Annu. Rev. Biochem. 1999. 68:649–686

MCM Proteins in DNA Replication

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Key Words DNA synthesis, cell cycle regulation, origin usage, pre-initiation complex, replicaton licensing, eukaryotes

■ **Abstract** The MCM proteins are essential replication initiation factors originally identified as proteins required for minichromosome maintenance in *Saccharomyces cerevisiae*. The best known among them are a family of six structurally related proteins, MCM2–7, which are evolutionally conserved in all eukaryotes. The MCM2–7 proteins form a hexameric complex. This complex is a key component of the prereplication complex that assembles at replication origins during early G1 phase. New evidence suggests that the MCM2–7 proteins may be involved not only in the initiation but also in the elongation of DNA replication. Orchestration of the functional interactions between the MCM2–7 proteins and other components of the prereplication complex by cell cycle–dependent protein kinases results in initiation of DNA synthesis once every cell cycle.

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INTRODUCTION

Eukaryotic genomes are organized into multiple chromosomes because of their size and complexity. Each chromosome must be coordinately replicated within a defined period, the S phase, of the cell cycle. During the development of multicellular organisms, cell proliferation is programmed to produce a finite number of cells at an appropriate time in the appropriate spatial arrangements to shape the final organism. The duration of cell cycles and therefore S phases can vary significantly depending on the stages of development of the organism. The flexibility in meeting these differing time constraints is afforded by using different numbers of replication initiation sites at different developmental stages (1). In insect cells, DNA replication is regulated at the level of origin usage. Many more origins are used in rapidly dividing embryonic nuclei than in slowly dividing somatic cells (2). Thus, initiation of DNA synthesis in eukaryotes is regulated both at the level of the cell cycle and at the level of organism development. During each cell cycle, initiation events are tightly coordinated with stages of the cell cycle by positive and negative regulation. Positive regulation is necessary for signaling the initiation of DNA synthesis. Negative regulation is imposed to prevent the reinitiation of DNA synthesis within a single cell cycle. In addition, the initiation of DNA synthesis at the multiple replication origins in each chromosome follows a defined temporal order within the cell cycle (3, 6). This prescribed order appears to be influenced by the patterns of gene expression of differentiated cells (7, 8).

The unit of DNA replication, a replicon, consists of two regulatory components: a *cis*-acting element, known as the replicator, and a *trans*-acting element, known as the initiator (9). Initiation of DNA synthesis is effected by the binding of the initiators to the replicators. Studies of replicators from fungi to metazoans suggest that the DNA sequences for the initiation of DNA replication have diverged in evolution to a state that confounds their ancestral derivation. In early embryos of *Xenopus laevis*, replication initiation appears to occur randomly without any sequence specificity (10). Although it is possible to show that replication initiation begins from preferred chromosomal locations in a broad zone in mam-

malian cells (11), the demonstration of a specific sequence that functions as a replication origin in isolation has been lacking. Instead, degenerate sequences appear to serve as origins of replication (ORIs) on plasmids when transfected into mammalian cells (12). The observation in the yeast *Saccharomyces cerevisiae* is quite different. Genomic sequences, known as autonomously replicating sequences (ARSs), that support the autonomous replication of plasmids are also ORIs on the native chromosomes (13–15). The work of a number of laboratories contributed to our current understanding of the anatomy of yeast ORIs. ARSs are defined as A-T–rich sequences of between 150 and 200 base pairs that show no obvious homology to one another except for an essential consensus element of 11 base pairs, called the A element or ARS consensus sequence (16). Flanking the A element is the essential B domain, which consists of nonconserved elements that are functionally interchangeable between ARSs (15, 17–21). Defined sequences also serve as ORIs in the fission yeast *Saccharomyces pombe*, but the organization of the essential elements of these origins appears to be more complex (22).

Identification of sequence-specific replicators or ARSs in *S. cerevisiae* allowed the purification of initiator proteins. The origin recognition complex (ORC), a complex of six subunits that binds specifically to the A element of ARSs, was purified by using a DNase1 footprinting assay (23). This ORC was later shown to bind to the A element of replication origins, not only in vitro but also in vivo (24). The binding of ORC to replication origins appears to occur at most if not all stages of the cell cycle, suggesting that the binding of ORC alone to replication origins is not sufficient to induce replication initiation (25); however, additional proteins are necessary.

Subsequent studies show that the initiation of DNA synthesis in eukaryotes is a complex, multistep process that requires the participation of a number of proteins. This process involves the binding of ORC to replication origins (26), the recruitment of Cdc6 and Mcm2–Mcm7 (Mcm for minichromosome maintenance) to form the prereplicative complex (pre-RC) (27), and the activation of pre-RC by Cdc7 and Cdc28 protein kinases to initiate DNA synthesis (28). Working in series or in parallel, these protein kinases ensure that DNA replication occurs only once at a specified time in every cell cycle. To dissect this multistep process of replication initiation, it is important to identify the protein factors that are involved in each of these steps. Whereas it is possible to identify structural protein components that bind tightly to origin DNA, as shown in the purification of the ORC complex in *S. cerevisiae* (23), regulatory proteins that interact transiently with replication origins cannot be easily identified without an in vitro system for replication initiation. Genetic approaches provide a powerful alternative and sometimes the only alternative for the identification of regulatory factors. It is based on genetic approaches that components of the pre-RC, including Cdc6 and MCM2–7, the cell cycle-regulated protein kinases Cdc7 and Cdc28, and other regulatory factors such as Cdc45 and Mcm10 were identified in *S. cerevisiae*. Thus, in the replicon model for *S. cerevisiae*, the replicator is a defined DNA sequence that binds the multicomponent initiator complex that includes ORC, Cdc6, and MCM2–7, a family of six proteins. Although the organization of the replicators in complex

eukaryotes remains unclear, mounting evidence indicates that the initiators, in particular ORC, Cdc6, and MCM2–7, are evolutionarily conserved from yeasts to humans (29). The studies of these initiator proteins are likely to lead to a unifying mechanism for the initiation of DNA replication in all eukaryotes and perhaps the ancestral origin of the degenerate replicators.

The study of DNA replication initiation in eukaryotes has seen an explosion in activity in the last 6–8 years as we have identified some of the key components of the replication initiation complex, first in the yeast *S. cerevisiae* and subsequently in metazoans, most notably *Xenopus laevis*. This newfound attention has prompted numerous comprehensive reviews on the current status of the field of DNA replication in eukaryotes (27, 29–33). It would be futile to duplicate these insightful and scholarly works. However, as the MCM2–7 proteins gain importance and recognition as the universal replication initiators in the eukaryotic world, I offer in this review an account of the conceptual models that motivated the search for these proteins in the first place. I highlight the key experiments that have influenced our current thinking on the subject and forecast the impact that studies of the MCM proteins may bring in elucidating the molecular mechanism of replication initiation in relation to the biology of cell division, of organism development, and ultimately of cancer. For a complete and exhaustive account on the explosion of information related to the MCM proteins, I refer the reader to a recent review by Kearsey & Labib (29).

Mcms (minichromosome maintenance) were first revealed to be involved in DNA replication as the result of a genetic screen for mutants defective in minichromosome maintenance (34, 35). The best known among these are the MCM2–7 proteins, a family of six conserved proteins that are the key components of the replication initiation complex that initiates DNA synthesis in all eukaryotes (36–38). Mcm1 is a global transcription factor (39) that regulates expression of some of the *MCM* genes and other DNA replication genes (40). A direct role for Mcm1 in the regulation of replication initiation remains to be investigated (41). Mcm10 is another replication initiation factor that intimately interacts with the MCM2–7 proteins in replication initiation (42). It should be pointed out here that Mcm1 and Mcm10 bear no sequence homology to the MCM2–7 family. Other Mcm proteins that bear the name are involved in chromosome segregation rather than replication initiation (43, 44). This review is devoted mostly to the studies of the MCM2–7 proteins, referred to as the MCMs throughout. New findings and implications for the role of Mcm10 in replication initiation are also discussed.

MCM2–7—A FAMILY OF SIX

Sequence Conservation Between Family Members in *S. cerevisiae*

Members of the MCM2–7 family include Mcm2/BM28, Mcm3/P1, Mcm4/Cdc21, Mcm5/Cdc46, Mcm6/Mis5, and Mcm7/Cdc47. A catalog of the

MCM2–7 proteins from bacteria to protists to metazoans has been compiled (29). Genes encoding these proteins were first identified in the budding yeast and the fission yeast either as genes required for the replication of minichromosomes [*MCM* (34, 45, 46) or *mis+* (47)] or genes required for the progression of the cell division cycle [*CDC* (48) or *nda+* (49)]. Initial characterization of Mcm2, Mcm3, and Mcm5/Cdc46 in *S. cerevisiae* implicated the roles of these proteins in DNA replication and indicated that they are related to each other in structure based on sequence similarities (45, 46, 48, 50, 51). Since then, this family of proteins has expanded to include Mcm4/Cdc21 (52, 53), Mcm6/Mis5 (47), and Mcm7/Cdc47 (54). A search of the *Saccharomyces* genome data base indicates that there are only six members in this family. The sizes of the family members range from 776 to 1017 amino acids, with Mcm5 being the smallest and Mcm6 the largest (Figure 1). A comparison of the amino acid sequences of members of the MCM2–7 family suggests that there are several regions of conservation. The largest and most conserved is a stretch of about 200 amino acids in the central region, which includes an element that is similar to the A motif of the Walker-type nucleoside triphosphate-binding sequence GXXGXGKS. This region shows moderate similarity with the NtrC family of bacterial transcription factors (55), which are putative ATPases that facilitate DNA melting at promoters (56). In contrast, the N-terminal and C-terminal conserved regions show interspersed similarities. The overall similarities among members of the MCM2–7 family are about 20%–30%. A potential zinc finger motif of the type $CX_2CX_nCX_2C$ is found at the N-terminal conserved region of Mcm2 (Figure 1). The importance of this putative zinc finger motif ($CX_2CX_{19}CX_2C$) in Mcm2 function has been suggested by mutagenesis (45). A variation of this motif in the form of $CX_2CX_{18}CX_4C$ is found in Mcm4, Mcm6, and Mcm7 (Figure 1).

MCMs are nuclear proteins (50, 51, 57). A bipartite nuclear localization sequence (58), similar to that found in Swi5, has been identified in a non-conserved region of Mcm3 (59). Swi5 is a transcription factor that regulates the expression of cell cycle-specific genes by virtue of its cell cycle-regulated nuclear import (60). Analysis of this Mcm3 nuclear localization sequence indicates that it is essential for the function of Mcm3. As expected of NLS, it is both necessary for the translocation of Mcm3 into the nucleus and sufficient for directing a reporter protein to the nucleus (59). Similar studies of the *S. pombe* Mcm2/Cdc19 protein suggest that spMcm2 also contains an NLS that is essential for the translocation of spMcm2 and perhaps cotranslocation of other members of the MCM2–7 family (S Pasion & S Forsburg, personal communication). A search for potential Cdc28 phosphorylation sites [T/S P X K/R (61)] in the MCM protein family indicates that Mcm3 has five Cdc28 consensus sequences all clustered around the nuclear localization sequence (59). Although some of these sites appear to be phosphorylated in vivo, substitution of the T/S with alanine has little or no effect on cell growth or the nuclear localization of Mcm3 (59). A motif search indicates that Mcm4 has two potential Cdc28 consensus sequences. The *Xenopus* Mcm4 protein has been shown to be a substrate for Cdc2 phosphorylation in vitro and in vivo (62). In general, MCMs are hydrophilic proteins that

contain stretches of negatively charged amino acids, which may account for their anomalous mobilities on sodium dodecyl sulfate gels. Although similar in structure, members of this family are not redundant in function. Each member is believed to perform functions that are essential for cell viability (36, 48, 63; Y Kawasaki & B Tye, unpublished results).

Global Membership in Eukaryotes

Homologs of the MCM2–7 proteins have been identified in all eukaryotes from yeasts to humans (Figure 2). So far, only six classes of MCM proteins corresponding to the six MCM2–7 family members have been identified, suggesting that the functions of these proteins have not diversified further since the emergence of eukaryotes in primordial evolution. Thus, the MCM2–7 protein family forms a superfamily of proteins with six distinct classes. Phylogenetic analysis of eukaryotic MCM sequences shows that each of the six classes of MCM proteins is approximately equally conserved (Figure 2). The species origins of this superfamily of MCM proteins are denoted by a prefix (sc for *S. cerevisiae*, etc). The sequence conservation of MCMs in human and yeast cells within each class varies between 450 and 600 amino acids, which represents 50%–70% of these protein sequences (see colored code in Figure 1).

Identification of the MCM2–7 proteins in higher eukaryotes has been facilitated by three properties of these proteins: their evolutionary conservation, their abundance in proliferating cells, and their physical associations with each other. Cross-reactivities observed in antibodies raised against MCMs from one species with MCMs from heterologous species are common. For example, antibodies

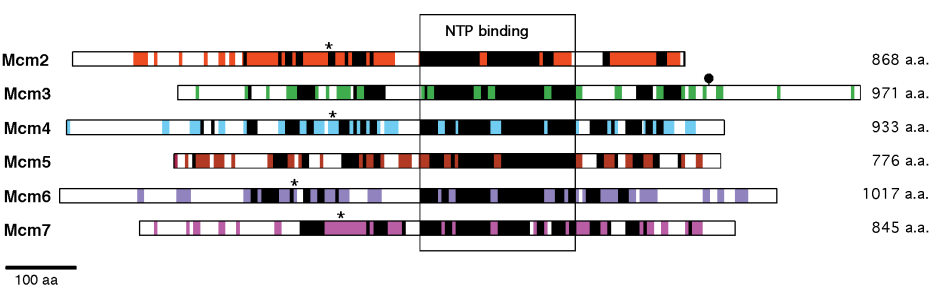

Figure 1 Sequence conservation among *S. cerevisiae* MCM proteins. *Black bars* represent regions conserved between *S. cerevisiae* MCMs and the single MCM protein of *M. thermoautotrophicum*, and *colored bars* represent regions conserved between yeast and mammalian MCMs of the same class. The largest conserved domain contains the nucleoside triphosphate-binding motif. *Asterisks*, putative zinc finger motifs; *solid circle*, nuclear localization sequence of Mcm3; aa, amino acids; NTP, nucleoside triphosphate. This figure was generated by using Sequence Similarity Presenter (202) and is adapted from Reference 29.

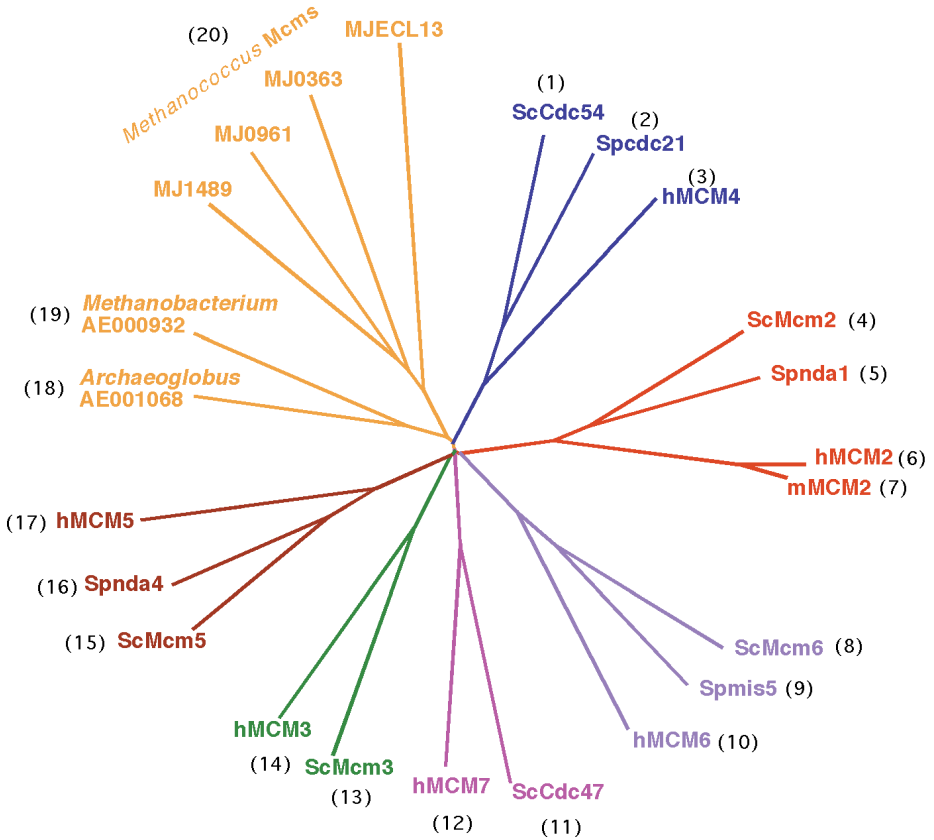

Figure 2 Phylogenetic tree of MCM protein sequences (reproduced from Reference 29). The tree was created from a CLUSTALW alignment, and the figure was generated by using PHYLODENDRON. Sources of sequence data [branch of tree, Reference(s)]: (1), 53; (2), 52, 96, 160; (3), 115, 203; (4), 45, 50, 59, 75, 89; (5), 47, 49, 204, 205; (6), 88, 95; (7), 206; (8), 42; (9), 47; (10), 71, 207, 208; (11), 54; (12), 209; (13), 45, 50, 59, 76, 89; (14), 57, 66, 210; (15), 46, 48, 51; (16), 49, 63; (17), 203; (18), 74; (19), 73; (20), 72.

raised against scMcm3 cross-react with the *Xenopus* xMcm3 (64). Similarly, antibodies raised against hMCM3 had been used to identify the xMcm3 (65). The abundance in the expression of MCM RNAs and MCM proteins in proliferating cells has also facilitated the identification of these proteins. For example, hMcm3/P1 was identified as a contaminant in DNA polymerase α preparations (57, 66). The physical associations between members of the MCM family have aided the identification of family members by copurification (67) or coimmunoprecipitation (68–71).

Ancestral Origin Preceding the Emergence of Eukaryotes

Identification of *MCM* genes was restricted to eukaryotes until recently, when the entire genome sequences of three archaebacteria became available (29). However, unlike eukaryotes, Archaea do not have six *MCM* genes. The *Methanococcus jannaschii* genome has four *MCM* genes, all of which are more closely related to each other than are the six classes of *MCM* genes found in eukaryotes (72). The *MCM* genes of *Methanobacterium thermoautotrophicum* (73) and *Archaeoglobus fulgidus* (74) are of particular interest. Each has only one *MCM* gene. All of these Archaea MCM proteins are marginally more related to Mcm4 than the other MCMs (Figure 2) but can be considered as the generic MCM proteins (Figure 1). A comparison of the sequence conservation between the six MCMs of *S. cerevisiae* and the single MCM protein of *M. thermoautotrophicum* is shown in Figure 1 (*black bars*). Interestingly, the putative zinc finger motif ($CX_2CX_nCX_2C$) characteristic of Mcm2 is conserved in each of the respective MCMs of *M. thermoautotrophicum* and *A. fulgidus* but in only one of the four MCMs of *M. jannaschii.* The conservation of the putative zinc finger motif in a nonconserved region of the Archaea MCM proteins and the eukaryotic Mcm2 protein supports the notion that this motif is essential for Mcm function. The occurrence of MCMs in Archaea suggests that the functions of MCMs may have evolved before the emergence of eukaryotes. These organisms may offer a simplified model for studying the biochemistry of the complex MCM family in eukaryotes.

THE ROLES OF MCM2–7 IN DNA REPLICATION

Origin Usage in the Minichromosome Maintenance Mutants

Identification of some of the MCM2–7 proteins in *S. cerevisiae* was initially motivated by a search for replication initiator proteins that would influence origin usage. The notion that the rate of DNA replication in developing organisms is regulated by the number of origins activated gives rise to the idea that regulation of origin usage might take place at the level of initiator-replicator interaction. It was argued that mutants with defective replication initiation factors should affect the initiation of DNA synthesis at replication origins and should do so with differential effects on individual origins (34). The analogy was drawn between these replication initiation factors and basal transcription factors that act on all promoters but exert different effects on individual promoters. These arguments were the premise for the isolation of the *mcm* mutants (34).

The *mcm* mutants are defective in the maintenance of minichromosomes that contain a centromere and a single replication origin. Although these minichromosomes are excruciatingly sensitive to the *mcm* mutations, natural chromosomes that contain multiple replication origins are only marginally affected (75, 76). This phenotype of the *mcm* mutants has two implications: first, that the *mcm* defect

is probably directed not at centromeres but more likely at replication origins, and, second, that the activation of one or a few replication origins on each chromosome is necessary and sufficient for the complete replication of that chromosome.

The *mcm* mutants so isolated were then subjected to a secondary screen and were tested for differential effects on individual ARSs on minichromosomes (Table 1). Several mutants exhibited this ARS-specific *mcm* defect. Although all minichromosomes were destabilized in these *mcm* mutants, their effects seemed to be more accentuated for minichromosomes carrying certain ARSs. The best example is the *mcm2-1* mutant. At 30°C, a permissive growth temperature for *mcm2-1*, the activities of all ARSs tested are diminished, but, at room temperature, only ARS1 and the telomeric ARS131 are selectively affected. This trend is pervasive in all of the *mcm* mutants. In general, the activity of ARS1 is supersensitive to all of these *mcm* mutations, whereas that of ARS121 is relatively insensitive. Mutants that showed an ARS-specific *mcm* phenotype were then tested for conditional lethality. The original *mcm2*, *mcm3*, and *mcm5* mutants all showed an ARS-specific *mcm* defect, but only *mcm2* and *mcm3* were conditionally lethal and exhibited a cell division cycle arrest in S phase with a nearly doubled (2C) DNA content (76, 77). It is interesting that these *mcm* mutants were virtually identical in phenotypes to the mutants of Cdc46, Cdc47, and Cdc54, which were later shown to be members of the MCM2–7 protein family. Even though these mutants were sought by using very different criteria, the *mcm* mutants showed *cdc* arrests (45, 76), and the *cdc* mutants showed an *mcm* defect (see *cdc46-1* in Table 1; 46) that can be suppressed by inserting multiple ARSs on the minichromosome (78). The *cdc* phenotype (S phase arrest with DNA content of between 1C and 2C) in most of these mutants is consistent with the initiation of DNA synthesis at a small subset of replication origins, resulting in incomplete replication (34, 76). Another interpretation is that these *mcm* mutants may be defective in replication elongation as well as replication initiation.

The properties of these ARS-specific *mcm* mutants suggest that the Mcm proteins are required for the initiation of DNA synthesis at all replication origins. The observed ARS-specificity of the *mcm* mutants was attributed to the inherent differences of replication origins. Consistent with this hypothesis are the major differences in the anatomy of two origins that have the most extreme responses to the *mcm* mutations. ARS1 has a single ARS consensus sequence, a B1 and a B2 element, and a single B3 element, which the transcription factor, ABF1, binds (79–82). ARS121 has four near matches to the 11-base-pair ARS consensus sequence, an enhancer region that may serve the same functions as B1 and B2, and two ABF1 binding sites (18). Interestingly, all *mcm* mutants appear to have a more adverse effect on the same set of ARSs, leading to the suggestion that these proteins may function as a complex to generate the similar effects (34, 45).

Early evidence for a role of the MCM proteins in the initiation of DNA replication comes from two-dimensional DNA gel analysis, in which initiation events can be detected as replicative intermediates that migrate as “bubble” structures (13). Mutants of *mcm2*, *mcm3* (50, 77), and *mcm7* (B Tye & L Homesley,

TABLE 1 Loss rates of minichromosomes in autonomously replicating sequence–specific *MCM* mutants[a]

	Loss of YCp minichromosomes (%) with ARS:				
Strains	**<1>**	**<121>**	**<H2B>**	**<TEL120>**	**<TEL131>**
Wild type (r.t.)	<0.02>	<0.02>	<0.01>	<0.01>	<0.04>
(30°C)	0.05	0.01	<0.01	<0.01	0.03
mcm1-1 (r.t.)	0.21	0.06	0.14	0.09	0.22
Mcm2-1 (r.t.)	0.40	0.02	0.02	0.05	0.36
(30°C)	0.44	0.21	0.20	0.25	0.46
mcm3-1 (r.t.)	0.34	0.18	0.36	0.15	0.35
(30°C)	0.33	0.34	0.46	0.27	0.45
mcm5-1 (r.t.)	0.04	0.02	0.03	0.01	0.06
(30°C)	0.22	0.02	0.03	0.04	0.14
cdc46-1 (r.t.)	0.09	0.03	0.03	0.02	0.08
(30°C)	0.21	0.03	0.12	0.07	0.13
mcm10-1 (r.t.)	0.04	0.03	0.04	0.03	0.05
(30°C)	0.18	0.06	0.19	0.13	0.18

[a]YCp minichromosomes were transformed into different yeast strains, and the loss rate of each plasmid was calculated as previously described (39). Loss rates shown are averages from testing at least two independent transformants whose loss rates varied by < 20%. ARS1, ARS121, and ARSH2B (ARS associated with the histone H2B gene) are single-copy ARSs. ARSTEL 120 and ARSTEL131 are subtelomeric ARSs associated with the repeated X sequences (201). Loss rate is calculated as $1-(F/I)^{1/n}$ where F is the final number of cells bearing plasmids, I is the initial number of cells bearing plasmids, and N = the number of cell divisions. Maximum loss rate per cell division is 0.5.

unpublished results) have been analyzed by two-dimensional DNA gels and shown to have diminished frequency of replication initiation at chromosomal replication origins. Furthermore, as predicted from minichromosome stabilities, ORI1 (chromosomal location of ARS1) appears to be more sensitive to these mutations than ORI121 (chromosomal location of ARS121), suggesting that the effect of the *mcm* mutations on the activity of ARSs may be extrapolated to their activities on chromosomes. However, caution must be taken in correlating ARS activities on plasmids with origin activities on chromosomes, because chromatin organization of replication origins is context dependent (see below). Studies have shown that some origins are active on plasmids (45) but are silent when organized in heterochromatin on chromosome III (83).

MCMs in Replication Licensing

Early mammalian cell fusion experiments suggested that chromatin exists in two distinct replication states during the cell cycle: a replication-competent state and

a replication-incompetent state. In these experiments, G1 nuclei enter S phase and replicate their DNA when fused with S-phase cells. In contrast, G2 nuclei fail to replicate their DNA when fused to S-phase cells (84). These experiments led to the concept that G1 chromatin is modified or "licensed" to replicate and that G2 chromatin is barred from this licensing process to prevent unscheduled DNA synthesis (85). The involvement of MCMs in replication licensing was first suggested by studies with *Xenopus* egg extracts that allow self-assembled nuclei one round of DNA synthesis. In these studies, xMcm3 was identified, based on its abundance in G1 chromatin and its absence from G2 chromatin (86). Furthermore, in a search for replication factors that confer one round of DNA synthesis to assembled nuclei, xMcm3 was purified as an essential component of this replication activity (65, 87). Associations of Mcm3 with other MCMs and with G1 chromatin was also observed in HeLa cells (86, 88) and yeast cells (89). A consistent theme in these studies is that the precise timing of association between the MCMs and chromatin during the cell cycle is critical for the initiation of DNA replication. The MCMs associate with chromatin during G1 phase and dissociate from chromatin during S phase (88–92). These observations led to the hypothesis that the periodic association of MCM proteins with G1 chromatin and their dissociation from S chromatin may play an important role in the restriction of DNA synthesis to once per cell cycle.

Although the integrity of the nuclear envelope (93) and the nuclear import (36, 50, 94) of the MCM proteins have been suggested to be a determining factor in the association of the MCMs to chromatin, subsequent experiments show that MCMs are constitutively nuclear (57, 89, 90, 95, 96). The periodic fluctuation of nuclear MCM proteins visualized by indirect immunofluorescence (50, 51) or green fluorescent protein microscopy (J Li, personal communication) in *S. cerevisiae* is best explained by the clustering of fluorescence caused by the binding of MCM proteins to chromatin (89). The nuclear import of these proteins appears not to be the controlling factor in regulating the association of the MCMs to chromatin (59, 97).

MCMs and Cell Proliferation

If the Mcms play a critical role in initiation of DNA synthesis, their expression is expected to correlate with cell proliferation when DNA replication must precede each cell division. Indeed, the expression of the *DmMCM2* gene in developing *Drosophila* embryos follows a pattern that corresponds to rapidly dividing cells (98). Early embryo replication is supported by maternal *MCM* transcripts, which disappear on cellularization. Zygotic transcription is initially uniform and then follows a pattern of expression that is limited to developing tissues such as the central and peripheral nervous system and then the gut. Inactivation of the *DmMCM2* gene inhibits proliferation of cells in the imaginal discs and central nervous system and causes an apparent prolongation of S phase in the embryonic and larval CNS. A similar study of the *Arabidopsis MCM7* gene indicated that *MCM7* is required for megagametophyte and embryo development and that it is

expressed in dividing cells throughout the plant (99). Another report implicated a role for Mcm3 in the limb development of *Xenopus* (100). Thus, the regulated expression of *MCM* genes in proliferating cells is critical to the development of vertebrates, invertebrates, and plants. The recent identification of two different forms, maternal and zygotic, of Mcm3 and Mcm6 in *Xenopus* suggests that specific forms of MCMs may be used during different stages of development (101; H Takisawa, personal communication).

Although yeast does not have a developmental program to speak of, expression of Mcm3 is restricted to proliferating cells. Mcm3 is abundant in logarithmically growing cells but is down-regulated during diauxic shift and completely shutoff in stationary, starved, or quiescent cells (89). Similarly, expression of *hMCM3* in growth arrested human fibroblasts is low but can be stimulated by serum (57). The correlation between cell proliferation and the abundance of MCMs has been used as an indicator for neoplasticity and may prove to be an effective diagnostic tool for certain cancers (102, 103; R Laskey, personal communication).

Effect of Dosage on Origin Usage

If the level of expression of the MCM proteins correlates with cell proliferation, one might ask whether the level of expression of MCMs correlates with origin usage, a determining factor for the rate of DNA replication. The effect of dosage on the activity of ARSs was examined in the budding yeast, which expresses all six MCM proteins abundantly in logarithmically growing cells. Mcm3 is present in ~2×10^5 molecules per cell, and Mcm2 is present in ~4×10^4 molecules per cell, both in vast excess (100- to 500-fold) over the total number of replication origins in yeast (104). However, only ~10% of the Mcm2 and Mcm3 proteins are in the nucleus, and only half of these nuclear MCM proteins are chromatin bound (89). By this estimation, there would be ~5–25 Mcm2 or Mcm3 molecules per replication origin. Using different extraction procedures, similar amounts of Mcm5 and Mcm7 were estimated to be associated with chromatin (29, 105).

Diploids containing only one copy of *Mcm2*, *Mcm3*, or *Mcm6*, each of which expresses a half dose of one of the Mcm proteins, were tested for origin usage. Interestingly, reducing the dosage of Mcm2 by half results in diminished usage of specific ARSs (104). Similar dosage effects of Mcm6 on origin usage were observed (Y Kawasaki & B Tye, unpublished results). In contrast, reducing to half the dosage of Mcm3, the most abundant member of the Mcm family, appears to have little or no effect on origin usage. These results suggest that a significant molar excess of MCM proteins relative to replication origins is required for the proper initiation of all replication origins. The simplest explanation is that, whereas individual MCM proteins may be present in excess, the assembled complex involving all six MCMs that bind to replication origins is present in limiting quantities. If so, then the subunit present in the lowest concentration and/or with the highest dissociation constant would be limiting for replication initiation. The relationship between

MCM concentrations and origin usage may provide a molecular basis for the abundant expression of MCMs in proliferating cells (98, 99, 106, 107).

BIOCHEMICAL PROPERTIES OF MCM PROTEINS

Interactions Between Members of the MCM2–7 Family

Interactions between Mcm4 and Mcm5 were first suggested genetically by the allele-specific suppression of *cdc54-1* (*mcm4*) by *cdc46-5* (*mcm5*) (108). Physical interactions between members of the MCM proteins were later demonstrated by using different methods in different organisms, including human (109), *Xenopus* (67, 70, 110), *Drosophila* (111), the budding yeast (104, 112), and the fission yeast (68). Immunoprecipitation of one of the MCM proteins often leads to the coprecipitation of all six members of the MCM (67, 68, 70, 110). Two-hybrid analysis and glutathione-S-transferase affinity chromatography indicate that MCMs show self interactions and interactions with each other in different pairwise combinations, although affinities for different partners may vary significantly (104; Y Kawasaki & B Tye, unpublished results). Sedimentation velocity or gel filtration studies indicate that the MCM proteins cosediment as large complexes that are consistent with hexamers (104, 109, 113) and smaller complexes that are consistent with tetramers (67, 113, 114), trimers (115), and dimers (66, 104). The stability of the larger complexes appears to be salt sensitive (66); at 0.5 M NaCl, hMcm3 and hMcm5 sediment mostly as dimers, but, at 0.15 M NaCl, they sediment in larger complexes of about 560 kDa. The extent of sensitivity to salt concentration seems to be dependent on the anion type; the Mcm complex is more stable in sodium acetate or sodium glutamate than in NaCl (68). In addition, the Mcm3 protein associated with complexes of different sizes appears to be phosphorylated to different extents (104). Thus, the oligomerization states of the MCM proteins may be regulated by their phosphorylation states. It is unclear whether these different oligomerization states represent distinct complexes that have specific functions or subcomplexes that are intermediates in the assembly or disassembly of the larger complexes. The first steps toward differentiating these possibilities are to examine the structure and composition of these complexes and then investigate whether any of the complexes have enzymatic activities or bind DNA. It is important to bear in mind that, although the MCM complex may be a key component of the replication initiation machinery, without its accessory proteins, MCM complexes may be devoid of DNA binding, ATPase, or helicase activities.

So far, the only reported enzymatic activities associated with MCM proteins have been observed with a multimeric complex containing hMcm2, hMcm4, hMcm6, and hMcm7. This complex is purified from HeLa cell extracts by histone H3 affinity chromatography based on the electrostatic interactions of the negatively charged Mcm2 and the positively charged histone H3 protein (116).

Interestingly, this tetrameric complex is devoid of activity unless Mcm2 is removed. The resulting complex containing hMcm4, hMcm6, and hMcm7 is associated with an ATPase activity and a weak helicase activity that unwinds a 17mer duplex but not a 34mer duplex (114). The physiological significance of these MCM-associated activities requires further investigation.

The MCM Hexamer

The MCM complex with the highest oligomerization state involving all six members has been the focus of attention. Stable hexameric complexes with identical subunits are likely to exist in one of two conformations, a globular hexamer (Figure 3*A*) or a planar hexamer (Figure 3*B*). Ultrastructural studies on the MCM complexes were carried out by using *S. pombe* proteins extracted from G2-phase cells and purified by a combination of gel filtration and immunoaffinity chromatography (68). The purified complex has a molecular mass of about 560 kDa and a Stokes radius of 76 Å, containing nearly equal quantities of each of the six MCM proteins. Electron microscopic images showed that the MCM complex has a globular shape of about 27-nm diameter (68), which may or may not contain a central cavity (Y Adachi, personal communication). Images containing a central cavity that reveal fine details suggest that, depending on the axis of viewing, these globular structures show two (Figure 3*C*, part *c*), three (Figure 3*C*, parts *a* and *e*), four (Figure 3*C*, part *b*), or six (Figure 3*C*, part *d*) subdomains. A structural model for a hexamer that has a globular shape and subdomain structures consistent with these electron-microscopic images is the staggered double trimer (Figure 3*A*). This globular structure, an octahedron with six vertices and eight faces, has a deep cavity when viewed along any one of the four axes perpendicular to the plane of any one of its eight faces. However, this hexamer would appear as a solid globular structure when viewed along any one of the three axes that pass through two of the subunits on opposite sides. In this model, the MCM heterohexamer is composed of two staggered heterotrimers (Figure 3A, upper right), three heterodimers (Figure 3A, lower right), or a heterotetramer with two monomers on opposite sides (Figure 3A, lower left). The apparently different electron-microscopic images shown in Figure 3 can be interpreted as subcomplexes of the hexamer (Figure 3*C*, *a* and *b*) or different views of the hexamer (Figure 3*C*, *c–e*). These features of the hexameric complex are consistent with many of the properties of the MCM complexes. For example, the spatial arrangement of the subunits in this model requires the contact of every subunit with four other subunits. Indeed, experimental results suggest that each Mcm protein can interact with multiple partners to form dimers (66, 104). In addition, MCM proteins are often associated with smaller complexes that are consistent with subcomplexes of the hexamer. Several reports suggested that Mcm2, Mcm4, Mcm6, and Mcm7 are associated with one another in tetramers (67, 113, 114) and that Mcm4, Mcm6, and Mcm7 are associated with each other in trimers (71, 114). Similarly, Mcm3 and Mcm5 appear to have a special affinity for each other (66, 67, 104, 117). It is conceivable that these subcomplexes represent preassembled or disassembled segments of the larger hexameric MCM complex. Because both

Mcm2 and Mcm3 contain an NLS, it is possible that subcomplexes of Mcm3 and Mcm5 and subcomplexes of Mcm2, Mcm4, Mcm6, and Mcm7 enter the nucleus as preassembled dimers and tetramers, respectively, which are then assembled to form the hexameric complex in the nucleus (S Forsburg, personal communication).

The conserved ATPase motif shared by members of the MCM family suggests that the hexameric complex may be associated with ATPase and/or helicase activities. However, analysis of these complexes has so far tested negative for these enzymatic or DNA-binding activities (68). These results are perhaps not surprising because these complexes are purified from asynchronous cultures of *S. pombe* and therefore from predominantly G2-phase cells when MCMs are not associated with chromatin. However, MCM hexamers purified from cells synchronized with hydroxyurea at the beginning of S phase are also devoid of any enzymatic activities (69). Similar studies on human and *Xenopus* MCM complexes also showed no DNA binding or ATPase activities (R Knippers & J Blow, personal communications).

The Active MCM Complex Is Likely To Be Localized to Chromatin

The vast abundance of MCM proteins relative to the total number of replication origins in budding yeast suggests that, if the function of MCM proteins is to initiate DNA replication, the active form of MCM complexes must represent only a minor species of the total MCM proteins that are present during the G1 phase. Studies in the budding yeast showed that, although the transcription of the *MCM* genes is periodic in the cell cycle, reaching the peak at M phase (51, 53, 54), the cellular MCM proteins are constitutively present in high abundance in proliferating cells (89, 105). These studies also showed that Mcm2 and Mcm3 proteins are distributed to both the cytoplasm and nucleoplasm in relatively constant levels throughout the cell cycle. However, about 5%–10% of these proteins are tightly associated with chromatin from early G1 phase to the beginning of S phase, when replication initiation occurs (85). This chromatin-bound form of Mcm proteins is resistant to 2 M salt washes and would be released from the chromatin fraction only by DNase treatment (50, 89, 105).

There is some evidence that the MCMs bind to chromatin as a complex. Immunoprecipitation of Mcm7 cross-linked to chromatin in budding yeast indicates that binding of Mcm7 to replication origins requires the function of Mcm5 (118). Chromatin immunoprecipitation in HeLa cells at the G1- to S-phase transition indicates that all six MCMs colocalize on sheared DNA fragments of ~500 base pairs, suggesting that they are bound to chromatin as a multimeric complex containing all six subunits (119). MCM proteins exist in distinct isoforms that have different phosphorylation states in different phases of the cell cycle. G1-phase Mcm3 proteins appear to be less phosphorylated than S- or M-phase Mcm3 proteins (89). Chromatin-bound forms of human Mcm2 and Mcm3 proteins are also hypophosphorylated compared with the S-phase–soluble Mcm2 and Mcm3 proteins (88, 89). Thus, phosphorylation appears to play

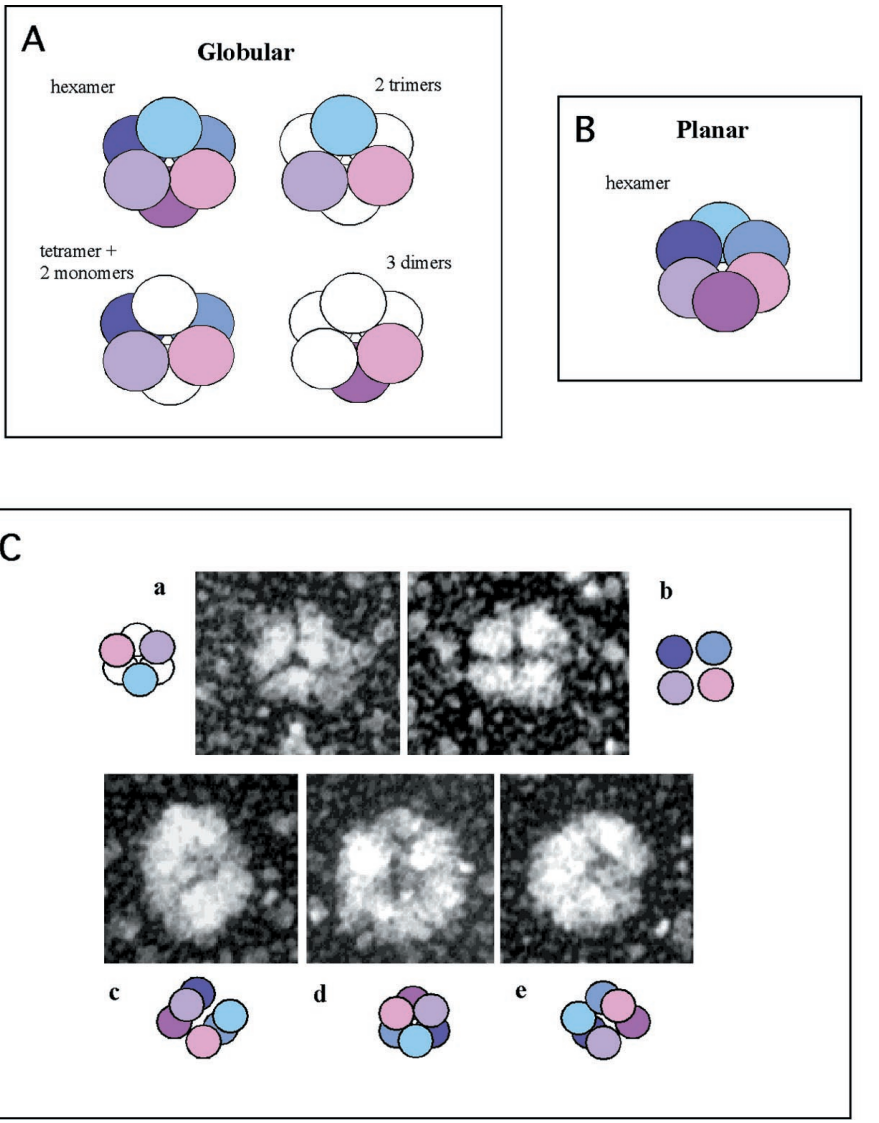

Figure 3 Structural models for the hexameric MCM complex. (*A*) A globular hexamer that consists of a staggered double trimer, three dimers, or a tetramer with two monomers. (*B*) A planar hexamer. (*C*) Electron micrographs of MCM complexes visualized by rotary shadow. The complex has a globular shape that shows three (*a*), four (*b*), two (*c*, *e*), or six (*d*) subdomains. The cartoon captions are interpretations for the structure of the images. The diameter for images *a* and *b* is about 23 nm, and the diameter for images *c*, *d*, and *e* is 27 nm. Electron micrographs are provided by Y Adachi and reproduced from Reference 68.

an important role in the regulation of the activities as well as the oligomerization states of the MCM complexes. Taken together, these observations suggest that, because MCMs are abundant proteins that are present in both the cytoplasm and the nucleus at all stages of the cell cycle, purification of the active form of MCMs may require extraction from chromatin during the G1- to S-phase transition.

A TWO-STEP MECHANISM IN THE REGULATION OF ONCE-PER-CELL CYCLE REPLICATION

The concept that chromatin exists in two different replication states, a replication-competent state and a replication-incompetent state, suggests that a temporal separation of these two states may provide the mechanism for restricting DNA synthesis to once per cell cycle (27). The molecular detail for this mechanism is embodied in the assembly of a pre-RC at replication origins before the initiation of DNA synthesis and the removal of the pre-RC after the initiation of DNA synthesis. If the window of opportunity for the assembly of the pre-RC is restricted to only the G1 phase, then DNA synthesis can only occur once per cell cycle. These cyclical events proceed like clockwork under the cues of two cell cycle-regulated protein kinases, Cdc28-Clb and Cdc7-Dbf4 (Figure 4). The picture that has emerged in the last several years suggests that the recruitment of Mcm proteins to replication origins is synonymous with the replication competence of replication origins. In *Xenopus* terminology, MCMs are the key components for the replication licensing of chromosomes.

The Assembly of the Prereplicative Complex

The ORC, like the MCMs, is evolutionarily conserved from yeasts to humans (32). When the ORC was purified as a complex of six subunits that bind to the consensus elements of yeast replication origins, it was believed to be the eukaryotic initiator for DNA replication. Mutations in the subunits of ORC exhibit phenotypes consistent with initiator mutants: *orc2*, *orc5*, and *orc6* mutants arrest at the beginning of S phase (78, 120, 121), and they show an *mcm* defect (122). Thus, the ORC proteins fit the classical definition of a replication initiator; they bind specifically to replication origins, and they are essential for replication initiation. However, studies in budding yeast indicated that ORC is essential but not sufficient for replication initiation. ORC binds replication origins constitutively, and, therefore, its binding to origins alone cannot be the determining factor for initiating DNA synthesis. On the other hand, the MCM proteins, which have not been reported to contain DNA-binding activity, either individually or in different states of oligomerization, bind chromatin in a very defined period of the cell cycle between early G1 phase and the beginning of S phase. The recruitment of MCMs to replication origins appears to depend on a short-lived protein, Cdc6,

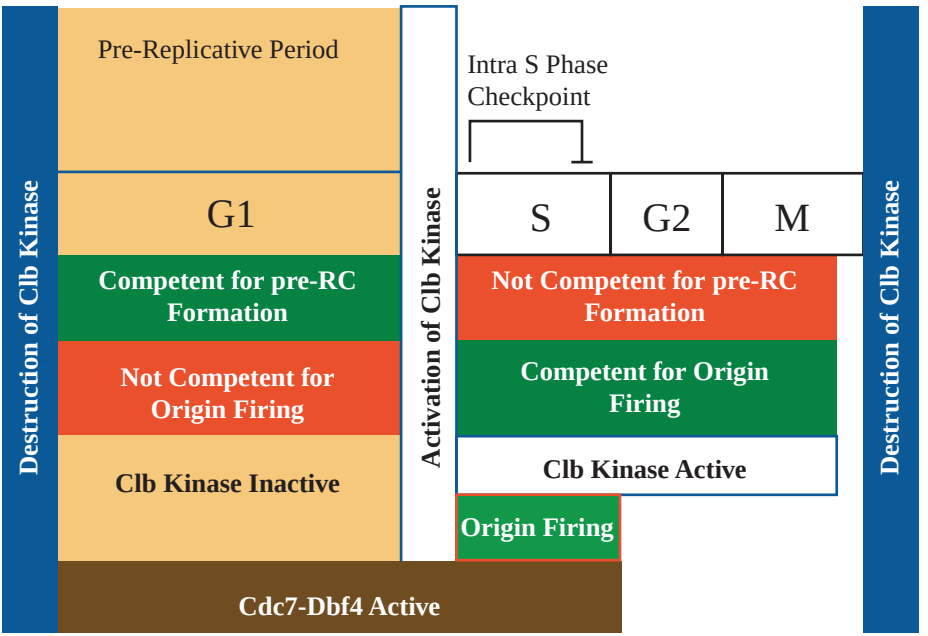

Figure 4 Restriction of DNA replication to once per cell cycle by the temporal separation of the two replication states of chromatin organization at replication origins. Oscillation between the replication-competent and replication-incompetent states is regulated by fluctuations in the cyclin B (Clb) kinase activity, which couple S phase and mitosis. Assembly of the pre-RC during the G1 phase begins with the degradation of M-phase Clb kinases that negatively regulate the assembly of the pre-RC. Cdc7-Dbf4 kinase is active during G1 and S phase, whereas S-phase Clb kinases are active during S phase. When both Cdc7-Dbf4 and S-phase Clb kinases are active, replication initiation or origin firing occurs. This period defines S phase. Assembly of the pre-RC is prevented when Clb kinases are active during S, G2, and M phases. The intra–S-phase checkpoint imposed by Mec1, Rad53 provides temporal regulation of origin firing during S phase. This figure is adapted from Reference 27.

which is expressed only during the G1 phase (123–125). This alternating process of assembly and disassembly of the pre-RC at replication origins was verified by footprinting analysis (24, 25, 126). During the S phase or M phase, replication origins are occupied by a complex known as the post-replicative complex (post-RC), which shows a DNase1 protection pattern that is similar to that of ORC-bound naked origin DNA (Figure 5, *iv*). During G1 phase, replication origins are occupied by the pre-RC which shows an enlarged footprint, suggesting that additional protein factors have been recruited to the replication origins (Figure 5, *i*). The enlarged footprint is formed only if ORC and Cdc6 are present and active. The contribution of the recruited MCM proteins to this enlarged footprint is sub-

tle (127). However, the presence of the MCMs in the pre-RC can be inferred from chromatin-binding experiments first demonstrated in *Xenopus* (124) and then in yeast (89, 105, 128) and later verified by the in vivo cross-linking of MCMs to replication origins (92, 118). The protein assembly that produced this footprint is defined as the pre-RC (25). Other proteins that are recruited to the pre-RC after the binding of MCMs appear not to affect the footprinting pattern of the pre-RC (25, 127). These include Cdc45 and Cdc7-Dbf4. All of the proteins known to be recruited to the pre-RC have been shown to interact with MCM2–7 genetically (32). In addition, Cdc45 (128, 129) and Cdc7-Dbf4 (77) have been shown to physically interact with the MCMs. After the assembly of the pre-RC, the actions of two S-phase–promoting protein kinases, Cdc28-Clb5/6 and Cdc7-Dbf4, are required before the recruitment of DNA polymerase α-primase to the replication origins (130). Figure 5 summarizes the interactions between the components of the pre-RC, their order of assembly during G1 phase, and the sequence of events involving Cdc45 and the cell cycle-regulated protein kinases at the G1- to S-phase transition that culminated in the initiation of DNA replication. The replication initiation machinery is reset when ORC rebinds origin DNA immediately after initiation to form the post-RC. In this section, I focus on the proteins that are involved in the assembly of the pre-RC and their functional relationship with the MCMs. The roles of the two S-phase kinases in the activation of the pre-RC are also discussed.

The Origin Recognition Complex

Replicon Size Determination If the major roles of ORC are to mark the sites of replication initiation and to recruit the Mcm proteins, then it is conceivable that ORC could play a direct role in determining origin usage via its interactions with chromatin and/or Mcms and their chaperones. The identification of ORC in *Xenopus* oocytes (131), which have no preferred initiation sites for DNA replication, provides a means to investigate the mechanism that determines origin usage or replicon size in higher eukaryotes. Only two of the ORC genes, xORC1 and xORC2, have been cloned, but both xOrc1 and xOrc2 are associated with a complex of six proteins, which presumably are the other subunits of the xORC (132). In *S. cerevisiae*, the ORC is a very stable complex that is estimated to be present at between 400 and 600 copies per cell. By this estimation, there is about one ORC per replication origin in yeasts (133). It is estimated that there are about 3.5×10^5 chromatin-bound xOrc2 protein molecules per nucleus in *Xenopus* early embryos, which translates to about one ORC complex per 8 kilobases (kb) of DNA (134). The replicon size for early embryos before the midblastula transition is about 7.2 kb (135). In contrast, the replicon size in somatic cells is about 172 kb (136). This transition in replicon size probably occurs sometime during midblastula transition. With the *Xenopus* cell-free system, the ratio of nuclei to cytoplasm can be manipulated to reflect the early development of *Xenopus* (134). At 2000 nuclei/μl of extract, the replicon size is estimated to be about 7.2 kb. At > 2000 nuclei/μl of extract, the replicon size increases, reaching an average of about 59

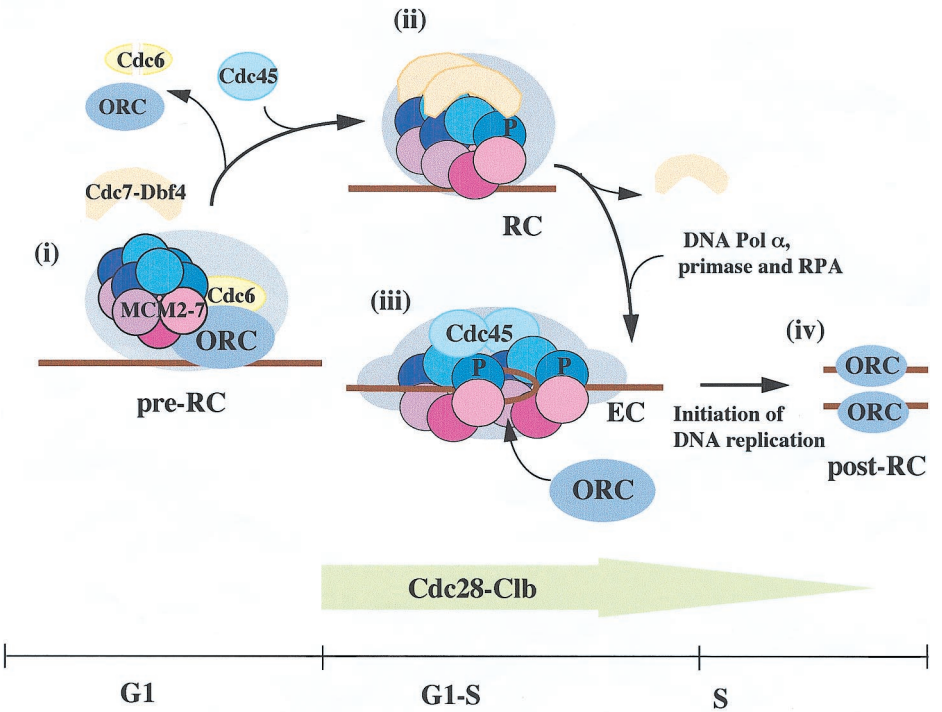

Figure 5 The sequence of events during the G1-phase to S-phase transition, which leads to the initiation of DNA replication. (*i*) The pre-RC is assembled during the G1 phase. The order of assembly of the pre-RC is ORC, Cdc6, and then MCM2–7. During G1 phase, Clb kinases are inactive, but Cdc7-Dbf4 kinase is active, and Cdc6 loads MCM2–7 onto replication origins, presumably by interacting with ORC, to form the pre-RC. (*ii*) Once the pre-RC is assembled, Cdc6 and ORC are dispensable and may leave the replication origin. As the cell progresses from the G1 phase to S phase, Cdc7-Dbf4 interacts with the MCM complex. Phosphorylation (P) of Mcm2 by Cdc7-Dbf4 induces a conformational change in the MCM complex that is critical for the subsequent melting of origin DNA. At this point, activation of the S-phase Clb kinases may be required for the recruitment of Cdc45 to the pre-RC. (*iii*) Cdc45 physically interacts with the MCM complex and is believed to move from the replication origin to inter-origin regions possibly as a component of the elongation complex (EC). Initiation of DNA synthesis occurs when RPA, primase, and DNA polymerase α are recruited to the melted replication origin. (*iv*) ORC is likely to rebind the vacated replication origin to form the post-RC as the RC transforms into the EC. *Gray shapes* represent complexes that may include factors that have yet to be identified.

kb at 10,000 nuclei/μl. However, ORC did not become stoichiometrically limiting for initiation in these experiments. Similar amounts of ORC bind to chromatin regardless of replicon size, suggesting that, at the optimal condition, all chromatin-bound ORC is active for replication initiation, but, as the nuclei-to-cytoplasm ratio increases, some factor other than xORC becomes limiting. Because neither xCdc6 nor xMcms appeared to be limiting in these experiments, it was concluded that replicon size is controlled by yet another factor that becomes limiting at the midblastula transition. In the budding yeast, although chromatin-bound MCMs appear to exceed the total number of replication origins (89), varying gene dosages showed that Mcm2 and Mcm6 are limiting for replication initiation (104) (Y Kawasaki & B Tye, unpublished results). A plausible explanation is that not all chromatin-bound MCMs are components of the pre-RC or engaged in the same activity. There is some evidence that may link hMcm7 to other functions (137). Thus, caution should be taken in equating individual chromatin-bound MCM proteins with active MCM complexes in the pre-RCs in titration experiments.

Where is the Origin Recognition Complex at the G1- to S-Phase Transition? Although it is generally accepted that ORC binds replication origins throughout the cell cycle, it is important to revisit those experiments that led us to this conclusion. The postreplicative footprints of replication origins from S phase to M phase closely resemble the in vitro footprints of ORC on naked DNA. Thus, there is little doubt that ORC represents at least part of the postreplicative complex that binds origin DNA from S to M phase (24). However, the expanded footprints of the pre-RC observed at replication origins during the G1 phase give very little information about which components of the pre-RC actually contact DNA (24, 25). Three recent experiments suggest that ORC may not necessarily be localized at replication origins during the brief period after the assembly of the pre-RC but before the initiation of DNA replication (Figure 5 *ii*). First, chromatin-binding studies in *S. cerevisiae* suggest that, after the MCMs have been loaded onto chromatin, Cdc6 and ORC can be removed by salt washes without affecting the binding of the MCMs (105). This result suggests that the anchoring of the MCMs to origin DNA is not mediated by Cdc6 or ORC once they are recruited to the origins of replication. Second, studies in *Xenopus* cell-free egg extracts showed that removal of Cdc6 and ORC after the loading of MCMs by immunodepletion does not affect the binding of MCMs to chromatin nor the subsequent activation of replication initiation by Cdk2 (138). This result suggests that Cdc6 and ORC are required for the recruitment of MCMs but dispensable for replication initiation. Third, chromatin immunoprecipitation experiments indicate that late in G1 phase, cross-linking of ORC to origin DNA becomes greatly reduced until the beginning of S phase (92). However cross-linking of ORC to origin DNA during G1 phase is enhanced when Cdc6 or Mcm5 is inactivated. An interpretation of these results is that ORC is present and may be required at the origin only up to the point when the pre-RC is assembled. These three results together suggest that, after the assembly of the pre-RC, ORC and Cdc6 are no longer required at

replication origins and ORC may briefly leave the replication origins with Cdc6 before reassociation with replication origins (Figure 5 *iii, iv*). Further experiments are necessary to dissect the sequence of events that take place during the G1- to S-phase transition.

Cdc6/Cdc18—the Matchmaker

The Cdc6 protein is essential for the formation of pre-RCs at replication origins. Formation of the pre-RC, which is characterized by an additional protected region adjacent to the ORC-binding site, occurs at a time when Cdc6 first appears during late anaphase or early G1 phase (123, 126). In the absence of de novo synthesis of Cdc6 during the G1 phase, pre-RCs are not formed. Cdc6p is required for both the establishment and the maintenance of the pre-RC: Pre-RCs are thermolabile in a *cdc6* temperature-sensitive mutant (125). There is a point of no return in late G1 phase after which expression of Cdc6 will no longer promote pre-RC formation. That point appears to coincide with the expression of the S-phase cyclins Clb5 and Clb6 (125, 139). Deletion of *CLB5* and *CLB6* delays the point of no return so that the window of opportunity for Cdc6 action is prolonged (28). *CDC6* interacts genetically with ORC, and recombinant Cdc6p can interact in cell extracts with ORC (140). Cdc6p is required for the loading of MCMs on prereplicative chromatin, a mechanism that is conserved from yeast to *Xenopus* (105, 118, 124). These observations suggest that Cdc6 is a component of the pre-RC and that its major role, in cooperation with ORC, is to recruit the MCMs to replication origins. Thus, understanding the properties of Cdc6 may provide useful information on the functions of the MCMs.

Cdc6 is related to two subunits of the ORC, Orc1p (141) and Orc4p (142), both of which contain a nucleotide-binding-site motif. Although direct binding of nucleoside triphosphate by Cdc6 has not been demonstrated, mutagenesis of the conserved motifs suggests that Cdc6 is a nucleotide-binding protein (143). The Walker A and B motifs of Cdc6 have two genetically separable functions: The A motif is essential for productive interaction with replication origins, and the B motif is essential for the subsequent loading of MCMs onto chromatin. In addition to Orc1 and Orc4, Cdc6 shows significant sequence similarities to a superfamily of loading factors that load ring-shaped DNA polymerase processivity factors onto DNA (143). These loading factors include subunits of the replication factor C (RF-C) (144, 145), which loads the eukaryotic sliding-clamp protein proliferating-cell nuclear antigen (PCNA) (146) onto DNA. PCNA, the processivity factor of the DNA polymerase δ holoenzyme, is a trimeric ring-shaped complex that has a pseudo-sixfold symmetry (Figure 3*B*). In a reaction that may be analogous to the loading of MCM complex by Cdc6, the clamp loader RF-C first forms a labile complex with PCNA in a reaction that is dependent on the binding of ATP (147, 148). In a second step, RF-C forms a stable complex with PCNA at the primer terminus in a reaction that involves the breaking of the PCNA ring and inserting of DNA into the central cavity of the ring. The breaking

and resealing of the sliding clamp require hydrolysis of ATP. The analogy between the loading of PCNA by RF-C and the loading of the Mcm complex by Cdc6 onto chromatin may be instructive in understanding the function of the chromatin-bound MCM complex. Helicases and sliding clamps that grip DNA and move along it often are ring-shaped oligomers that have a six fold symmetry (Figure 3*B*). However, a major difference between the two is that helicases, such as the simian virus 40 (SV40) large-T antigen (149, 150) and the *Escherichia coli* branch migration protein RuvB (151), can assemble onto DNA without the aid of a second protein factor to get on and off DNA. Based on this analogy, the MCM complex, in the form that is being loaded onto chromatin by Cdc6, may be more similar to a sliding clamp than a helicase.

What exactly is the role of Cdc6 in the initiation of DNA replication? Recent studies suggest that Cdc6 is required only for the loading of the MCM complex but is dispensable for the subsequent initiation event (105, 138). This property of Cdc6 is similar to that of a class of proteins known as the molecular matchmakers, which, in an ATP-dependent reaction, brings two compatible but otherwise solitary macromolecules together, promotes their association by a conformational change, and then leaves the complex (152). Examples of molecular matchmakers include MutL in the mismatch repair of *E. coli* (153), DnaC in the assembly of primosomes that synthesize primers at origins and for Okazaki fragments (154), and the clamp loaders for the DNA polymerase clamps (155). In making these conceptual comparisons, it is important to remember that, although there is mounting evidence that Cdc6 and ORC are required for the loading of MCM proteins on chromatin, there is no direct evidence for the physical interaction between MCM2–7 and Cdc6. Future objectives of investigation would be to identify the match of the MCMs that Cdc6 made—for example, do the MCMs make contact with origin DNA, histones (116), or other protein factors (42); to characterize the conformational change in MCMs induced by Cdc6 that results in the tight binding of MCMs to chromatin; and to dissect the molecular events that accompany the binding and hydrolysis of ATP by Cdc6.

Mcm2–Mcm7, a Role in Initiation As Well As Elongation

Evidence for the binding of MCM2–7 to replication origins has been difficult to produce because thermolabile MCM proteins do not significantly affect the DNase1 protection pattern of the pre-RC at replication origins (127). Experiments to determine the order of assembly of the pre-RC were based on chromatin binding not specific to replication origins. Simple calculations allow one to estimate the number of MCM complexes that may be localized at replication origins. In budding yeast, each MCM is present in ~10^4–10^5 molecules per cell (29), but only about 5%–10% of the MCM proteins are chromatin bound (89). Based on this premise, if the number of hexameric complexes formed is bound by the availability of the subunit in the lowest concentration (104) or with the

highest dissociation constant, then there could be as few as one to two MCM complexes per replication origin. We note that, although estimates of the total amount of MCM proteins per cell are in general agreement between different groups (29), estimates of the percentage of total cellular MCM proteins that are chromatin bound vary for the different MCM proteins by different groups (89, 105). These variations may be real if different MCM proteins have functions other than their roles as components of the pre-RC.

With chromatin immunoprecipitation, MCM proteins could be cross-linked in vivo to origin DNA by formaldehyde (92, 118, 128). The cross-linking of the MCMs to replication origins is dependent on a functional replication origin, a functional ORC, and a functional Cdc6. In a time course experiment in which synchronized cells are allowed to traverse from G1 phase into S phase, the localization of the MCM proteins based on cross-linking appears to move from replication origins to interorigin regions, before the Mcms are finally released from chromatin. The localization of Mcms at replication origins during the G1 phase and the dependence of this localization on ORC and Cdc6 support the notion that the MCMs are components of the pre-RC. However, the observation that at least two of the MCMs, Mcm4 and Mcm7, are also localized at interorigin regions at increasing distances from the replication origins during S phase suggests that the MCMs may have functions beyond their roles as components of the pre-RC in the initiation of DNA replication (see discussions in 33, 92).

What might be the other roles for the MCMs? One possibility is that, analogous to the large-T antigen of SV40, the MCMs may be involved not only in the initial binding (156, 157) and melting of replication origins (158) but may also act as a helicase at the elongation forks (159). This hypothesis is consistent with the conserved ATPase domain shared by all members of the MCM2–7 family (29, 55), and the weak helicase activity reported to associate with a hexamer that contains three of the MCMs, Mcm4, Mcm6, and Mcm7 (114). It also explains the kinetics of cross-linking of Mcm4 and Mcm7 with interorigin DNA (92). Current results, limited to the study of only selective members of the MCM proteins, do not exclude the possibility that only a subset of the MCM proteins acts as helicase in a subsequent step. This hypothesis, however, does not explain the observations suggesting that Mcm4 (160) and Mcm5 (51) are not required after the hydroxyurea block in budding yeast and fission yeast. Perhaps the mutant alleles analyzed in these studies are defective only in initiation but not elongation, even though the wild-type proteins are required for both functions. The isolation of new *mcm* mutant alleles that specifically disable each of these functions would address the question of whether the MCM proteins are involved in multiple steps during DNA replication.

An alternative explanation for the cross-linking of MCM proteins to interorigin DNA during S phase is that MCM proteins are bound not only to replication origins but also along the chromosome at interorigin regions. As the replication forks migrate along the interorigin region, the MCM proteins become more susceptible to cross-linking (33). The binding of MCM proteins along the entire length of

chromosomes has been observed in *Xenopus* self-assembled nuclei (93, 161) and in mammalian cells (88, 90). In HeLa cells, each MCM protein is present in ~10^5–10^6 molecules per cell (29) and therefore in about one hexameric complex per 10 kb of DNA if each complex contains one of each MCM protein. Double-labeling immunofluorescence microscopy in mammalian cells and *Xenopus* egg extracts suggests that the bulk of the chromatin-bound MCMs do not colocalize with sites of nucleotide incorporation of nascent DNA or "replication foci" (124, 161). However, other proteins involved in DNA replication, such as Orc2p, and Cdc6p, also fail to colocalize with replication foci. To investigate whether MCMs and ORC colocalize at the same sites on chromatin in vertebrates, it is important to use approaches that yield a resolution better than that attained by indirect immunofluorescence microscopy.

Two experiments with HeLa cells address the question whether hMCMs and hORC are bound to chromatin in juxtaposition to one another. In the first set of experiments, hMCM proteins and hORC are released from nuclease-digested chromatin with different kinetics. hMCMs are released with mild treatment of micrococcal nuclease, whereas hORC proteins are released only after extensive nuclease digestion (162). In the second set of experiments, hORC and hMCMs were cross-linked to chromatin by formaldehyde in vivo in thymidine-starved cells. Specific immunoprecipitations showed that cross-linked nucleoprotein fragments carried either hMCM proteins or hOrc2p but not both (119), suggesting that MCMs and ORC do not colocalize on DNA fragments of ~500 base pairs. An alternative explanation for these results is that subsaturating conditions were used in cross-linking, resulting in no more than one protein-DNA cross-link per DNA fragment. Although cross-linking of multiple subunits of the MCM complex to the same DNA fragment was detected, cross-linking of subunits of a complex may be mediated by protein-protein cross-linking. Further experimentation is required to provide insight into the relationship between ORC and MCMs in the organization of replication initiation sites in higher eukaryotes.

Cdc45—a Role in the Transition of Prereplicative Complex to Replicative Complex

Cdc45 is a protein essential for the initiation of DNA replication. The direct interaction between Cdc45 and the MCM proteins suggests that its role in replication initiation must be coupled to the actions of the MCM proteins at replication origins. Mcm5 and Mcm7 were initially identified by their mutant alleles that specifically suppress the *cdc45* mutation (48). Allele-specific extragenic suppression is often the result of the restoration of physical interactions between two mutant proteins (163, 164). Subsequent experiments showed that Cdc45 coimmunoprecipitated with Mcm2, Mcm5, and Mcm7 (128, 129, 165). Cdc45 is associated with chromatin after "Start" in late G1 and during the S phase of the cell cycle. Tight binding of Cdc45 to chromatin is dependent on the Cdc28-Clb kinase activity as well as functional Cdc6p and Mcm2p (128). The timing of the association

of Cdc45 with the pre-RC and the activation of Cdc28-Clb suggest that Cdc45 is recruited after the assembly of the pre-RC and the activation of the S-phase Cdc28 kinase. Indeed, genomic footprinting analysis indicates that the DNase1 protection pattern of the pre-RC is insensitive to thermolabile Cdc45 (166). When the temporal order of actions of Cdc45 and Cdc7 was examined in a genetic experiment in which each gene was inactivated in sequence, it was found that the action of Cdc45 is dependent on a functional Cdc7 and vice versa (166). Because the action of Cdc7 is required for the entry of S phase, these results suggest that the action of Cdc45 is coincident or nearly coincident with the entry into S phase (Figure 5 *ii*, *iii*). During S phase, Cdc45 physically interacts with the MCM proteins on chromatin; however, dissociation kinetics of Cdc45p from chromatin are slower than those of MCMs, suggesting that the proteins are released by different mechanisms (128). On the other hand, in chromatin cross-linking studies, Cdc45 appears to change its localization from replication origins at late G1 phase to interorigin regions during S phase, with similar kinetics to that of polϵ, suggesting that, like the MCM proteins, Cdc45 may also be associated with the elongation machinery (92; Figure 5 *iii*).

Homologs of Cdc45 have recently been identified in *Xenopus* and in human cells (167). By using *Xenopus* egg extracts, it was shown that xCdc45 is essential for DNA replication in that depletion of xCdc45 abolishes replication activity. xCdc45 physically interacts with DNA polymerase α in the extract, and they become associated with chromatin at approximately the same time after the loading of xMCMs. These findings, together with the apparent requirement of S-phase cdk activity for the loading of xCdc45, suggest that xCdc45, under the control of S-phase Cdk, plays a pivotal role in the loading of DNA polymerase α onto chromatin.

Mcm10—the New Kid on the Block

Mcm10 is a recently identified nuclear factor that is essential for the initiation of DNA replication in *S. cerevisiae* (42). The *mcm10-1* mutant was identified in the same genetic screen for minichromosome maintenance that gave rise to the *mcm2*, *mcm3*, and *mcm5* mutants. Although mutants of Mcm10 have many of the same phenotypes as mutants of the MCM2–7 family, Mcm10 shares no sequence conservation with MCM2-7. *MCM10* encodes a basic protein (pI 9.7) of 571 amino acids. It is identical to *DNA43*, a gene identified independently for its putative role in replicating DNA (168). A homolog of Mcm10 has been identified in *S. pombe*. Although the *S. pombe* Cdc23 protein is only 17% identical in sequence to Mcm10, it complements an *mcm10* null mutation when expressed on a high-copy-number plasmid (Y Kawasaki & H Tanaka, unpublished results).

The requirement of Mcm10 for replication initiation has been demonstrated in two ways. Like the other *mcm* mutants, *mcm10-1* affects plasmid replication in an ARS-specific manner (Table 1; 42). Two-dimensional gel analysis shows that the *mcm10-1* lesion causes a dramatic reduction in DNA replication initiation at chro-

mosomal origins, including ARS1. In addition, the *mcm10* mutant has a unique phenotype not shared by other replication initiation mutants. The *mcm10-1* lesion causes replication forks to pause during elongation through replication origins that failed to initiate. The pausing of elongation forks at ARS1 in the *mcm10* mutant is dependent on the integrity of the essential A element that ORC binds, and two of the three B elements, which the pre-RC occupies (Y Kawasaki & B Tye, unpublished results). Thus, pausing of elongation forks in the *mcm10-1* mutant is contingent on a functional replication origin in which pre-RCs are assembled, suggesting that a barrier at replication origins results from a defective Mcm10 protein. These results are consistent with Mcm10 playing a role not only in the assembly of pre-RCs but also the disassembly process. Two-hybrid analysis and glutathione-S-transferase affinity chromatography indicate that Mcm10 physically interacts with at least five members of the Mcm2–7 family, supporting the notion that Mcm10 plays a role in replication initiation that requires physical contact with the MCM complex. The role of Mcm10 in replication initiation will become clear when more information about the properties of Mcm10 is available.

Positive and Negative Control by Cell Cycle–Regulated Protein Kinases

Cdc7-Dbf4 Kinase Cdc7-Dbf4 is a serine/threonine protein kinase whose cell cycle-regulated activity is required for the onset of DNA synthesis (169–172). Cdc7, the catalytic subunit, is expressed at a constant level throughout the cell cycle. On the other hand, Dbf4, the regulatory subunit, is expressed periodically from G1 phase throughout the S phase of the cell cycle (172–174). Homologs of both Cdc7 and Dbf4 have been identified in the fission yeast (69, 175) and in human cells (176, 177). The activity of Cdc7-Dbf4 peaks at the G1- to S-phase transition, at which point action of Cdc7-Dbf4, presumably in coordination with the activation of Cdc28–S-phase cyclin (Clb5 and 6), triggers a cascade of reactions that leads to the initiation of DNA replication (Figure 4). Identification of the target of regulation/phosphorylation by Cdc7-Dbf4 is crucial to the elucidation of this cascade.

A role for Cdc7-Dbf4, which requires the recruitment of this cdk-like kinase to replication origins, was suggested in an earlier experiment. By using the one-hybrid assay, a library of genomic fusions to the activation domain of Gal4 was screened for candidates that can activate a reporter gene containing the ARS consensus sequence of a yeast replication origin. In this screen, Dbf4 was identified, suggesting that Dbf4 is recruited to replication origin directly or indirectly via the ARS consensus sequence, which ORC binds (178).

An intimate relationship between Cdc7-Dbf4 and the MCM proteins was suggested in another experiment in which extragenic suppressors of *cdc7* mutations were sought. A mutant allele of *mcm5* (*mcm5-bob1*) was identified as a suppressor of *cdc7*. Remarkably, the *mcm5-bob1* mutation was able to suppress all mutations in *CDC7* or *DBF4*, including null mutations of these two genes, suggesting

that *mcm5-bob1* bypasses the essential function of Cdc7-Dbf4 (179). In other words, an alteration in Mcm5 was sufficient to fulfill the essential function of Cdc7-Dbf4. Surprisingly, Mcm5 does not appear to be a substrate for phosphorylation by the purified Cdc7-Dbf4 kinase in vitro (77), so how might Cdc7-Dbf4 act through the MCM complex in the initiation of DNA replication.

The answer came in yet another extragenic suppressor screen, but this time suppressors of the *mcm2-1* mutation were sought. In this search, an allele of *dbf4*, known as *dbf4-6*, was identified (77). Suppression of *mcm2-1* by *dbf4-6* is allele specific, and the suppression of defects was reciprocal because individual mutations were lethal at high temperature but together conferred viability. Two-hybrid analysis indicated that Mcm2 physically interacts with Cdc7-Dbf4, but this interaction was curtailed in the Mcm2-1 protein. Consistent with the notion that interaction between Mcm2 and Cdc7-Dbf4 is required for the phosphorylation of Mcm2, Mcm2-1 is a poor in vitro substrate for Cdc7-Dbf4 in comparison with Mcm2. Together, these results, in addition to the dispensability of Cdc7-Dbf4 in the *mcm5-bob1* mutant, suggest that the only essential function of Cdc7-Dbf4 in DNA replication is mediated through the MCM complex and that Mcm2 may be the only essential target of regulation for Cdc7-Dbf4. This regulation by Cdc7-Dbf4 could be effected by phosphorylation of or physical contact with Mcm2. In support of the notion that Mcm2 may be the only Mcm phosphorylated by Cdc7-Dbf4, when Hsk1-Dfp1, the *S. pombe* homolog for Cdc7-Dbf4, was used to phosphorylate the hexameric spMCM complex in vitro, spMcm2 was the predominant, if not only, substrate (69). Although Mcm3, Mcm4, and Mcm6 individually are also phosphorylated by the insect cell-expressed Cdc7-Dbf4, it is unclear whether phosphorylation of these MCM proteins by Cdc7-Dbf4 occurs in vivo (77). A simple model that can explain these results and the fact that the *mcm5-bob1* mutation can bypass the requirement of Cdc7-Dbf4 is that the Mcm complex as a whole is inactive until it comes into contact with Cdc7-Dbf4 or until Mcm2 becomes phosphorylated. This physical and biochemical interaction results in a conformational change of the MCM complex that leads to the activation of the MCM complex (Figure 5 *ii*). The Mcm5-bob1 mutation may alter the structure of Mcm5 in a way that locks the MCM complex in a constitutively active conformation that no longer requires the action of Cdc7-Dbf4. This model explains why Cdc7-Dbf4 becomes dispensable in the *mcm5-bob1* mutant even though Mcm5 may not be a direct target of phosphorylation by Cdc7-Dbf4.

Cdc28-Clb Kinases The involvement of Cdc28 and the six B-type cyclins in regulation of DNA replication is much more complex because these cyclins serve redundant functions both in the activation of initiation and in the prevention of re-initiation (27, 28, 139). DNA synthesis requires the activation of the S-phase cdk, Cdc28-Clb5 and 6.

Removal of these cyclins will delay initiation only until the M-phase cyclins Clb1–4 are expressed. Furthermore, the activation of Cdc28 by any one of the B-type cyclins, Clb1–4, is sufficient to prevent reinitiation, presumably by prevent-

ing the assembly of the pre-RC (Figure 4). The role of Cdc2 and mitotic cyclins in the prevention of rereplication has also been suggested by a number of observations in the fission yeast (180, 181). Heat inactivation of thermolabile Cdc2 or Cdc13 (mitotic cyclin B) in certain *cdc2* or *cdc13* mutants results in a block in G2 phase. When these cells are returned to permissive temperature, they proceed to S phase without an intervening mitosis (182, 183). Similarly, cells deleted for *cdc13* undergo successive S phases without intervening M phases. Overexpression of *rum1*, the inhibitor of *cdk1* (*cdc2/cdc13*) in fission yeast, induces repeated rounds of S phase without M phase (184, 185). These results suggest that Cdk1 positively regulates mitosis but negatively regulates the initiation of DNA synthesis. Figure 5 summarizes the coordinated actions between Cdc7-Dbf4 and Cdc28-Clb kinases in restricting DNA synthesis to once per cell cycle.

Origin firing can occur only when Cdc7-Dbf4 and Clb-kinase are both active (Figure 4). That period of activity is S phase. The Clb kinase is active from the beginning of S phase until late anaphase when the cell exits mitosis (30, 186). On the other hand, Cdc7-Dbf4 is active only during G1 phase and S phase. Initiation of DNA synthesis occurs at the G1- to S-phase transition, when both Cdc7-Dbf4 and Cdc28-Clb kinases are activated. The apparently normal cell cycle of the Δ*cdc7 mcm5-bob1* mutant suggests that Cdc28-Clb5 alone is sufficient to regulate entry into S phase if the MCM complex has already undergone a conformational change similar to that induced by Cdc7-Dbf4. Thus, activation of not one but both kinases during S phase is required for the initiation of DNA synthesis. In support, two recent reports suggest that expression of Cdc7-Dbf4 is required throughout S phase for the firing of late replication origins (187, 188). The same is likely to be true for Cdc28-Clb.

To visualize the sequence of events coordinated by Cdc7-Dbf4 and Cdc28-Clb5 that culminate in the initiation of DNA synthesis at replication origins, in vivo substrates for these kinases must be identified. Having shown that Mcm2 is likely to be the only essential substrate for Cdc7-Dbf4 in this sequence of events, the focus now turns to the substrates for Cdc28-Clb5. Although we do not have the answer to these questions yet, accumulating data, when pieced together, provide some insight into the intricate timing of events coordinated by these two kinases. First, the recruitment of Cdc45 to the pre-RC occurs at a time when Cdc28-ClbS is activated. Second, the action of Cdc45 is dependent on an active Cdc7-Dbf4, and the action of Cdc7-Dbf4 is dependent on an active Cdc45. Third, the recruitment of RPA to replication origins occurs downstream of the action of Cdc7-Dbf4 and the recruitment of Cdc45 to replication origins (130). Finally, Cdc45 appears to play a direct role in recruiting polymerase α (167). Keeping in mind that Mcm2 is the target of initiation activation by Cdc7-Dbf4, this sequence of events can be ordered as shown in Figure 5.

The origin recognition complex is associated with replication origins during most of the cell cycle. During the G1 phase, assembly of the pre-RC is initiated by the direct interaction of Cdc6 with ORC. Cdc6 acts as a chaperone of the MCM2–7 complex by escorting the MCM complex to the replication origin

(Figure 5 *i*). Recruitment of the MCM2–7 to replication origins results in the formation of the pre-RCs. It is unclear whether the interaction of MCM2–7 with replication origins occurs via the direct contact of MCM and DNA or via a yet-to-be-identified protein. It is unlikely that the MCMs are anchored to origin DNA via ORC or Cdc6 (105). Whether Cdc6 or ORC, which are dispensable after the assembly of the pre-RC (138), remains associated with the pre-RC (92) requires further investigation (Figure 5 *ii*). The fact that the MCM proteins once loaded onto chromatin can withstand salt washes of 2 M NaCl and would be released from chromatin only by DNase treatment suggests a major change in conformation or biochemical properties of the MCM complex (50, 89). Interactions between Cdc7-Dbf4 and the MCM complex may induce a second conformational change required of the MCM complex before the activation of replication initiation. This second conformational change probably occurs sometime during the G1- to S-phase transition when Cdc28-ClbS is activated and at a time when the action of Cdc45 is executed (Figure 5 *ii*, *iii*; 128, 166). This Cdc7-Dbf4–induced conformational change in the MCM complex appears to be a prerequisite for downstream events such as the melting of origin DNA and the recruitment of primase (130) and DNA polymerase α (167). The MCM complex as well as Cdc45 may play a critical role in the transition of the RC to the elongation complex (EC) by transferring from the RC to the replisome. Rebinding of ORC at the replication origin probably occurs immediately after the conversion of the RC to the EC (Figure 5 *iii*) as suggested by the cross-linking of ORC to origin DNA at the beginning of S phase (92).

Temporal Regulation of Origin Firing

The sequence of events that describes an early firing replication origin during the G1- to S-phase transition (Figure 5) is probably not different from the firing of late origins that are activated during S phase. The temporal order for the firing of replication origins most likely is not regulated at the level of the timing of the formation of pre-RCs, because footprints characteristic of pre-RCs are evident during the G1 phase in late or silent replication origins (189). Although footprints alone are not informative about the presence or absence of MCMs at late origins during the G1 phase, the mere fact that Cdc6 is absent during S phase suggests that MCMs cannot be loaded onto late origins during S phase. It seems unlikely that the MCM proteins play a role in determining the timing of origin usage because the selective usage of replication origins in the *mcm* mutants seems to be a phenomenon that correlates with inherent differences of individual replication origins rather than the temporal order of origin usage. For example, two replication origins, ARS1 and ARS121, which respond differently to the same *mcm* defects (45, 104) are both replicated early during S phase (S Hunt & B Brewer, personal communications). Accumulating data suggest that the temporal firing of replication origins is influenced by chromatin context (4, 190). Position effect on the delayed firing of replicating origins is best illustrated by the effect

of telomeres on the timing of firing of an adjacent replication origin that has been separated from the telomeric chromatin at different points of the cell cycle (5). A late replicating origin that is under the influence of telomeric heterochromatin will replicate late if separated from the telomeric chromatin at Start in G1 phase. In contrast, the same origin, if separated from its native chromatin context during M phase, will fire early in the next S phase. Thus, the late replicating program must be set up sometime between M phase and Start of the G1 phase at a time coincident with the formation of pre-RCs. It is possible that chromatin context dictates the accessibility of the pre-RCs to factors required for downstream events, such as the loading of Cdc45 or the cell cycle-regulated protein kinases Cdc7-Dbf4 and Cdc28-Clb. Because the Mcms are known to interact with some of these downstream factors, regulation through modification of MCMs cannot be ruled out. Recent studies suggest that two protein kinases, Mec1 and Rad53, which mediate the checkpoint sensing mechanism for DNA damage and incomplete DNA replication, negatively regulate the firing of late origins (130, 191, 192). In the absence of Mec1 and Rad53, late origins are fired early, suggesting that Mec1 and Rad53 directly or indirectly inhibit the activation of pre-RCs that are assembled in the late chromatin context. Interestingly, the essential functions of Mec1 and Rad53 can be bypassed by overexpression of ribonucleotide reductase, a key enzyme in the biosynthesis of deoxyribonucleotides. These results suggest that the late firing program may be designed to regulate origin usage and therefore the number of elongation forks in response to changing nucleotide pools (193; J Newport, personal communication). In this model, Mec1 and Rad53, which act as an intra–S-phase checkpoint (Figure 4), negatively regulate the firing of late origins in response to fluctuations in the nucleotide pool and to the chromatin context of late origins.

Targets for the Positive and Negative Regulation by Cdc28-Clb

Identifying the targets of regulation by Cdc28-Clb will help elucidate the molecular mechanism that restricts DNA replication to once per cell cycle. So far, there are many suspects but no culprit. In principle, any one of the components of the pre-RC assembled at the replication origins could be a target of positive regulation by Cdc28-Clb. Modifications after the assembly of the pre-RC could be a signal for the activation of the pre-RC. For example, phosphorylation of chromatin-bound Cdc6 could be a signal for the removal of Cdc6 from the pre-RC and for activation of downstream events. Indeed, Cdc6 binds tightly to Cdc28-Clb (194), and phosphorylation of Cdc18/Cdc6 by Cdc2-cyclin B/Cdc28-Clb signals degradation of chromatin-bound Cdc18p (195). In addition, Cdc45 or Cdc7-Dbf4, which acts after Start, could also be a substrate for positive regulation by Cdc28-ClbS. Similarly, any one of the components of the pre-RC could be a substrate for the negative regulation of Cdc28-Clb, which prevents reinitiation of DNA synthesis by inhibiting assembly of the pre-RC until G1 phase (Figure 5). For example, phosphorylation

of spOrc2 or Cdc18/Cdc6, which interact with Cdc2 and have multiple Cdc2 consensus phosphorylation sites (196), could prevent the association of Cdc6 or ORC with the MCMs when Cdc2-cyclin B is active. Alternatively, phosphorylation of any one of the MCM proteins during S phase could preclude their association with Cdc6 (62). Elucidation of the mechanism that imposes the temporal separation of the replication-competent and replication-incompetent states of replication origins awaits identification of the targets of regulation by Cdc28-Clb.

THE SV40 LARGE-T ANTIGEN AS A PARADIGM FOR EUKARYOTIC MCM2–7 PROTEINS

Much of what we know today about the biochemistry of the initiation of DNA replication in eukaryotes comes from studies in SV40 (197, 198). Only three proteins are needed for the initiation and elongation of DNA synthesis in SV40, the large-T antigen–-the initiator protein, RPA–-the single-stranded DNA-binding protein, and DNA polymerase α. The simplicity of the viral system, which has a single replication origin that permits multiple rounds of replication of the viral genome in a single S phase, provides a framework for understanding the complexity of the eukaryotic host system.

The large-T antigen, which forms a double hexamer of two rings each consisting of six identical subunits (156), serves the functions performed by the combined efforts of at least 20 proteins in eukaryotes. The large-T antigen recognizes the replication origin (199), binds and melts the origin DNA (158), recruits DNA polymerase α (200), and then acts as a helicase (159) to unwind replication forks in concert with polymerase α. In contrast, in the budding yeast, ORC, a complex of six subunits, recognizes and binds specifically to origin DNA. Cdc6, which shows sequence conservation with RF-C, delivers the MCM complex to replication origin. Activation of the pre-RC by Cdc7-Dbf4 and Cdc28-Clb occurs simultaneously with the loading of Cdc45. Recruitment of polymerase α by Cdc45 is followed by the actual melting of replication origins, presumably by MCM2–7. Only at this point, RPA is recruited to the replication origin. Thus, the combined efforts of ORC, Cdc6, MCMs, and Cdc45 are equivalent to the function of a single protein that can initiate multiple rounds of replication within a single S phase. The SV40 model suggests that the machinery for replication initiation is very simple. The complexity of the eukaryotic host is evolved with the need to replicate large chromosomes that have multiple replication origins, which must be initiated no more than once per cell cycle. To do so, additional proteins are needed to temporally separate the process of assembly of the pre-RC from the initiation event. Clearly, Cdc7-Dbf4 and Cdc28-Clb serve a regulatory role in this process. In an MCM-centric view, each of the replication initiator proteins serves a supporting role to the leading role performed by the MCMs. ORC can be viewed as a component of the replication origin that marks the site where the MCMs must land, Cdc6 as the chaperone for delivering MCMs on to replication origins, and Cdc45 as the facilitator that escorts the MCMs from the ini-

tiation complex to the elongation complex. The conservation of the MCMs from Archaea to *Homo sapien* may provide a unique retrospective to the evolution of a fundamental biochemical process that has gained complexity by developing six related MCM proteins from a single MCM progenitor.

ACKNOWLEDGMENTS

I thank Ming Lei, John Lis, and Judy Campbell for their critical reading of this manuscript. I am indebted to members of my lab for their encouragement and discussions, especially on the many relevant papers that were cited in this review. I thank Philip Pian and Sara Sawyer for their help in computer analysis of sequences. I am eternally grateful to Stephen Kearsey and Karim Labib for providing me with Figures 1 and 2. Last but not least, I thank Y. Adachi and M. Yanagida for making available the hundreds of original EM images of the Mcm complexes and for allowing me to reproduce some of those beautiful images shown in Figure 3. This work is supported by grants from the American Cancer Society (CB-191) and the National Institutes of Health (GM34190)

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