 (cdc46-2) | ts | ++ | − | ++ |
| mcm5-463 (cdc46-3) | ts | ++ | − | ++ |
| mcm5-465 (cdc46-5) | ts | ++ | − | ++ |
| mcm7-1 (cdc47-1) | ts | ++ | ++ | ++ |
| cdc45-1 | cs | − | − | − |

The phenotype of each parental strain is shown, as well as the phenotypes of double mutants obtained by crossing to mcm10-1. The mcm10-1 mutant is temperature-sensitive with a restric- tion of 37°C. We see strong suppression between mcm10-1 and mcm7-1, weak suppression between mcm10-1 and mcm5-461, and synthetic lethality between mcm10-1 and cdc45-1. The original cdc notations used for the mcm4, mcm5, and mcm7 mutants are shown in parentheses.

²(ts) Heat sensitive, (cs) cold sensitive.
³(+) growth, (−) no growth. (*) The restrictive temperature for mcm2-1 is 38°C.
mcm10-1, the two heat-resistant spores from a typical TT tetrad were crossed back to wild-type cells individually. From these crosses, one of the spores [Fig. 4B, a] from each tetrad always gave back only wild-type spores, implying that it itself is wild type (data not shown). When the second spore [Fig. 4B, b] was crossed to wild-type cells, TT tetrads yielded temperature-sensitive spores that could be complemented by transformation with either MCM10 or MCM7 on a single copy plasmid (data not shown). Therefore, we concluded that the second spore was the double mutant. Although mcm10-1 and mcm7-1 were independently isolated, our data suggest that they synthetically cosuppress the heat-sensitive defects of both parents [Fig. 4B,C].

The mutual suppression phenotype of the mcm10-1 mcm7-1 double mutant was checked for growth at 37°C on plates [Fig. 4C] and by FACS analysis [data not shown]. At the restrictive temperature for the single mutants, the double mutant grew well [Fig. 4C]. By FACS analysis, mcm10-1 and mcm7-1 both arrested with nearly two genome equivalents (2C) of DNA after 3 hr at 37°C. However, the double mutant did not arrest and cells with a DNA content of 1C and 2C were well represented in the population under the same conditions.

Mutual suppression was also seen in mcm10-1 mcm5-461 [cdc46-1] double mutants, although the growth rate of this double mutant at 37°C is lower than wild-type levels (Table 1; Fig. 4C). There is some suppression of the temperature-sensitive phenotype in this double mutant because no growth was apparent on plates containing double mutants of mcm10-1 paired with other mutants of the MCM2–7 family, including mcm2-1, mcm3-1, and mcm4-1 [cdc54-1] (Table 1). In addition, the mcm5-461 mcm10-1 strain has a shorter doubling time at 37°C than either mcm10-1 or mcm5-461 alone [Fig. 4D]. This suppression is allele specific; a double mutant of mcm10-1 with other alleles of mcm5 did not show suppression of the temperature sensitivity [Table 1], nor did double mutants of mcm5-461 with another mcm10 allele [mcm10-43; data not shown].

Double mutants of mcm5-461 mcm7-1 are synthetically lethal [Hennessy et al. 1991] suggesting that two defective subunits of the MCM2–7 complex are detrimental to yeast cells. Because mcm10-1 is able to suppress each of these mutations individually, we tested the combined effects of the triple mutant mcm10-1 mcm5-461 mcm7-1. Interestingly, the triple mutant was viable [Fig. 4C], indicating that the specific defects of the Mcm5 and Mcm7 mutant proteins could be simultaneously suppressed by the altered Mcm10-1 protein within the same cell. We also noted that the suppression of the mcm10-1 heat-sensitive phenotype was more complete in the triple mutant than in the mcm10-1 mcm5-461 or mcm10-1 mcm7-1 double mutant as shown by growth on plates and in culture [Fig. 4C,D]. The doubling time of the triple mutant at 30°C and 37°C was 2.6 and 2.9 hr, respectively, compared to 3.3 and 5.1 hr for the mcm5-461 mcm10-1 mutant, and 2.7 and 4.6 hr for the mcm7-1 mcm10-1 double mutant [Fig. 4D].

Replication initiation is restored and fork barriers are obliterated in the mcm10-1 mcm7-1 double mutant

The role of Mcm10 and other MCM proteins in DNA replication were first identified by the poor ability of

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**Figure 4.** Tetrad analyses of diploids from a cross between R125A22-2C [mcm10-1] and DBY2029 [mcm7-1]. (A) Segregation patterns of phenotypes indicate that the double mutant is wild type, although both parental strains are temperature sensitive [ts]. (TT) Tetrad type; (PD) parental ditype, (NPD) nonparental ditype. Both parental strains from a tetrad always gave back only wild-type spores, implying that it itself is wild type (data not shown).
their mutants to maintain minichromosomes, which are plasmids containing an origin of replication (ORI) and a centromere (CEN) [Maine et al. 1984]. To determine whether the high plasmid loss phenotypes of the mcm10-1 and mcm7-1 strains were alleviated in the double mutant, plasmid stability assays were performed. Using minichromosomes carrying autonomously replicating sequence (ARS) fragments from different regions of the genome, minichromosome maintenance defects were observed for both mcm10-1 and mcm7-1 [Fig. 5A]. However, double mutants of mcm10-1 mcm7-1 derived from spores of two different tetrads support the replication of plasmids at wild-type levels [Fig. 5A]. This result suggests that the replication defects of the single mutants are suppressed in the double mutant.

To investigate whether the double mutant both restores the competence to initiate DNA synthesis at replication origins and abolishes the pausing of elongation forks, the activities of ORI1 in the double mutant were examined. Two-dimensional gel analysis showed that both mcm10-1 and mcm7-1 have reduced origin firing, which was visualized by a reduced intensity of the bubble arc signal (Fig. 5B). In contrast, the mcm10-1 mcm7-1 double mutant restores this origin firing to a level comparable to that of the wild-type strain. Two species of Y form replicative intermediates that accumulate in two spots of intense signals, one strong and one weak, in the mcm10-1 mutant, as indicated by arrows. These intense signals result from the pausing of migrating replication forks as they pass through ORI1 from opposite directions. However, pause signals are completely abolished in the double mutant, indicating that the block to replication forks traversing through the origin is removed. Thus, mcm10-1 mcm7-1 mutants do not display any of the defects that are observed in each of the single mutants.

![Figure 5](image-url)

**Figure 5.** Replication defects of the mcm10-1 and mcm7-1 single mutants are corrected in mcm10-1 mcm7-1. (A) Stability of plasmids in single and double mutants of mcm10-1 and mcm7-1. Plasmid loss rates were determined for the single mutants mcm7-1 and mcm10-1, as well as for two double mutants [LHY1A and LHY5C] and a wild-type strain [8534-8c] at 30°C. Five different minichromosomes each containing a different ARS were used in this assay. The corresponding ARS of each minichromosome (e.g., ARS121, ARS120) is indicated on the x-axis. The y-axis shows the rates of plasmid loss per cell per generation. (B) The double mutant relieves the pause phenotype of mcm10-1. Two-dimensional DNA gel analysis of mcm10-1 mcm7-1. Genomic DNA was isolated from the wild-type [8534-8c], mcm10-1, mcm7-1, and mcm10-1 mcm7-1 strains. Genomic DNA was digested with NcoI, separated on two-dimensional gel, blotted, and probed with 32P-labeled ORI1 DNA. Bubble and Y arcs are indicated by cartoons on the blot showing the DNA from the wild-type strain. Pause signals that result from accumulation of specific species of replication intermediates are indicated by arrows.
Mutual suppression is dominant

The mutual suppression between mcm10-1 and mcm7-1 could be the result of the neutralization of two antagonists. If so, suppression is due to a loss of function and therefore, would be recessive. On the other hand, the mutual suppression could be the result of the restoration of physical contact between two interacting proteins, a gain of function scenario. In this case, the suppression should be dominant. To determine whether the mutual suppression between mcm10-1 and mcm7-1 is dominant, diploid strains heterozygous for mcm10-1 and homozygous for mcm7-1 or heterozygous for mcm7-1 but homozygous for mcm10-1 were constructed. The resulting diploids both grew well at 37°C (data not shown) indicating that the mutual suppression is dominant and likely due to the restoration of physical contact between the two mutant proteins.

Overexpression studies can also lend support for a physical interaction model. An increase in the level of one of two poorly interacting proteins can provide more opportunity for the crippled physical interaction to occur. We overexpressed Mcm7 in a mcm10-1 strain and Mcm10 in a mcm7-1 strain by transforming the strains with high copy plasmids. In both cases, an increased level of growth was seen for the transformants at 37°C (data not shown). These results also support a model in which increased physical contact between the two mutant proteins alleviates their individual defects.

Mutual suppression of mcm10-1 and mcm7-1 is due to the restoration of physical interaction between the two mutant proteins

The restoration of physical interaction between the mutant proteins is illustrated in Figure 6A. Mcm10 and Mcm7 (XY) were shown previously to physically interact by two-hybrid analysis (Merchant et al. 1997), therefore it is possible that the mutations in each protein individually abolish this interaction [Xy or yX]. When both proteins are altered in a specific manner, however, perhaps the interaction can be restored (xy). To test this hypothesis, two-hybrid analysis was performed. Fusion proteins were created between each mutant or wild-type protein and the Gal4 activation domain (GAD) or the Gal4-binding domain (GBD). These were transformed by pairs into PJ69-4a cells containing the β-galactosidase reporter.
gene downstream of a GAL4-inducible promoter [James et al. 1996]. The levels of interaction between the mutant and wild-type proteins were measured by β-galactosidase activity as shown in Figure 6, B and C.

The physical interactions between Mmc10 and Mmc7 were tested in two different orientations in the two-hybrid system. In one orientation, Mmc7 proteins were fused to the GAD and Mmc10 proteins fused to the GBD [Fig. 6B]. In another orientation, Mmc10 proteins were fused to the GAD and Mmc7 proteins to the GBD [Fig. 6C]. To test the specificity of interaction, two mcm10 mutant alleles were used: mcm10-1 and mcm10-43 [Fig. 6C]. There is a significant decrease in the level of interaction between the proteins when either Mmc10 or Mmc7 is mutated (Fig. 6B,C). A specific mutant pair, Mmc7-1 and Mmc10-1, however, interacts as strongly as the two wild-type proteins [Fig. 6B,C]. This strong interaction is observed in both orientations of activation and binding domain fusions. In contrast, when the mutant pair Mmc10-43 and Mmc7-1 was tested, the interaction is comparable to that observed when only one of the proteins is mutant but significantly less than that observed between the wild-type proteins or the Mmc10-1 and Mmc7-1 combination [Fig. 6C].

This result suggests that restoration of interaction between Mmc10 and Mmc7 mutant proteins is allele specific. If interaction between Mmc10 and Mmc7 is a prerequisite for normal functions of these proteins, then the mcm10-43 mcm7-1 double mutant should not be viable at the restrictive temperature of the single mutants. To test this, we crossed the mutant strains of mcm7-1 and mcm10-43 and dissected tetrads. The TT tetrads yielded one wild type spore and three Ts spores, indicating that the double mutant is temperature sensitive for growth [Fig. 6D]. Similar results were obtained in a cross between mcm5-461 and mcm10-43, suggesting that there is no suppression between these mutant alleles [data not shown].

**Amino acid changes that affect interactions between Mmc10 and Mmc7**

The mcm7-1 mutation has been shown previously to be a change from serine to tyrosine at position 286 [Dalton and Hopwood 1997] in the amino-terminal portion of Mmc7. We have now determined the sequence of the two mcm10 mutant alleles—mcm10-1, which was identified initially as a minichromosome maintenance defective mutant [Maine et al. 1984; Merchant et al. 1997], and mcm10-43, alias dna43, which was originally identified for its defect in DNA synthesis [Solomon et al. 1992]. Both alleles are the result of single nucleotide substitutions resulting in single amino acid changes. The mcm10-1 mutation is a proline to leucine substitution at amino acid 269 and mcm10-43 is a cysteine to tyrosine change at residue 320 [Fig. 6E]. Both mutations disrupt the interaction between Mmc10 and Mmc7, but only one of them, P269L, is suppressed by the mcm7-1 mutation, suggesting that the restoration of interaction between Mmc10 and Mmc7 may be defined by specific contacts between the two proteins.

Recently, a homolog of MCM10 has been identified in Schizosaccharomyces pombe (Aves et al. 1998). The cdc23+ gene of S. pombe has been shown to complement a Saccharomyces cerevisiae mcm10 null strain [Y. Kawasahi, unpubl.]. The Cdc23 ORF encodes a putative protein of 593 amino acids that is only 17% identical but almost 60% similar to Mmc10. An ORF [con4-2907] in the Candida albicans genome database and another [WPY47D3] in the Caenorhabditis elegans genome database show significant similarities with Mmc10 in this region of about 85 amino acids [Fig. 6E]. Alignment of these four sequences indicates that P269 is conserved in the yeast homologs, though not in C. elegans. However, C320 is conserved in all four sequences in a region that encodes a putative zinc finger motif of the type Cx9,Cx12,Cx2H [Fig. 6E].

**Genetic interactions between MCM10 and CDC45**

The two mutant alleles, mcm5-461 and mcm7-1, which suppress the heat-sensitive growth defect of mcm10-1 but not that of mcm10-43, were identified originally as extragenic suppressors that alleviate the cold-sensitive growth defect of the cdc45-1 mutant [Moir and Botstein 1982, Fig. 7A]. Cdc45 is a protein factor essential for DNA replication initiation. The cdc45 mutant allele shows a cell cycle arrest phenotype that is typical of replication initiation mutants, that is, large budded cells with a DNA content of 1C [Hennessy et al. 1991]. Physical interactions of Cdc45 with Mmc5 and Mmc7 have been demonstrated previously using the two-hybrid system [Dalton and Hopwood 1997] and by immunoprecipitation [Hopwood and Dalton 1996]. The suppression of mcm10-1 by the suppressors of cdc45-1 led us to examine the genetic interactions between CDC45 and MCM10.

To construct a double mutant that contains both the cdc45-1 and mcm10-1 mutations, we crossed the single mutant strains. Diploids were sporulated, and the results of the tetrad analysis are shown in Figure 7B. A majority of the resulting tetrads contained only three live spores. Several tetrads contained only two live spores, and in these cases the two spores were wild type for growth at 37°C and 14°C. The only four-spore tetrads contained two heat-sensitive and two cold-sensitive spores, the phenotype of a parental ditype (PD) tetrad. The pattern of spore viability of the tetrads was consistent with synthetic lethality of the mcm10-1 and cdc45-1 mutations [Fig. 7A]. Further tetrad analysis of diploids carrying a MCM10-containing plasmid confirms the lethality of the mcm10-1 cdc45-1 double mutant [data not shown].

**Discussion**

Mmc10 is an essential protein that plays a pivotal role in DNA replication. The identification of Mmc10 ho-
subunits of the complex during G1 phase. Cdc45 is recruited to these origins by interactions with Mcm7 and possibly other members of the MCM2–7 complex with a replication origin is mediated by Mcm10. Contacts Mcm10 is also used to contact Cdc45, although these contacts are required for the initiation of DNA synthesis. Interaction between Mcm10 and Mcm7, a subunit of the MCM2–7 complex, is required for the initiation of DNA synthesis at replication origins. Furthermore, this interaction contributes to the structure of the pre-RC by directly or indirectly removing a block to elongating replication forks that migrate through replication origins.

We showed that the pause phenotype of mcm10-1 is dependent on the specific interaction established between Mcm10 and Mcm7. However, disruption of interaction between Mcm10 and Mcm7 alone is not sufficient to cause the pause phenotype as the mcm7-1 mutant does not demonstrate replication fork pausing. Previously, we have shown that Mcm10 interacts with all but one subunit, Mcm5, of the MCM2–7 complex [Merchant et al. 1997]. The allele-specific suppression of mcm10-1 by mcm5-461 shown in this study suggests that Mcm10 and Mcm5 may also physically interact. The observation that defects of the mcm10-1 mutant can be compensated for by changes in more than one subunit of the MCM2–7 complex is consistent with the view that Mcm10 contacts several subunits of the MCM2–7 complex at the same time. Whereas a mutation in MCM10 might simultaneously affect all interactions between Mcm10 and the MCM2–7 complex, mutations in any one of the subunits of the MCM2–7 complex may only affect that specific contact. This explanation is supported by the observation that the triple mutant mcm10-1 mcm7-1 mcm5-461 grows significantly better than either of the double mutants (Fig. 4C,D). Restoration of interactions between Mcm10 and only one subunit of the MCM2–7 complex is not as desirable as the restoration of interactions between Mcm10 and two or more subunits of the MCM2–7 complex. Obviously, there are other plausible explanations for the pause phenotype that is unique to mcm10 mutants. It is possible that the interaction between Mcm10 and replication origins may also be an important factor. Testing these hypotheses will help us elucidate the full range of functions performed by Mcm10.

Suppression of the mcm10-1 phenotypes was not limited to a change in the Mcm7 protein, as we also observed partial suppression by a specific change in the Mcm5 protein. Furthermore, suppression of the mcm10-1 defects by mutations in MCM5 and MCM7 was allele specific. What is remarkable about these observations is that neither mcm7-1 nor mcm5-461 was initially isolated as a suppressor of mcm10-1. We believe that this is not coincidence, but rather that mcm7-1 and mcm5-461 contain functional elements that are normally occupied by Mcm10.
mcm5-461 were preselected for compensation of the mcm10-1 mutation. The mcm7-1 and mcm5-461 mutations can be traced to a single origin; they were both isolated as suppressors of the cold-sensitive cdc45-1 mutation (Fig. 7A) (Moir et al. 1982, Hennessy et al. 1991). Cdc45 is the last known factor to be recruited to the pre-RC just before the initiation of DNA synthesis [Zou et al. 1997; Zou and Stillman 1998]. Previously, Cdc45 has been shown to physically interact with Mmc5 and Mmc7 [Hopwood and Dalton 1996; Dalton and Hopwood 1997]. In this paper we have shown a synthetic lethal effect between cdc45-1 and two different alleles of mcm10, indicating an important link between the two gene products. All of these physical and genetic interactions suggest that association of the MCM2–7 complex with Mmc10 and Cdc45 may be critical steps in the initiation of DNA synthesis. We noticed that both suppressors of the mcm10-1 mutation [mcm5-461(C183Y)] [Hopwood and Dalton 1996] and mcm7-1(S286Y) [Dalton and Hopwood 1997] are changes that result in a tyrosine substitution at the amino-terminal portion of Mmc5 and Mmc7, respectively.

To put into perspective the functional relationship between subunits of the MCM2–7 complex, Mmc10, and Cdc45, we will first consider the known facts about each of these proteins. Our results suggest that association of the MCM2–7 complex with Mmc10 and Cdc45 is a key component of the pre-RC just before the initiation of DNA synthesis (Zou et al. 1997; Tanaka et al. 1997). After replication initiation, the MCM2–7 complex is believed to leave the replication origins as a processive helicase at the G1 phase (Lei et al. 1997). Mcm10, the late arrival of Cdc45 at replication origins during the G1 to S phase [Aparicio et al. 1997]. The synthetic lethality conferred by cdc45-1 and two different alleles of mcm10 is consistent with the critical roles played by Cdc45 and Mmc10 in the same pathway. This model also provides an explanation for the pause phenotype of the mcm10-1 mutant by suggesting that failure to dislodge certain factors at replication origins may create temporary passive replication blocks at replication origins. Our results do not exclude the possibility that interactions between Mmc10 and MCM2–7 may persist beyond the initiation step into the elongation phase. Further studies will inform other roles that Mmc10 might play in DNA replication.

Materials and methods

Strains and plasmids

Plasmids and strains used in this study are listed in Table 2. Yeast transformation was performed using the lithium acetate method [Ito et al. 1983]. The Escherichia coli strain used for routine cloning was DH5α.

Isogenic ori1 mcm10-1 strains were constructed as follows: a Δtrp1 Δori1 strain [YK15, a his3 is14 len2 ura3 mcm10-1 Δtrp1Δori1::URA3] was first constructed by disrupting the TRP1 ORI1 locus with URA3 in the mcm10-2c strain [Merchant et al. 1997]. Various ori1 alleles were constructed by replacing the disrupted ori1 locus with the TRP1–ARS1 fragments isolated from pTA1 (for mcm10-1 ORI1), pPAR1/865-872 [for mcm10-1 ori1-a], pTA1/756–758, 798–805 [for mcm10-1 ori1-b263], pTA1/835-842 [for mcm10-1 ori1-b1] or pTA1/756–758 [for mcm10-1 ori1-b3], respectively.

Chromatin immunoprecipitation

Formaldehyde cross-linking and immunoprecipitation were carried out as described [Aparicio et al. 1997; Tanaka et al. 1997]. The strain (a bar1 ade2 can1 his1 trpl2 leu2 2 mcm10::MCM10–9MYC) was constructed as follows: an amino terminus truncated MCM10 gene was cloned in pRS406. A 9x Myc epitope cassette [a gift from K. Nasmyth, IMP-Research Institute of Molecular Pathology, Vienna, Austria] was inserted at the 3′ end of the gene as a Nor1 fragment. The plasmid was then linearized by Neol digestion and integrated at the MCM10 locus of W303bar1. The primers used for the amplification of ORI1 and the 240-bp sequence were 5′-GAAATAGGTTATTACTGAGTAG/5′- CACTCGGATGTATATATTTGGTCAT and 5′-CATCAATTCTG- CACTCGGAC/5′-GAACACCGCAATTCAGTGG, respectively. The condition for PCR was 1× [2 min at 95°C, 3 sec at 55°C, 6 sec at 72°C], 28× [30 sec at 95°C, 30 sec at 55°C, 60 sec at 72°C], 1× 4 min at 72°C. PCR products were separated in a 6% PAGE and visualized with ethidium bromide.

Plasmid stability assays

Yeast minichromosomes were transformed into yeast using the lithium acetate method [Ito et al. 1983]. Transformants were plated onto YEPD, and grown for 11–16 generations until individual colonies were visible. Individual colonies were resuspended in total, and plated on both complete and selective plates after appropriate dilutions. The total numbers of cells in each colony that grew on complete and selective media were determined and plasmid loss rates were calculated. Loss rates per cell per generation were calculated using the equation per cell per generation were calculated using the equation...
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Table 2.  Plasmids and strains

| Strain | Genotype | Source |
|--------|----------|--------|
| W303bar1 | a bar1 ade2 can1 his3 trp1 ura3 leu2 | Y. Kawasaki [unpubl.] |
| 8534-M2 | α leu2-3,112 ura3-52 his4Δ34 mcm2-1 | Yan et al. [1993] |
| R61-3C | α leu2-3,112 ura3-52 his4Δ34 mcm3-1 | Gibson et al. [1990] |
| DBY2035 | α his4-619 ura3-52 mcm4-1(cdc54) | Moir et al. [1982] |
| BY2028 | a ura3-52 ade2-1 lys2-801 leu2-3,112 mcm5-461(cdc46-1) | Moir et al. [1982] |
| 434 | a ade2-1 mcm5-462(cdc46-2) | Moir et al. [1982] |
| 436 | a ade2-1 his4Δ34 mcm5-463(cdc46-3) | Moir et al. [1982] |
| 437 | a ade2-1 mcm5-465(cdc46-5) | Moir et al. [1982] |
| DBY2029 | a ade2-1 lys2-801 leu2-3,112 ura3-52 mcm7-1(cdc47-1) | Hennessy et al. [1991] |
| DBY2027 | a ade2-1 lys2-801 leu2-3,112 ura3-52 cdc45-1 | Moir et al. [1982] |
| LHY1A | a leu2-3,112 his3 his4 mcm10-1 mcm7-1 | this study |
| LHY5C | α leu2-3,112 ura3-52 ade2-1 mcm10-1 mcm7-1 | this study |
| LHY4B | α leu2-3,112 lys2-801 ura3-52 mcm10-1 mcm5-461 | this study |
| LHYTM18C | α leu2-3,112 cdc45-1 mcm5-461 mcm10-1 | this study |
| BTY103 | a ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-52 mcm10-43 | this study |
| BTY106 | a his4Δ34 ura3-52 leu2-3,112 mcm10-43 | this study |

Plasmids Description Source

| Plasmid | Description | Source |
|---------|-------------|--------|
| pLitmus28–MCM10 | pLitmus28 with EcoRI/XbaI MCM10 fragment | this study |
| pLitmus28–mcm10-1 | pLitmus28 with EcoRI/XbaI mcm10-1 fragment | this study |
| pRS315–MCM10 | ARSH4 LEU2 CEN6 MCM10 | this study |
| pRS316–MCM10 | ARSH4 URA3 CEN6 MCM10 | this study |
| pRS316–MCM7 | ARSH4 URA3 CEN6 MCM7 | this study |
| pNKY51 | URA3-hisG cassette for disruption | Alani et al. [1987] |
| pUC119–MCM10 | pUC119 with MCM10 | this study |
| YEp24–MCM10 | 2µ ori URA3 MCM10 | this study |
| YEp24–MCM7 | 2µ ori URA3 MCM7 | this study |
| pGAD–C2–MCM10 | pGAD–C2 with GAL4AD–MCM10 fusion | this study |
| pGAD–C2–mcm10-1 | pGAD–C2 with GAL4AD–mcm10-1 fusion | this study |
| pGBKU–C2–MCM10 | pGBKU–C2 with GAL4BD–MCM10 fusion | this study |
| pGBKU–C2–mcm10-1 | pGBKU–C2 with GAL4BD–mcm10-1 fusion | this study |
| pGAD–C3–MCM7 | pGAD–C3 with GAL4AD–MCM7 fusion | this study |
| pGAD–C3–mcm7-1 | pGAD–C3 with GAL4AD–mcm7-1 fusion | this study |
| pGBKU–C3–MCM7 | pGBKU–C3 with GAL4BD–MCM7 fusion | this study |
| pGBKU–C3–mcm7-1 | pGBKU–C3 with GAL4BD–mcm7-1 fusion | this study |

1 – \((F/I)^{1/2}\), where \(I\) is the total number of cells [those growing on complete media] after \(N\) generations, and \(F\) is the number of cells that retain the plasmid [those growing on selective media] after \(N\) generations. All loss rates were the average of at least three data points.

Two-dimensional DNA gel electrophoresis

Two-dimensional DNA gel electrophoresis was performed according to the neutral–neutral method [Brewer and Fangman 1987]. Purified DNA was digested to completion with NcoI. To enrich the sample for replicating DNA, digested DNA was passed through BND cellulose [Sigma, St. Louis, MO] columns as described in [Dijkwel et al. 1991]. ORI1 probes were made by isolating the 4.7-kb NcoI fragment of ORI1 and radiolabeling using random hexamer primers [Pharmacia, Uppsala, Sweden]. Radiolabeled probe at 2.5 × 10⁷ cpm was added to 20 ml of hybridization solution and incubated with the membrane at 42°C for 24 hr.

Yeast two-hybrid analysis

MCM10 and mcm10-1 genes were amplified by PCR from yeast genomic DNA using primers constructed to contain BgIII sites, then cloned into Litmus 28. All inserts were fully sequenced before continuing with the cloning procedure. The genes were then digested from Litmus 28 with BglII and cloned into pGAD-C2 and pGBKU-C2 [James et al. 1996] at the BglII site. The 5’ ends of MCM7 and mcm7-1 were amplified from plasmid DNA using BgII/SmaI-containing primers and cloned into the SmaI/BgII sites in pGBKU-C3 and pGBKU-C3. The 3’ end of the gene was ligated into the resulting plasmid by cloning a BgII/BamHI fragment into the BgII site. All of the resulting plasmids were checked for complementation of temperature-sensitive mutants. Wild-type genes complemented the temperature-sensitive mutants, whereas mutant genes did not. Plasmids were transformed into Pf69-4a [James et al. 1996] and selected for on complete media lacking leucine and uracil. Colonies were then assayed for β-galactosidase activity in liquid cultures as previously described [Ausubel et al. 1998]. At least three colonies [two dilutions each] were tested for each plasmid combination and an average taken.

Sequencing of the two mcm10 mutant alleles

The approximate locations of the mcm10 mutations were first mapped using gapped plasmid repair [Sambrook et al. 1987] and then the exact locations were determined by PCR sequencing.
Genomic PCR of the MCM10 gene was performed in both mcm10-1 and mcm10-43 (dna43-1) strains using the following primers: 5’-TCGTAATCGATGATCATTCCTCGGAA-3’ (5’ end of gene), and 5’-ACAGCTGACATTTTATAT-TATCTCGAGATC (3’ end of gene). Sequencing of at least two independent JCR products for each strain was performed, and mutations were confirmed by sequencing of both Watson and Crick strands, using primers complementary to both 3’ end and 5’ end of the gene as well as internal gene sequence.

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