Phosphorylation of the Replicative Helicase by the S-phase Kinase, Dbf4-Cdc7, at Origins of Replication in *Saccharomyces cerevisiae*

by

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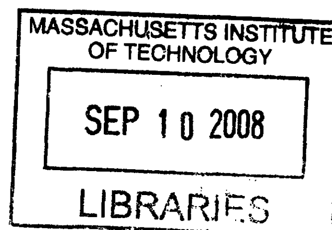

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Abstract

In eukaryotic cells, events such as DNA replication and mitosis must be carefully coordinated in the cell cycle to ensure that the entire genome is duplicated before cells undergo cell division. In particular, initiation of DNA replication occurs in two temporally distinct steps, selection of sites of initiation (called origins) and activation of these selected sites. Selection of origins occurs in late M- and early G1-phase and is mediated by the origin DNA-binding protein called the Origin Recognition Complex (ORC). ORC recruits several proteins to the DNA, including the Mcm2-7 replicative helicase, to form the pre-Replicative Complex (pre-RC). Origins are activated by recruitment of other initiation factors to the pre-RC to form the pre-Initiation Complex (pre-IC) and this event requires two kinases, Cyclin Dependent Kinase (CDK) and Dbf4-Dependent Kinase, Dbf4-Cdc7 (DDK). These events lead to activation of the Mcm2-7 helicase and recruitment of DNA synthesis machinery

The S-phase kinase, DDK, phosphorylates the Mcm2-7 helicase, and is thought to be required either for helicase activation or for recruitment of pre-IC factors (or both). To gain a better mechanistic understanding of the role of DDK in initiation, we developed a biochemical assay to examine the DDK phosphorylation of pre-RC-linked Mcm2-7 complex. We found that DDK specifically targets the MCM complex and is recruited to the origin by the MCM complex. DDK preferentially phosphorylates the MCM complexes most tightly linked to the DNA. Moreover, prior phosphorylation of the MCM complex is required for DDK binding to, and phosphorylation of, the MCM complex suggesting that another, as yet unknown kinase, is also required to initiate DNA replication.

To further understand the multiple phosphorylation events that may be required for activation of the helicase, phosphorylation sites were mapped on pre-RC-linked MCM complexes. Each phosphorylated MCM subunit contained both DDK-dependent and -independent phosphorylation sites except Mcm5. In sum, the work described herein provides a model for how DDK specifically targets those MCM complexes taking part in DNA replication. Moreover, this work provides a stepping off point for many future mechanistic studies to understand the role of DDK, and other kinases, in initiation of DNA replication.

Thesis Supervisor: Stephen P. Bell
Title: Professor of Biology

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As I always say, science is not done in a vacuum, so although all the work I report in this thesis was done (mostly) by me, this thesis would not have happened without the help and support of so many people.

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Of course, I have to thank my husband, Myckey, for following me to Boston back when our relationship was still in its infancy. He's been incredibly supportive throughout my (long) graduate school career. And I have to thank Quinn for being such a good baby and toddler. He did make me pretty ill through the first few months of my pregnancy, and pretty uncomfortable through the last few months, but his great personality, sleep schedule, and overall happy demeanor made it easier to be able to concentrate on finishing up my degree (and he just makes life pretty fun). I thank my mom for always encouraging me to follow my own path, and for taking care of Quinn for the last year…for free. And last but not least, thanks to all my friends and family who have been very supportive and haven't asked too many questions (like, "so *how* long have you been in grad school?!?").

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Chapter I

Introduction

Overview

Each time a cell divides it must carefully coordinate cellular events to ensure that the entire genome is duplicated before cells undergo chromosome segregation. Cells employ many methods to control the cell cycle, including regulation of protein synthesis, degradation, and phosphorylation. Of these, protein phosphorylation has the advantage that it can rapidly change the function of existing proteins. The added phosphates can alter protein function in many ways, such as altering protein conformation or creating a new protein binding site. Kinases are the primary triggers driving the G1- to S-phase transition in eukaryotic cells. In *S. cerevisiae*, the transition from G1- to S-phase, and the concomitant activation of replication origins, is coordinated through the action of two S-phase kinases, the Cyclin-Dependent Kinase, Clb5/Clb6-Cdc28 (CDK) and Dbf4-dependent kinase, Dbf4-Cdc7 (DDK) (Bell and Dutta, 2002).

Initiation of DNA replication is divided into two temporally distinct events: selection of sites of initiation and activation of those selected sites. In G1-phase of the cell cycle, before CDK and DDK are active, sites of initiation (also called origins of replication) are selected by the formation of pre-Replicative Complexes (pre-RCs) (Takeda and Dutta, 2005). This process begins with the recognition of each origin of replication by the eukaryotic initiator protein, the Origin Recognition Complex (ORC), which is constitutively bound to origin DNA in *S. cerevisiae*. In late M-phase and early G1-phase, ORC recruits Cdc6, Cdt1 and together these three proteins load the Mcm2-7 replicative helicase onto the origin DNA (Mendez and Stillman, 2003). At the G1- to S-phase transition, CDK and DDK activities are activated, leading to activation of existing pre-RCs by the coordinated action of the two kinases. CDK and DDK activate pre-RCs by modifying components of the pre-RC and by modifying and recruiting a variety of additional replication initiation proteins. Together, these events lead to the formation of a pre-initiation complex (pre-IC). Formation of the pre-IC activates the Mcm2-7 DNA helicase causing unwinding of the origin DNA. The resulting single-stranded DNA (ssDNA) facilitates the recruitment of DNA polymerases and polymerase-accessory factors resulting in the formation of a pair of bi-directional replication forks (Bell and Dutta, 2002).

Cdc7 and its activating subunit, Dbf4, were discovered in genetic screens for genes involved in cell cycle progression (Masai and Arai, 2002). The Cdc7 kinase activity is dependent upon the cyclical appearance of Dbf4, akin to the dependence of CDK activity on the cyclins. Dbf4 protein levels are controlled by regulated proteolysis in late M- and early G1-phase, with Dbf4p appearing in late G1-phase and peaking in S-phase. Cdc7 kinase activity coincides with the cyclical appearance of the Dbf4 protein levels, also peaking in S-phase. Dbf4-Cdc7 appears to be required at individual origins as they initiate during S-phase (versus being a global activator at the G1-S transition) (Bousset and Diffley, 1998; Donaldson et al., 1998). Consistent with this mechanism of action, DDK is recruited to origins of replication during S-phase, although the mechanism of this recruitment remains unclear (Jares and Blow, 2000; Walter, 2000).

The Mcm2-7 complex is the most important, if not the only, substrate of the Dbf4-Cdc7 kinase. This hypothesis is supported by both *in vivo* and *in vitro* evidence. *In vivo,* an allele of *MCM5* (*mcm5-bob1*) has been identified that bypasses the requirement for Cdc7 and Dbf4 (Hardy et al., 1997). *In vitro* experiments with individual MCM subunits show that DDK can phosphorylate all of the MCM subunits except for Mcm5 (Weinreich and Stillman, 1999). These data lead to the hypothesis that the point mutation in the *mcm5-bob1* allele causes a conformational change in the MCM complex that mimics a possible conformational change brought about by DDK phosphorylation of other subunits in the wild-type MCM complex. DDK-dependent phosphorylation has been observed for Mcm4, and this phosphorylation preferentially targets chromatin associated MCM complexes (Masai et al., 2006; Sheu and Stillman, 2006). Experiments with purified human Mcm2-4-6-7 subcomplex suggest Mcm2, 4 and 6 are targets (Masai et al., 2000). Although both approaches strongly support the MCM complex as a DDK target, there is conflicting *in vivo* and *in vitro* data as to which MCM subunits are phosphorylated by DDK. Despite this, the most compelling data suggests Mcm2, Mcm4 and Mcm6 as the most likely DDK targets (Masai and Arai, 2002).

The consequences of DDK phosphorylation of the MCM complex are poorly understood. One hypothesis is that phosphorylation effects a conformational change in the Mcm2-7 complex that activates its helicase activity (Sclafani, 2000). Another hypothesis is that phosphorylation leads to binding of additional factors to the Mcm2-7

complex that activate its helicase activity. In support of the latter hypothesis, the purified Mcm2-7 complex has little helicase activity in biochemical assays; however, a complex of Cdc45, GINS and Mcm2-7 purified from *Drosophila* egg extracts was shown to have helicase activity (Moyer et al., 2006). Importantly, Cdc45 binding to chromatin requires DDK function (Zou and Stillman, 1998), and Cdc45 has been shown to bind the MCM complex (Hopwood and Dalton, 1996; Zou and Stillman, 1998, 2000), therefore it is thought that DDK phosphorylation of the MCM complex leads to Cdc45 binding to the MCM complex.

This thesis focuses on the mechanisms of DDK binding to, and targeting of, DNA-bound Mcm2-7 complexes. To understand how the cell controls these events, I have taken a biochemical approach using pre-RCs purified from G1 extracts as the substrate in DDK kinase assays. I have determined which components of the pre-RC are modified by DDK in this context and which components of the pre-RC are important for recruiting DDK. Most interestingly, my studies show that, as is thought to occur *in vivo*, DDK preferentially targets a specific DNA-linked population of MCM complexes and suggest a mechanism for this targeting. To further understand how DDK and other kinases trigger initiation of DNA replication, I have collaborated with a post-doc in the lab, John Randell, to map the sites of Mcm2-7 phosphorylation. Using mass spectrometry we have identified the sites of Mcm2-7 phosphorylation before and after DDK phosphorylation. Our studies reveal that DDK specifically targets the N-termini of the Mcm2, Mcm4 and Mcm6 subunits. Moreover, we identify several classes of DDK-independent target sites that occur . In this introduction, I will review the events and proteins necessary for initiation of DNA replication, with a special emphasis on the function of DDK and the Mcm2-7 complex.

Origins of DNA replication

Under most circumstances, DNA replication initiates from specific sites on a chromosome. Many bacteria with smaller genomes contain only one site of initiation. In contrast the larger genomes of eukaryotes demand a more distributive approach to replication initiation. These organisms have many origins on each chromosome. In the case of *S. cerevisiae*, the organism studied in this thesis, replication initiates from about

300 different sites to ensure that the entire genome is duplicated within the required amount of time (Nieduszynski et al., 2007). Moreover, not all origins initiate at the same time in early S-phase; instead, initiation is distributed temporally throughout S-phase, with specific origins initiating at characteristic times during each S-phase.

In budding yeast, origins of replication were originally identified as Autonomously Replicating Sequences, or ARSs (Stinchcomb et al., 1979). These sites were identified on the basis of their ability to confer stable replication to plasmids lacking an origin of replication. Subsequent studies found that the majority of ARS elements acted as origins in the chromosome as well (Raghuraman et al., 2001). A typical yeast origin contains several functional domains. The A element is defined by an 11-bp A/T-rich consensus sequence, also known as the ARS consensus sequence (ACS) (Broach et al., 1983). The ACS is the binding site for the initiator protein, the Origin Recognition Complex (ORC, see below) and is essential for origin function (Bell and Stillman, 1992; Marahrens and Stillman, 1992). Adjacent to the A element is the B-region of the origin which is typically composed of two to three smaller elements, depending on the origin. One of the most studied origins, *ARS1*, contains three smaller domains within the B element: B1, B2 and B3 (Marahrens and Stillman, 1992).

Although the sequence of these elements is not well conserved, these elements are functionally conserved between origins as they can be substituted for one another. Importantly, deletion of any of these smaller domains causes reduced origin function and removal of the entire B region eliminates initiation of replication. The B1 element is most proximal to the ACS and is known to stabilize ORC binding to the A element (Rao and Stillman, 1995). In contrast, the B2 element is important for recruitment of the Mcm2-7 complex to the origin (Lipford and Bell, 2001; Wilmes and Bell, 2002; Zou and Stillman, 2000). The B3 element is the binding site for the transcription factor Abf1 (Diffley and Stillman, 1988). It is not well understood what role Abf1 plays in DNA replication initiation and this element is not found at all origins, but one likely function is to facilitate the formation of a nucleosome-free region at the origin (Lipford and Bell, 2001). Genome-wide studies of nucleosome positioning show that origins are always found within a nucleosome free region of DNA (D. MacAlpine pers comm).

The Pre-Replicative Complex

As mentioned in the overview, the first step in initiation of DNA replication is the selection of the DNA sites that will act as origins of replication. This process requires the formation of a complex of proteins termed the pre-Replicative Complex (pre-RC) at each origin (Fig. 1). ORC, which is bound to DNA throughout the cell cycle, recruits the other pre-RC proteins during late M- and early G1-phase when CDK activity is low (see "CDK" section below). Cdc6 recruitment by ORC is required for subsequent recruitment of Cdt1 and the Mcm2-7 replicative helicase to the DNA. Both ORC and Cdc6 are members of the AAA+ family of ATPases and ATP hydrolysis by these two proteins catalyzes distinct steps in pre-RC formation (Bowers et al., 2004; Randell et al., 2006) (see below for details). Because pre-RC formation directs the Mcm2-7 helicase onto the origin DNA, pre-RC formation is the helicase loading event for eukaryotic cells. In addition, genome-wide analysis of pre-RC formation shows that pre-RCs mark all potential origins of DNA replication (Wyrick et al., 2001). Below, I will discuss in more detail how each of the pre-RC proteins contributes to pre-RC formation with particular attention on how ATP binding and hydrolysis contributes to MCM association with the DNA.

ORC

The Origin Recognition Complex is a six-subunit, highly conserved protein required for initiation of DNA replication in all eukaryotes tested. In *S. cerevisiae*, ORC binds DNA in a sequence specific manner at sites called the ARS consensus sequence (ACS - see above) and is required to recruit all other members of the pre-RC to the DNA (Bell and Dutta, 2002). ORC must bind ATP to interact with origin DNA in a sequence specific manner (Klemm et al., 1997). Moreover, the rate of ATP hydrolysis by ORC is reduced when ORC is bound to origin (but not non-origin) DNA. Three subunits in ORC, Orc1, Orc4 and Orc5 are clear members of the AAA+ family of ATPases and Orc2 and Orc3 are proposed to be more distantly related to this class of proteins (Erzberger and Berger, 2006). Mutational analysis of the ATP binding motifs of these three subunits indicates that only Orc1 and Orc5 bind ATP. *In vivo*, mutation of the Orc1 ATP binding

Figure 1.

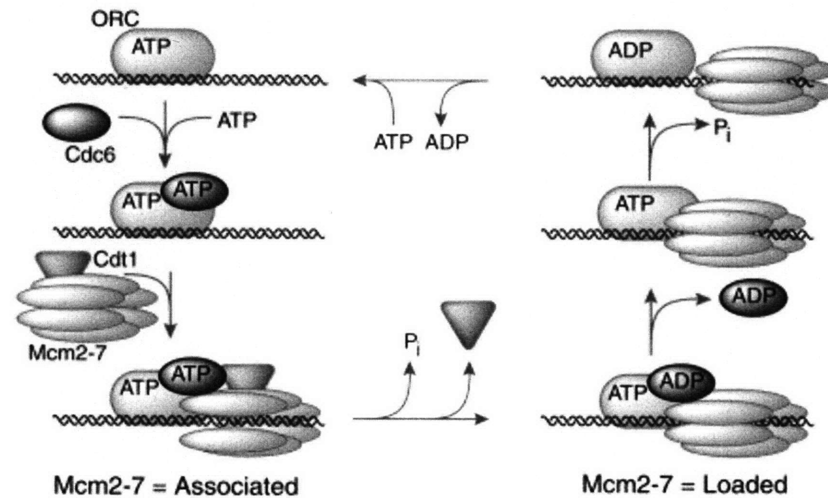


Figure 1. A Model for Mcm2-7 Loading by the ORC and Cdc6 ATPases. ATP bound ORC first binds origin DNA. Cdc6 then binds ORC and ATP. Cdt1 and Mcm2-7 (possibly as a complex) associate with ORC and Cdc6 at the origin. ATP hydrolysis by Cdc6 leads to the loading of Mcm2-7 complexes on DNA and the release of Cdt1 from the origin. Cdc6 association is destabilized by Cdc6 ATP hydrolysis. ATP hydrolysis by ORC completes the Mcm2-7 loading reaction allowing further rounds of Mcm2-7 loading. (Randell et al., 2006)

site is lethal whereas mutation of the Orc5 ATP binding has no effect on cell division or ORC function. Consistent with the more important role of the Orc1 ATP binding site *in vivo*, *in vitro* studies show that the Orc1 ATP binding site controls the ATP-dependence of ORC DNA binding and is solely responsible for ORC ATP hydrolysis (Klemm et al., 1997).

In vitro studies have shown that ORC ATP hydrolysis is required for a discrete step in pre-RC formation. Normally, multiple MCM complexes are loaded onto DNA during the pre-RC assembly reaction. However, if ORC is allowed to bind but not hydrolyze ATP during pre-RC formation, the resulting pre-RC complexes contain only a single loaded Mcm2-7 complex (Bowers et al., 2004). This finding indicates that the ability to load multiple Mcm2-7 complexes at the origin requires ORC ATP hydrolysis. These findings have led to the hypothesis that ATP hydrolysis by ORC is required to reset the pre-RC assembly machinery to a starting state. Once reset, the process of loading additional Mcm2-7 complexes at the origin can begin (Fig 1). Consistent with this hypothesis, the Mcm2-7:ORC ratio on chromatin *in vivo* has been shown to be much greater than 1:1 in all organisms tested. Indeed, although two Mcm2-7 complexes would seem to be adequate for each origin to generate a bidirectional pair of replication forks, the ratio of ORC:Mcm2-7 complexes is more typically >8:1 (Takahashi et al., 2005). It is not clear as to why more MCM complexes are loaded onto chromatin than should theoretically be needed, however, recent studies have suggested that the extra MCM complexes may be useful in dealing with replication stress such as inhibition of replication or DNA damage (see the "Mcm2-7" section below for further discussion) (Ge et al., 2007; Woodward et al., 2006).

Cdc6

Cdc6 is a second AAA+ ATPase required for pre-RC formation. Cdc6 association with origin DNA is dependent on ORC and binding to ORC induces substantial changes in Cdc6 activity. On its own, Cdc6 neither binds nor hydrolyses ATP. Instead, Cdc6 can only bind and hydrolyze ATP in the presence of ORC bound to origin DNA (Randell et al., 2006). Given that ATP binding and hydrolysis is critical for Cdc6 function (see below), the requirement of origin-bound ORC to activate Cdc6 for ATP binding and

hydrolysis creates a mechanism to prevent Cdc6 from acting away from the origin. Just as ORC ATP hydrolysis is not required for DNA binding, ATP hydrolysis by Cdc6 is not required for Cdc6 association with ORC (Mizushima et al., 2000; Randell et al., 2006). Once bound to ORC, Cdc6 is required to recruit Cdt1 and the Mcm2-7 complex to the origin.

Studies of pre-RC assembly show that ATP hydrolysis by Cdc6 is required for the Mcm2-7 complex to reach a state in which it associates with DNA independently of other pre-RC assembly factors (Randell et al., 2006) (Fig 1). In the absence of Cdc6 ATP hydrolysis, Mcm2-7 complexes are recruited to the origin DNA but the resulting association requires the continued association of ORC and Cdc6 with the origin DNA. If ORC and Cdc6 are removed from the DNA (e.g. by extraction with 0.5 M NaCl) at this stage, Mcm2-7 association with the origin is also lost. In contrast, if Cdc6 is allowed to hydrolyze its associated ATP, the association of the Mcm2-7 complex with origin DNA no longer requires the continued presence of ORC, Cdc6 or Cdt1. At this stage the same high-salt extraction removes ORC, Cdc6 and Cdt1 but the Mcm2-7 complex remains bound to the DNA. Importantly, *in vivo* experiments indicate that pre-RC associated Mcm2-7 complexes associate with origins in a manner that is independent of ORC and Cdc6 (Jares and Blow, 2000). These data indicate that ORC, Cdc6 and Cdt1 are Mcm2-7 loading factors but are not required to maintain Mcm2-7 association with the origin after loading is complete. This is consistent with studies indicating that ORC and Cdc6 are not required for the events of replication initiation that occur after pre-RC formation (Hua and Newport, 1998; Rowles et al., 1999).

Cdt1

Cdt1 is recruited to the origin by ORC and Cdc6 and is required for the initial association of the Mcm2-7 complex with the origin. Studies in several organisms suggest that ORC and Cdc6 recruit Cdt1, as inactivation or depletion of Cdt1 does not affect ORC and Cdc6 binding to the origin (Devault et al., 2002; Maiorano et al., 2000; Nishitani et al., 2000). Consistent with the hypothesis that Cdt1 is directly involved in recruiting the MCM complex to the pre-RC, Cdt1 has been shown to interact with the MCM complex in many organisms (Cook et al., 2004; Gopalakrishnan et al., 2001; Tanaka and Diffley,

2002). Moreover, in *S. cerevisiae*, Cdt1 and the MCM complex arrive at the origin at the same time (Randell et al., 2006), and Cdt1 and the MCM complex are dependent upon one another for localization to the nucleus (Tanaka and Diffley, 2002). Similarly, in *Xenopus* egg extracts Cdt1 associates with the origin after Cdc6 recruitment (Tada et al., 1999; Tsuyama et al., 2005) but either before or simultaneously with Mcm2-7. Together these data suggest that Cdt1 acts as a chaperone for the MCM complex. In *S. cerevisiae*, although Cdt1 is required for pre-RC formation, it is not a stable component of the pre-RC. *In vitro* pre-RC assembly assays show that Cdc6 ATP hydrolysis releases Cdt1 from the DNA, as Cdt1 becomes more stably associated with the pre-RC in a Cdc6 ATP hydrolysis mutant (Randell et al., 2006). Similarly, addition of ATP-γ-S (a non-hydrolyzable form of ATP) to Xenopus egg extracts stimulates the association of Cdt1 with chromatin (Gillespie et al., 2001).

Mcm2-7

The Mcm2-7 complex is the replicative helicase in eukaryotes. It is a six subunit protein complex, with all six subunits containing a highly related, central AAA+ ATPase motif and an OB-like fold domain (typically a single-stranded DNA binding motif) (Tye, 1999) (Fig 2). Interestingly, despite the high similarity of this central region, each individual MCM protein (e.g Mcm4) is more closely related to the same MCM subunit in another species than it is to the different MCM subunit (e.g. Mcm6) from the same species. This conservation allows the identification of Mcm2-7 orthologs in all eukaryotic species analyzed.

Structural studies of MCM complexes suggest that it adopts a hexameric ring-like structure (Fig 3). These studies are most evolved for the archaeal homologs of the Mcm2-7 complex. Archaeal MCM complexes are formed from a single MCM-related protein that forms a double hexamer with easily detectable helicase activity. A crystal structure of the N-terminal half of an archaeal MCM complex revealed a hexameric complex with a positively charged central channel large enough to accommodate either single-stranded- or double-stranded DNA (Fletcher et al., 2003). EM studies of the whole protein from the same organism also showed a hexameric complex (Pape et al., 2003) and EM studies with eukaryotic Mcm2-7 indicate that these heterohexamers form a

Figure 2.

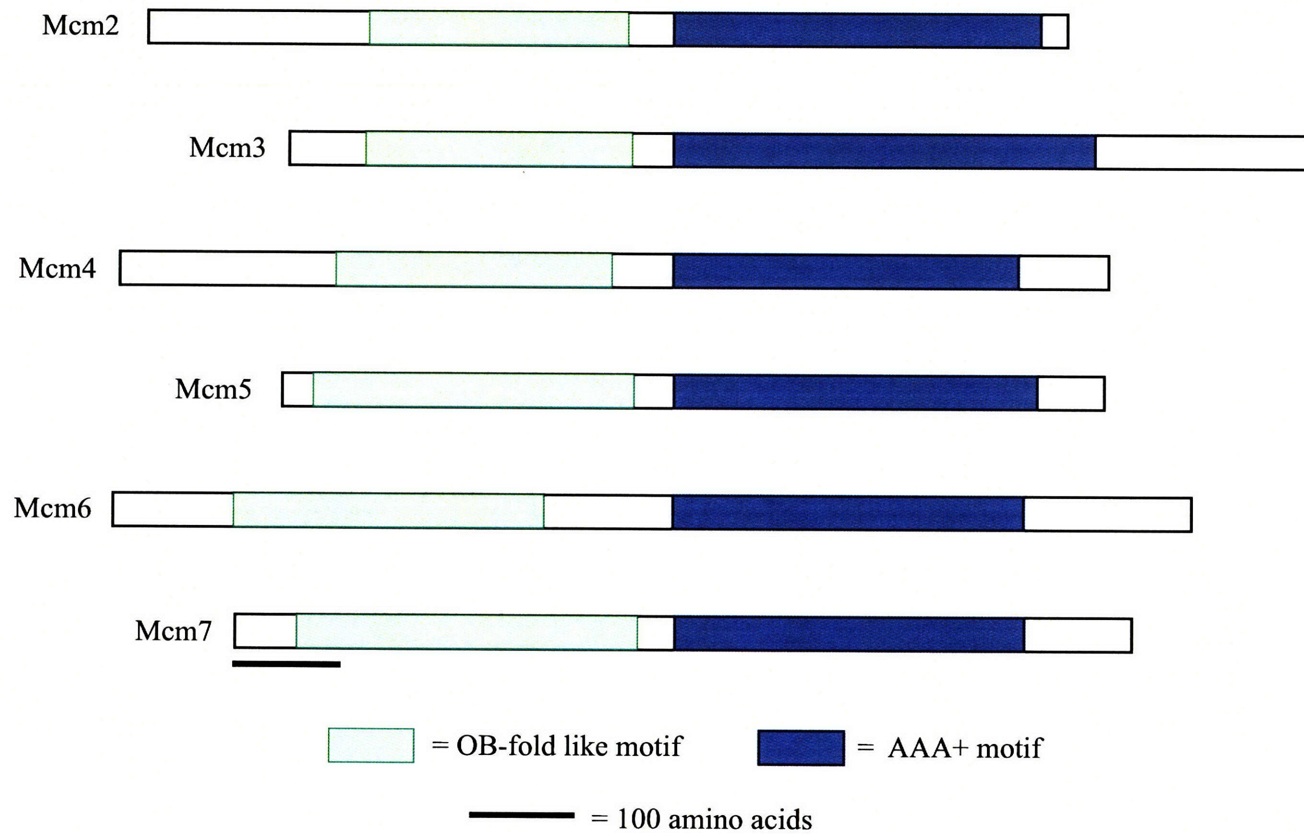


Figure 2. Location of conserved domains in *S. cerevisiae* Mcm2-7 proteins. The subunits are aligned according to where the AAA+ motif begins. The sizes of the Mcm subunits and conserved domains are to scale.

Figure 3.

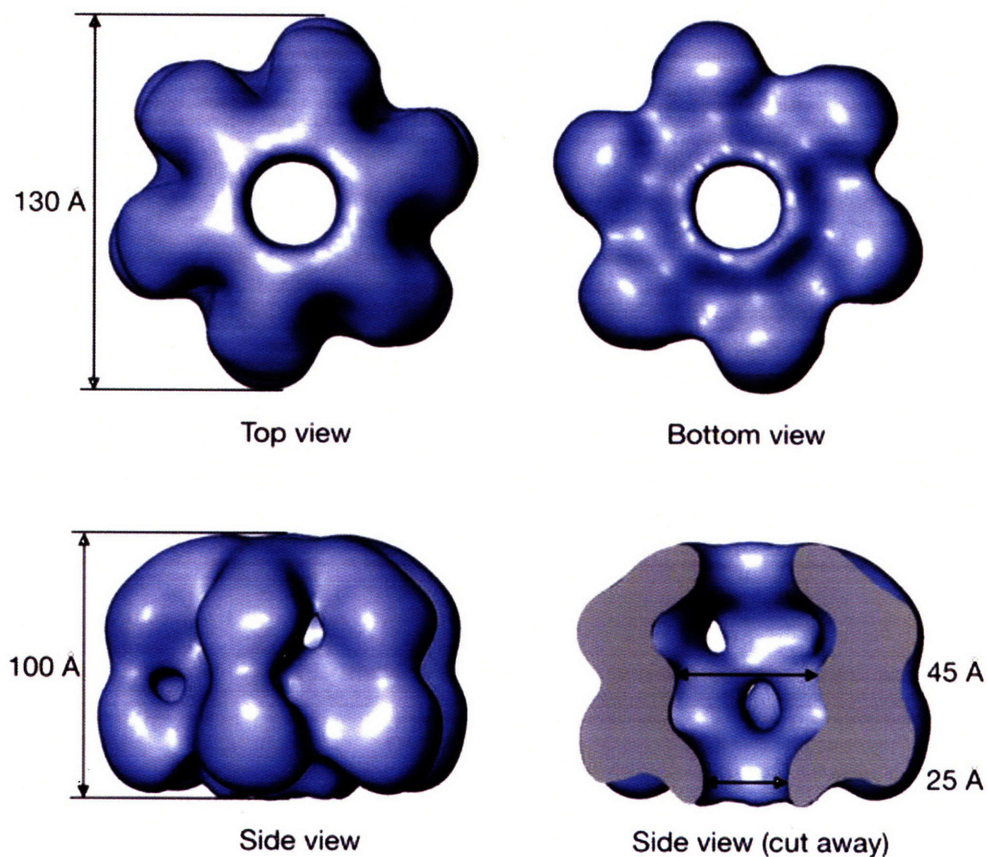


Figure 3. Three-dimensional reconstruction of MtMCM at 23-Å resolution. A surface representation including 100% of the expected volume of the electron density obtained from the three-dimensional reconstruction of the MtMCM complex is shown in different orientations. The overall dimensions for the complex are given. The protein monomers assemble into a hexameric ring around a wide central channel, with a clear asymmetry between the top and bottom views. A side view has been cut open to reveal the large central chamber and the channel spanning the entire length of the molecule. (Pape et. al., 2003)

ring-like structure as well (Adachi et al., 1997; Bochman and Schwacha, 2007; Sato et al., 2000).

A lack of *in vitro* helicase activity of purified Mcm2-7 complex confounded the field of replication for many years. Several studies suggest that all six subunits are required *in vivo*. For example, studies of mutations in the ATPase domains in each of the six subunits indicate that all of the subunits are required for viability and S-phase progression (Schwacha and Bell, 2001). A subcomplex of Mcm2-7 consisting of Mcm4, Mcm6 and Mcm7 (Mcm467) does display helicase activity and it was thought that this subset of the Mcm2-7 complex might be the replicative helicase *in vivo* (Aparicio et al., 2006), with Mcm2, 3 and 7 playing a regulatory role in initiation. However, the all six MCM subunits are loaded onto origins in G1 and move with replication forks in S-phase (Aparicio et al., 1997). Moreover, experiments using rapid degradation of Mcm2-7 subunits in S-phase have shown that all six subunits are required for elongation (Labib et al., 2000), suggesting that the full Mcm2-7 complex is the replicative helicase *in vivo*. More recent experiments described in the next paragraph have finally demonstrated helicase activity *in vivo* with the Mcm2-7 complex. Further studies with mutations in the ATPase domains of Mcm2, 3 and 5 suggest that these subunits may play a role in controlling helicase function by regulating the association of the MCM complex with the DNA (Bochman and Schwacha, 2007).

Although *in vivo* studies support the idea that the Mcm2-7 complex is the replicative DNA helicase, only more recently has the Mcm2-7 complex been shown to have helicase activity *in vitro*. The *Drosophila* Mcm2-7 complex shows robust helicase activity if it is associated with two additional initiation factors, Cdc45 and GINS, both of which bind to the pre-RC as cells enter S-phase (Moyer et al., 2006). Interestingly, Cdc45 and GINS both have been shown to travel with the replication fork during DNA replication (Calzada et al., 2005; Kanemaki and Labib, 2006), suggesting that these two proteins activate the Mcm2-7 helicase. Recent biochemical studies with purified *S. cerevisiae* Mcm2-7 complex suggest that there is a "gate" between Mcm2 and Mcm5 that regulates helicase loading onto DNA (Bochman and Schwacha, 2008). Importantly, conditions that are predicted to close this proposed gate also allow purified Mcm2-7 complexes to exhibit DNA helicase activity. These data suggest that the MCM helicase

activity is regulated at least partly by conformational changes and that binding of Cdc45 and GINS hold the Mcm2-7 complex in an active form.

Although structural studies suggest that the central channel of the MCM complex is purported to be large enough to encircle single- or double-stranded-DNA (Fletcher et al., 2003; Pape et al., 2003), it is not known how the MCM complex encircles the DNA at different stages of the cell cycle. The *E. coli* helicase, DnaB, translocates along single-stranded DNA, thus it was widely assumed that the eukaryotic helicase does so as well (Kaplan et al., 2003). However, EM and *in vitro* helicase studies suggest that SV40 T-antigen helicase forms a double hexamer, binds duplex DNA and extrudes single-stranded DNA from the interface of the two hexamers (Takahashi et al., 2005). Similarly, studies of the Mcm467 helicase and DnaB show that both proteins can move along double stranded DNA in a directional manner (Kaplan et al., 2003; Kaplan and O'Donnell, 2004). In contrast, studies of the E1 DNA helicase strongly support a model in which ssDNA is present in the central channel during helicase activity (Enemark and Joshua-Tor, 2006). Finally, it is worth noting that there is no evidence that origin DNA is unwound during pre-RC formation, suggesting that either the MCM complex is tightly bound to DNA (even at 0.5 M NaCl) through a DNA binding site outside of the central channel or that the MCM complex encircles dsDNA at this stage.

As mentioned in previous sections, association of the MCM complex with the DNA depends on ORC, Cdc6, and Cdt1. It is thought that these proteins act as a "helicase loader", akin to the sliding clamp loaders required for assembly of DNA sliding clamps (e.g. PCNA) onto DNA (Perkins and Diffley, 1998; Randell et al., 2006). Consistent with this hypothesis, several ORC subunits and Cdc6 have sequence and structural similarity to subunits of clamp loaders (Perkins and Diffley, 1998). Like the multiple AAA+ subunits of sliding clamp loaders, it has been proposed that ORC and Cdc6 come together to form a helicase-loading machine. Detailed mechanistic studies of ORC and Cdc6 ATP hydrolysis in *S. cerevisiae* suggested a hypothesis for how the ORC/Cdc6 clamp loader may work (also see ORC and Cdc6 sections). In this model, ORC and Cdc6 (with the assistance of Cdt1) recruit the MCM complex without hydrolyzing ATP. Cdc6 ATP hydrolysis is required for the MCM complex to encircle the DNA. ORC ATP hydrolysis is proposed to act downstream of Cdc6 ATP hydrolysis to release the recently

assembled Mcm2-7 complex and allow for a new round of MCM complex loading to begin (Randell et al., 2006)(Fig 1).

Interestingly, studies in both *S. cerevisiae* and in *Xenopus* suggest that more MCM complexes are loaded at onto DNA than are theoretically needed (i.e. only two per origin should be needed for formation of bidirectional replication forks) (Bell and Dutta, 2002). Moreover, studies in *Xenopus* egg extracts show that efficient replication can occur even if the number of MCM complexes is reduced. It is not currently understood why so many MCM complexes are loaded onto DNA, however, recent studies suggests that they may be needed in times of replication stress (Ge et al., 2007; Woodward et al., 2006). For example, when aphidicolin is used to inhibit active replication in Xenopus egg extracts, normally dormant origins can be used to initiate DNA replication. Furthermore, decreasing the number of available MCM complexes on the DNA increases the amount of DNA replication inhibition caused by replication inhibitors such as aphidicolin or Actinomycin D. These data suggest that the role of excess MCM complexes may be to help cells recover from replication inhibition or other replication stresses.

The Pre-Initiation Complex

After origins are selected by pre-RC formation, the assembly of additional replication factors at the G1 to S transition is required to activate DNA unwinding at the origin (Labib and Gambus, 2007). The proteins required for this event are called the pre-initiation complex (pre-IC) and its formation requires the actions of the two S-phase kinases, CDK and DDK (Fig 4). Pre-IC components can be divided into two classes: those that are required for both initiation and replication fork progression (and hence are destined to be components of the replication fork) and those that are required for initiation but are not required for replication fork progression.

Compared to the mechanistic understanding of pre-RC formation, much less is known about the formation of the pre-IC. The two S-phase kinases, CDK and DDK, both are required for association of pre-IC factors, however, how these kinases control the association of pre-IC components is less clear. Furthermore, efforts to purify pre-initiation complexes or to reconstitute their formation have yet to be successful, making

Figure 4.

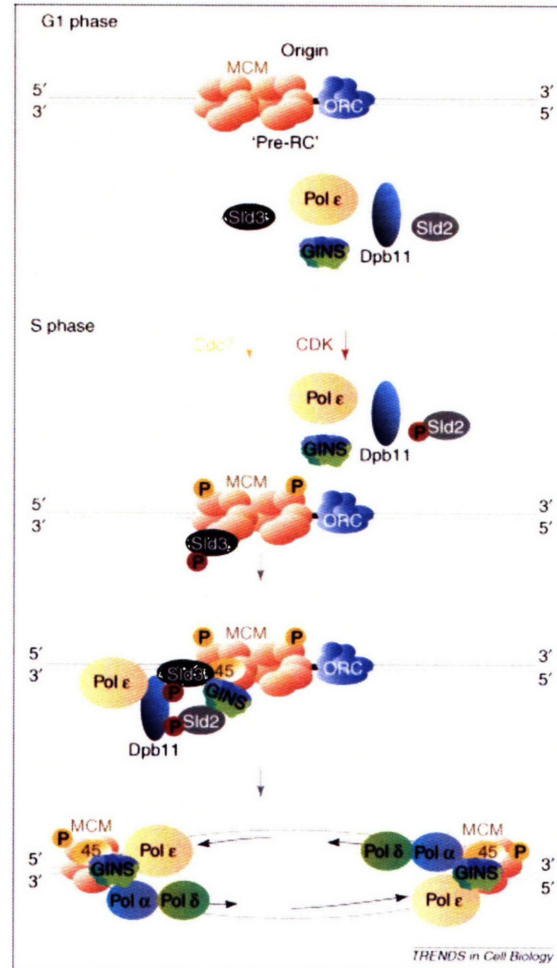


Figure 4. Major events during the initiation of chromosome replication in budding yeast. The origin becomes 'licensed' for replication during G1 phase by loading of inactive MCM complexes at origins to form pre-RCs. As cells enter S phase, CDK phosphorylates Sld2 and Sld3 (phosphorylations shown in red), which can then bind to Dpb11 and promote loading of other factors, such as GINS, Cdc45 and DNA polymerase by a mechanism that has yet to be determined. The Cdc7 kinase is also required for this step (phosphorylations shown in yellow), and MCM is a major target (probably the Mcm2, Mcm4 and Mcm6 subunits). The combined action of CDK and Cdc7 results in the formation of an active MCM complex associated with Cdc45, GINS and other factors. Note that other important proteins such as Mcm10 have been omitted from this scheme for the sake of simplicity, and the precise order of events might differ somewhat between early and late origins. Abbreviations: ORC, origin recognition complex; P, phosphate. (Labib and Gambus, 2007)

analysis of pre-IC formation more difficult to achieve. Nonetheless, I will provide an overview of the current model of pre-IC formation and then will discuss each of the pre-IC protein factors in more detail and how CDK and DDK are involved in controlling the association of these proteins.

The current working model for pre-IC formation begins with mutually dependent association of Sld3 and Cdc45 with the origin (Labib and Gambus, 2007) (Fig 4). Cdc45 has been shown to associate with the MCM complex, and Sld3 binds to Cdc45. Sld3 probably then recruits Sld2, and CDK phosphorylation of these two proteins recruits another protein, Dbp11. Importantly, Dpb11 interacts with DNA Pol ε and is thought to facilitate the recruitment of this polymerase to the origin. GINS, which was introduced earlier as a possible accessory factor of the MCM helicase, is recruited to the origin by Sld3, and GINS association with the origin causes Cdc45 to bind more tightly to the MCM complex. Based on observations that helicase activity can be detected when the Mcm2-7 complex co-purifies with Cdc45 and GINS, it is proposed that the recruitment of these two proteins to the origin is the critical event required to activate the Mcm2-7 helicase completing pre-IC formation.

CDK and DDK are proposed to exert their control over DNA replication initiation by directly phosphorylating the MCM complex (DDK) and Sld2 and Sld3 (CDK) and thereby influencing the recruitment of Sld2, Sld3, Dpb11, Cdc45 and GINS to the origin (Labib and Gambus, 2007). Cdc45 binding to the origin requires DDK activity, and it is thought that this effect is mediated through DDK phosphorylation of the MCM complex. CDK phosphorylation of Sld2 and Sld3 are required for Dpb11 association with the origin, however there is evidence that Sld3 associates with the origin first and recruits Sld2 and Dpb11 (via CDK phosphorylation). Association of GINS with the origin requires both CDK and DDK activity, though this is probably through CDK phosphorylation of Sld3 and DDK-mediated Cdc45 binding to the MCM complex. Moreover, tight association of Cdc45 with the origin requires CDK activity, but this again could be an indirect consequence of the phosphorylation of Sld3 and the subsequent recruitment of GINS.

One other protein, called Mcm10, has been shown to be involved in both initiation and replication fork progression. The role of Mcm10 in initiation and replication fork

progression has been somewhat controversial. Mcm10 interacts with the MCM complex and is required for Cdc45 association with the origins (Maiorano et al., 2006). However, more recent studies suggest that a second critical role of Mcm10 in DNA replication is to stabilize the interaction of polα/primase with the DNA (Ricke and Bielinsky, 2004, 2006).

As mentioned previously, some initiation factors are required for both initiation and for replication fork progression. These initiation factors are Mcm10, Cdc45, GINS and the MCM complex. Interestingly, other than Mcm10, these proteins (Cdc45, GINS and the MCM complex) are involved in active helicase complex formation (Aparicio et al., 2006). The role of Mcm10 in initiation of replication fork progression may be two-fold: aiding the formation of the active helicase by helping to recruit Cdc45, and stabilizing the association of polα/primase with the DNA (Maiorano et al., 2006).

Sld2 and Sld3

The *Sld* genes were identified in a screen for genes that are synthetically lethal with a mutation in *DPB11 (dpb11-1)* (Kamimura et al., 1998). Sld2 and Sld3 are required for initiation of replication. Sld3 binds to the origin before Sld2 and both proteins are phosphorylated by CDK. In their phosphorylated form Sld2 and Sld3 bind and recruit Dpb11 to the pre-IC (Tanaka et al., 2007; Zegerman and Diffley, 2007). Sld3 physically interacts with Cdc45 (Kamimura et al., 2001; Tanaka et al., 2007), and Psf1 (a member of GINS) (Takayama et al., 2003), and facilitates the recruitment of both proteins to the pre-IC.

GINS

The Go-Ichi-Ni-San (GINS) complex is the most recently identified component of the replisome and is required both for initiation of replication and for DNA replication progression (Labib and Gambus, 2007). The genes that encode the GINS proteins were each uncovered in other genetic screens and are called Sld5, Psf1, Psf2 and Psf3 (Go-Ichi-Ni-San is from the Japanese words for five-one-two-three). GINS was initially discovered in *S. cerevisiae* (Takayama et al., 2003) and *Xenopus* (Kubota et al., 2003), however orthologs have been found in many other organisms, including fission yeast (*S.*

pombe) (Gomez et al., 2005; Yabuuchi et al., 2006), mice (Kong et al., 2006; Ueno et al., 2005), humans (De Falco et al., 2007) and archaea (Marinsek et al., 2006). In *S. cerevisiae*, GINS is recruited to origins of replication at the time of initiation at each origin (Takayama et al., 2003). In *S. cerevisiae* and *S. pombe*, Sld3 is required to recruit GINS to the origin (Takayama et al., 2003) and GINS association with the origin is required for stable association of Cdc45 with the MCM complex (Kubota et al., 2003; Yabuuchi et al., 2006). Moreover, the loading of Psf2 has been shown to be dependent on both CDK and DDK activity (Kanemaki and Labib, 2006).

Cdc45

Cdc45 was initially discovered in a screen for mutants in cell-cycle progression (Moir et al., 1982). Like GINS, Cdc45 is conserved among eukaryotes and plays a role both in formation of the pre-IC and in replication fork progression (Labib and Gambus, 2007). Cdc45 physically and genetically interacts with members of the MCM complex (Hopwood and Dalton, 1996; Kamimura et al., 2001; Zou and Stillman, 1998), Sld3 (Kamimura et al., 2001), Psf2 (a member of GINS) (Seki et al., 2006), Mcm10 (Sawyer et al., 2004) and both DNA Polα and DNA Polε (Aparicio et al., 1999; Zou and Stillman, 2000). Tight association of Cdc45 with chromatin depends on both CDK and DDK activity. As discussed previously, Cdc45, along with GINS, is thought to be an accessory factor for the MCM helicase. Interestingly, all of the initiation and replication factors that Cdc45 interacts with are involved in replication fork progression, including DNA synthesis machinery. This suggests that Cdc45 could play a critical role in connecting the Mcm2-7 helicase and the DNA synthesis machinery, perhaps functioning in a manner analogous to the prokaryotic τ protein that coordinates helicase, polymerase and sliding clamp functions.

Dpb11

Dpb11 is essential for DNA replication initiation and was initially identified in a screen for multi-copy suppressors of mutations in DNA Pol ε (Araki et al., 1995). Subsequent studies showed that Dpb11 physically interacts with this polymerase (Masumoto et al., 2000). These studies have led to the hypothesis that the primary

function of Dpb11 is to recruit DNA Pol ε to the origin. Unlike GINS and Cdc45, Dpb11 is not required for progression of replication forks, indicating that Dpb11 is not required to maintain DNA Pol ε at the origin (Masumoto et al., 2000). Dpb11 is recruited to the pre-IC by binding to CDK phosphorylated Sld2 and Sld3 (Masumoto et al., 2002; Tak et al., 2006; Tanaka et al., 2007; Zegerman and Diffley, 2007). Because Dpb11 recruitment occurs prior to DNA unwinding, it is possible that it is only required to recruit DNA Pol ε in the absence of ssDNA template and that after DNA unwinding DNA Pol ε is held at the origin through interactions with its DNA substrate.

Mcm10

Recent studies suggest that, like the Mcm2-7 complex, Cdc45 and GINS, Mcm10 is required both for replication initiation and elongation (Maiorano et al., 2006). The association of Mcm10 with the pre-RC depends on the Mcm2-7 complex and Mcm10 is required for Cdc45 association with the origin. Mcm10 has also been shown to interact with DNA Polα/primase, and may be required to prevent DNA Polα degradation (Ricke and Bielinsky, 2004, 2006)

The role of CDK in the G1- to S-phase transition

Cyclin-dependent kinases (CDKs) control cell cycle progression in eukaryotes and are in turn controlled by regulation of their activating binding partners (cyclins). In budding yeast, cyclins each associate with the same kinase subunit, Cdc28 (aka Cdk1). In contrast, there are many different cyclins that appear at different times of the cell cycle. During G1, Cln3 accumulation is followed by the accumulation of Cln1 and Cln2. These CDKs stimulate both the synthesis of two B-type cyclins, Clb5 and Clb6, and the degradation of a potent inhibitor of B-type CDK activity, Sic1. As cells enter S-phase, Clb5- and Clb6-CDK activity are activated and trigger key events in S-phase (see below). Later in the cell cycle, Clb1-4 cyclins accumulate and stimulate the events of chromosome segregation. As cells exit mitosis, B-type CDK activity is eliminated and remains off until the following S phase.

The activation of Clb5-Cdc28 and Clb6-Cdc28 at the G1-S transition has two important consequences with respect to DNA replication. First, Clb5/6-Cdc28 activity is

required for origin activation in general and pre-IC formation in particular. Second, the increased B-type CDK activity present throughout S, G2 and M phase (including Clb1-4-Cdc28) inhibits new pre-RC formation. As cells exit mitosis, loss of B-type CDK activity allows a new round of pre-RC formation to be initiated. By oscillating between a high and low Clb-CDK state once per cell cycle, the cell ensures that pre-RCs can only be formed and activated once per cell cycle, which, in turn, ensures that the genome is replicated exactly once per cell cycle.

The essential function of S-phase CDK during replication initiation has recently been elucidated. Studies in budding yeast have identified the essential phosphorylation targets of S-phase CDK as Sld2 and Sld3 (Tanaka et al., 2007; Zegerman and Diffley, 2007). Sld2 contains 11 potential CDK phosphorylation sites (S/T-P, the minimal CDK consensus site) and Sld3 contains 12 sites, however, mutagenesis studies show that only one site on Sld2 and two sites on Sld3 are essential for growth. Recent studies hace shown that phosphorylation of Sld2 and Sld3 by CDK generates a binding site for Dbp11, which is a protein that contains four BRCT repeats. BRCT domains are known to bind phospho-peptides (Manke et al., 2003). The two C-terminal BRCT repeats in Dpb11 bind phosphorylated Sld2 and the two N-terminal BRCT repeats bind phosphorylated Sld3 (Zegerman and Diffley, 2007).

Further studies demonstrate that Sld2 and Sld3 are the only essential targets of CDKs during DNA replication (Tanaka et al., 2007; Zegerman and Diffley, 2007). Cells containing a phospho-mimetic allele of Sld2 and a fusion of Sld3 and Dpb11 (presumably bypassing the need for the normal phosphorylation-dependent Sld3-Dpb11 interaction) can replicate their DNA in the absence of any CDK activity. It is important to note, however, that although DNA replication occurs in these cells, there were other deleterious consequences of the lack of CDK activity. As mentioned above, CDK activity has also been shown to be important for efficient binding of Cdc45 to the chromatin, as well as the GINS complex (Zou and Stillman, 1998). These studies suggest that the CDK requirement for Cdc45 loading is indirect. The most likely explanation involves the role of Sld3 in recruiting GINS to the origin. It is likely that CDK phosphorylation of Sld3 either stabilizes Sld3 association with the origin or with GINS,

thus because GINS stabilizes Cdc45 origin association, CDK action would also indirectly facilitate Cdc45 origin association.

The role of DDK in the G1-S transition

Like CDK, DDK is also required for recruitment of pre-IC components. For example, several reports suggest that DDK is required for tight association of Cdc45 with chromatin in S-phase (Masai et al., 2006; Yabuuchi et al., 2006; Zou and Stillman, 2000). A possible mechanism for this recruitment comes from studies in both budding yeast and HeLa cells indicating that a chromatin-bound hyper-phosphorylated version of Mcm4 is required for Cdc45 association with chromatin (Masai et al., 2006; Sheu and Stillman, 2006). Moreover, both hyperphosphorylation of Mcm4 and stable interaction of Mcm4 with Cdc45 are compromised in cells lacking Cdc7 protein. What remains unclear is whether this effect is mediated by phospho-specific MCM subunit binding by Cdc45 or a more indirect effect of DDK phosphorylation on the Mcm2-7 complex. In this section, I will provide a detailed introduction to the Cdc7 and Dbf4 proteins, as well as evidence that DDK might be localized to origins of replication. I'll finish this chapter by describing the evidence that the MCM complex is the target of DDK for initiation of DNA replication.

Cdc7 and Dbf4 in S. cerevisiae *and homologs in other eukaryotes*

CDC7 was initially identified in *S. cerevisiae* in a screen for mutants that arrest at particular points in the cell cycle (Hartwell, 1971, 1973) and the *DBF4* gene was isolated in a screen for genes involved in initiation of DNA replication (Johnston and Thomas, 1982a, b). The first clues that these two proteins were somehow involved came with the observation that the *cdc7* and *dbf4* terminal phenotypes were almost identical. Analysis of the Cdc7 amino acid sequence showed it to be a serine-threonine kinase (Patterson et al., 1986), however by itself it is not active, and Dbf4 was shown to be required for Cdc7 kinase activity (Kitada et al., 1992; Yoon and Campbell, 1991; Yoon et al., 1993). A direct interaction between Dbf4 and Cdc7 has also been shown (Dowell et al., 1994; Varrin et al., 2005)

Homologs of DDK have been identified in a number of other eukaryotes. A Cdc7 homolog (called Hsk1) was first identified in fission yeast based on structural similarity to budding yeast Cdc7 (Masai et al., 1995). Based on the structural similarity between Cdc7 and Hsk1, other Cdc7 homologs were discovered in other organisms, including human, mouse and *Xenopus* laevis (Masai and Arai, 2002). Although Cdc7 is not generally well conserved, the Cdc7 kinase domains are fairly well conserved (60% identity between budding and fission yeast, 45% identity between budding yeast and human). Dbf4 is less conserved than Cdc7, however, homologs have been discovered using pulldown or two-hybrid screening in other species. As will be discussed in later sections, although the amino acids sequences have diverged, the functions of these DDK-related proteins appear to be conserved.

Does DDK localize to origins of replication?

Studies of DDK activity and localization suggest that DDK is required to activate origins throughout S-phase (Bousset and Diffley, 1998; Donaldson et al., 1998). Moreover, chromatin fractionation studies show that Cdc7 is bound to chromatin throughout the cell cycle, but Dbf4 binding to chromatin is only observed in late G1 and early S phase when Dbf4 protein is present (Weinreich and Stillman, 1999). These data suggest that Cdc7 is bound to chromatin in an inactive state and is then recruited to the origin in an active state by Dbf4. This hypothesis is supported by evidence that Dbf4 can bind to an origin of replication by one-hybrid assay (Dowell et al., 1994). Moreover, this study showed that mutations in Dbf4 that disrupted binding to Cdc7 did not disrupt Dbf4 binding to the origin, suggesting that Dbf4 recruits Cdc7 to the origin, rather than the opposite possibility.

There is conflicting data concerning what proteins are important for DDK recruitment to chromatin. *In vivo* interaction studies, such as two-hybrid and co-IP assays, indicate that Dbf4 binds to Orc2, Orc3 (Duncker et al., 2002; Varrin et al., 2005), Mcm2 (Varrin et al., 2005) and Mcm4 (Sheu and Stillman, 2006). Moreover, experiments in *S. cerevisiae* have shown that ORC is required for Dbf4 association with chromatin, but that the MCM complex is not required (Pasero et al., 1999). In contrast, studies in *Xenopus* egg extracts have shown that Cdc7 requires the presence of the MCM

complex on chromatin but not ORC or Cdc6 (Jares and Blow, 2000; Walter, 2000). Studies presented in this thesis will address this issue from a biochemical perspective.

Domain structure of Dbf4

Studies of the conserved regions of Dbf4 have identified domains involved in origin recruitment and Cdc7 binding. The conserved regions have been named motif N, motif M and motif C (Fig 5). One-hybrid analysis with Dbf4 deletion mutants in *S. cerevisiae* has shown that an N-terminal region of Dbf4 containing motif N is required for Dbf4 recruitment to origin DNA (Dowell et al., 1994). Two-hybrid experiments with Dbf4 deletion mutants showed that motif N also was important in binding Orc2. In contrast, similar two-hybrid studies showed that motif M was important for Dbf4 binding to Mcm2 (Varrin et al., 2005). The region of Dbf4 involved in binding the origin (N-terminus) is distinct from the regions of Dbf4 required to bind Cdc7 (middle and C-terminus) (Hardy and Pautz, 1996). Another study showed that N-terminal truncations including deletion of motif N could bind and activate Cdc7 kinase (Gabrielse et al., 2006) and experiments in *S. pombe* showed that the M- and C-motifs in spDbf4 were sufficient to bind and activate the spCdc7 kinase (Ogino et al., 2001).

Although most of the conserved regions of Dbf4 are not related to other proteins, a portion of Motif N is related to the BRCT motif. Within the 100 amino acids conserved in motif N, the N-terminal 60 amino acids are similar to the conserved BRCT (*BR*CA1 *C-t*erminal) motif. BRCT motifs typically contain about 100 amino acids, however, in Dbf4, the C-terminal 40 amino acid section diverges from the canonical BRCT motif but is conserved in Dbf4 homologs. This region has been termed the *BR*CT and *D*b*f*4 similarity (BRDF) motif (Gabrielse et al., 2006). Proteins with BRCT motifs are often found to be involved in DNA damage response (though not always – see Dpb11 above) and function as phospho-peptide binding domains. Cells lacking motif N, or larger N-terminal truncations that included the BRDF domain, are slow growing but viable and are sensitive to DNA damaging agents (Gabrielse et al., 2006; Varrin et al., 2005).

Figure 5.

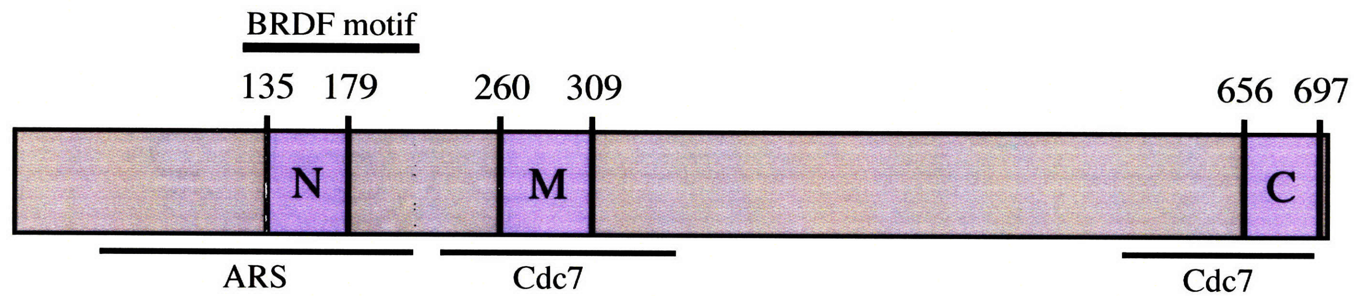


Figure 5. Dbf4 protein: Conserved elements and interaction domains. Locations of motifs N, M and C are shown, as well as the BRDF motif. Regions required for interaction with the origin (ARS) and Cdc7 are depicted by solid lines.

DDK phosphorylation of the MCM complex in S. cerevisiae

Both *in vivo* and *in vitro* studies suggest that the MCM complex is an important target of DDK. A genetic screen for suppressors of DDK function uncovered a mutant allele of *MCM5* (*mcm5-bob1*), with a point mutation in a conserved residue in the N-terminus (Hardy et al., 1997). Interestingly, *in vitro*, DDK has been shown to phosphorylate all of the individual MCM subunits except for Mcm5 (Weinreich and Stillman, 1999). A possible explanation for these data is that the mutation in the *mcm5-bob1* allele causes a conformational change in the MCM complex that mimics a conformational change caused by DDK phosphorylation of other MCM subunits, leading to an active kinase. But this hypothesis has yet to be proven. Other genetic interactions have been demonstrated between DBF4 and MCM2 (Lei et al., 1997). Moreover, the mobility of Mcm2 on 2D protein gels changes to a less phosphorylated form in a *Dbf4*ts mutant. Another study showed that an S-phase-specific hyperphosphorylated form of Mcm4 was found to be dependent on Cdc7 activity (Sheu and Stillman, 2006), suggesting that Mcm4 is also a target of DDK *in vivo.*

Studies in both yeast and mammalian DDK suggest that DDK targets the N-termini of Mcm2 and Mcm4 (Masai and Arai, 2002; Masai et al., 2006; Montagnoli et al., 2006; Sheu and Stillman, 2006). Although the N-termini of the MCM subunits are the least conserved regions of these protein families, the N-termini of Mcm2 and Mcm4 are enriched in S and T residues in all organisms. *S. cerevisiae* Mcm6 also has a very S/T rich N-terminus, however, this S/T richness is less conserved in other organisms. The enrichment of S/T residues without more precise conservation has led to the hypothesis that bulk phosphorylation by DDK, rather than phosphorylation of specific residues, is critical for Mcm2-7 activation. In support of this hypothesis, evidence from both budding and fission yeast (see below) indicates that the N-terminus of one MCM subunit can functionally replace the N-terminus of another subunit for DDK phosphorylation and for cell growth. In a more extreme example, in *S. cerevisiae*, an N-terminal portion of Mcm4 required for cell growth and for phosphorylation of Mcm4 by DDK could be functionally replaced by the HA epitope tag but only if several S residues were added to the endogenous sequence (Sheu and Stillman, 2006).

DDK phosphorylation of the MCM complex in other organisms

Studies in many other organisms using both *in vitro* kinase assays and phospho-specific antibodies *in vivo* suggest that Mcm2, Mcm4 and possibly Mcm6 are substrates of the Cdc7 kinase (Jares and Blow, 2000; Masai and Arai, 2002; Masai et al., 2006; Montagnoli et al., 2006; Tsuji et al., 2006). For example, in HeLa cells, like in budding yeast, Cdc7-dependent hyperphosphorylation of Mcm4 was observed. Furthermore, experiments using phospho-specific antibodies demonstrated that phosphorylation of chromatin-bound Mcm4 at a specific amino acid was dependent on Cdc7 (Masai et al., 2006). Again, as in budding yeast, phosphorylation of Mcm2 and Mcm4 by Cdc7 occurs in the N-termini of the proteins. Moreover, an N-terminal portion of fission yeast Mcm4 that was important for growth could be replaced by, in this case, an N-terminal portion of Mcm6, which restored growth. This hypothesis may also extend to the different MCM subunits. Combining individually viable deletion mutations and phosphorylation mutants in Mcm2, Mcm4 and Mcm6 in fission yeast caused cell death suggesting that Cdc7 phosphorylation of the N-termini of these MCM subunits is redundant.

Detailed mapping of DDK phosphorylation sites has shown that DDK prefers phosphorylating serine or threonine residues adjacent to acidic amino acids (i.e. S/T-D/E sites) (Cho et al., 2006; Montagnoli et al., 2006). Interestingly, it has also been reported that DDK preferentially phosphorylates the first S/T site in S/T-S/T-P sites in which the second S/T is phosphorylated (S/T-P in the canonical CDK phosphorylation site) (Cho et al., 2006; Masai et al., 2006; Montagnoli et al., 2006). This raises the possibility that CDK phosphorylation of the 2nd S/T site in an S/T-S/T-P sequence could facilitate DDK phosphorylation of the 1st S/T site leading to coordination of the phosphorylation activities of these two kinases *in vivo.*

Thesis Summary

In the first section of this thesis I describe the biochemical approach I used to study how DDK binds to and targets the Mcm2-7 complex in the context of the pre-RC. I first determined components of the pre-RC that are modified by DDK, and which components of the pre-RC are important for recruiting DDK. I also demonstrated that DDK specifically targets the population of MCM complexes most tightly bound to the origin, consistent with *in vivo* data that Mcm2-7 is only modified by DDK when bound to chromatin. Most interestingly, I showed that DDK requires prior phosphorylation of the pre-RC to bind to and phosphorylate the Mcm2-7 complex. These results suggest DDK specifically targets the small percentage of MCM complexes that take part in DNA replication and that DDK might coordinate its action with other kinases. In the second section of this thesis, I describe preliminary results of a collaborative effort undertaken with a post-doc in the lab, John Randell. We have used mass spectrometry to determine the sites of phosphorylation on the Mcm2-7 complex both before and after DDK phosphorylation. This analysis will almost certainly be helpful in further dissecting the mechanisms of activation of the replicative helicase.

References

Adachi, Y., Usukura, J., and Yanagida, M. (1997). A globular complex formation by Nda1 and the other five members of the MCM protein family in fission yeast. Genes Cells *2*, 467-479.

Aparicio, O.M., Stout, A.M., and Bell, S.P. (1999). Differential assembly of Cdc45p and DNA polymerases at early and late origins of DNA replication. Proc Natl Acad Sci U S A *96*, 9130-9135.

Aparicio, O.M., Weinstein, D.M., and Bell, S.P. (1997). Components and dynamics of DNA replication complexes in S. cerevisiae: redistribution of MCM proteins and Cdc45p during S phase. Cell *91*, 59-69.

Aparicio, T., Ibarra, A., and Mendez, J. (2006). Cdc45-MCM-GINS, a new power player for DNA replication. Cell Div *1*, 18.

Araki, H., Leem, S.H., Phongdara, A., and Sugino, A. (1995). Dpb11, which interacts with DNA polymerase II(epsilon) in Saccharomyces cerevisiae, has a dual role in S-phase progression and at a cell cycle checkpoint. Proc Natl Acad Sci U S A *92*, 11791-11795.

Bell, S.P., and Dutta, A. (2002). DNA replication in eukaryotic cells. Annu Rev Biochem *71*, 333-374.

Bell, S.P., and Stillman, B. (1992). ATP-dependent recognition of eukaryotic origins of DNA replication by a multiprotein complex. Nature *357*, 128-134.

Bochman, M.L., and Schwacha, A. (2007). Differences in the single-stranded DNA binding activities of MCM2-7 and MCM467: MCM2 and MCM5 define a slow ATP-dependent step. J Biol Chem *282*, 33795-33804.

Bochman, M.L., and Schwacha, A. (2008). The Mcm2-7 complex has in vitro helicase activity. Mol Cell *31*, 287-293.

Bousset, K., and Diffley, J.F. (1998). The Cdc7 protein kinase is required for origin firing during S phase. Genes Dev *12*, 480-490.

Bowers, J.L., Randell, J.C., Chen, S., and Bell, S.P. (2004). ATP hydrolysis by ORC catalyzes reiterative Mcm2-7 assembly at a defined origin of replication. Mol Cell *16*, 967-978.

Broach, J.R., Li, Y.Y., Feldman, J., Jayaram, M., Abraham, J., Nasmyth, K.A., and Hicks, J.B. (1983). Localization and sequence analysis of yeast origins of DNA replication. Cold Spring Harb Symp Quant Biol *47 Pt 2*, 1165-1173.

Calzada, A., Hodgson, B., Kanemaki, M., Bueno, A., and Labib, K. (2005). Molecular anatomy and regulation of a stable replisome at a paused eukaryotic DNA replication fork. Genes Dev *19*, 1905-1919.

Cho, W.H., Lee, Y.J., Kong, S.I., Hurwitz, J., and Lee, J.K. (2006). CDC7 kinase phosphorylates serine residues adjacent to acidic amino acids in the minichromosome maintenance 2 protein. Proc Natl Acad Sci U S A *103*, 11521-11526.

Cook, J.G., Chasse, D.A., and Nevins, J.R. (2004). The regulated association of Cdt1 with minichromosome maintenance proteins and Cdc6 in mammalian cells. J Biol Chem *279*, 9625-9633.

De Falco, M., Ferrari, E., De Felice, M., Rossi, M., Hubscher, U., and Pisani, F.M. (2007). The human GINS complex binds to and specifically stimulates human DNA polymerase alpha-primase. EMBO Rep *8*, 99-103.

Devault, A., Vallen, E.A., Yuan, T., Green, S., Bensimon, A., and Schwob, E. (2002). Identification of Tah11/Sid2 as the ortholog of the replication licensing factor Cdt1 in Saccharomyces cerevisiae. Curr Biol *12*, 689-694.

Diffley, J.F., and Stillman, B. (1988). Purification of a yeast protein that binds to origins of DNA replication and a transcriptional silencer. Proc Natl Acad Sci U S A *85*, 2120-2124.

Donaldson, A.D., Fangman, W.L., and Brewer, B.J. (1998). Cdc7 is required throughout the yeast S phase to activate replication origins. Genes Dev *12*, 491-501.

Dowell, S.J., Romanowski, P., and Diffley, J.F. (1994). Interaction of Dbf4, the Cdc7 protein kinase regulatory subunit, with yeast replication origins in vivo. Science *265*, 1243-1246.

Duncker, B.P., Shimada, K., Tsai-Pflugfelder, M., Pasero, P., and Gasser, S.M. (2002). An N-terminal domain of Dbf4p mediates interaction with both origin recognition complex (ORC) and Rad53p and can deregulate late origin firing. Proc Natl Acad Sci U S A *99*, 16087-16092.

Enemark, E.J., and Joshua-Tor, L. (2006). Mechanism of DNA translocation in a replicative hexameric helicase. Nature *442*, 270-275.

Erzberger, J.P., and Berger, J.M. (2006). Evolutionary relationships and structural mechanisms of AAA+ proteins. Annu Rev Biophys Biomol Struct *35*, 93-114.

Fletcher, R.J., Bishop, B.E., Leon, R.P., Sclafani, R.A., Ogata, C.M., and Chen, X.S. (2003). The structure and function of MCM from archaeal M. Thermoautotrophicum. Nat Struct Biol *10*, 160-167.

Gabrielse, C., Miller, C.T., McConnell, K.H., DeWard, A., Fox, C.A., and Weinreich, M. (2006). A Dbf4p BRCA1 C-terminal-like domain required for the response to replication fork arrest in budding yeast. Genetics *173*, 541-555.

Ge, X.Q., Jackson, D.A., and Blow, J.J. (2007). Dormant origins licensed by excess Mcm2-7 are required for human cells to survive replicative stress. Genes Dev *21*, 3331-3341.

Gillespie, P.J., Li, A., and Blow, J.J. (2001). Reconstitution of licensed replication origins on Xenopus sperm nuclei using purified proteins. BMC Biochem *2*, 15.

Gomez, E.B., Angeles, V.T., and Forsburg, S.L. (2005). A screen for Schizosaccharomyces pombe mutants defective in rereplication identifies new alleles of rad4+, cut9+ and psf2+. Genetics *169*, 77-89.

Gopalakrishnan, V., Simancek, P., Houchens, C., Snaith, H.A., Frattini, M.G., Sazer, S., and Kelly, T.J. (2001). Redundant control of rereplication in fission yeast. Proc Natl Acad Sci U S A *98*, 13114-13119.

Hardy, C.F., Dryga, O., Seematter, S., Pahl, P.M., and Sclafani, R.A. (1997). mcm5/cdc46-bob1 bypasses the requirement for the S phase activator Cdc7p. Proc Natl Acad Sci U S A *94*, 3151-3155.

Hardy, C.F., and Pautz, A. (1996). A novel role for Cdc5p in DNA replication. Mol Cell Biol *16*, 6775-6782.

Hartwell, L.H. (1971). Genetic control of the cell division cycle in yeast. II. Genes controlling DNA replication and its initiation. J Mol Biol *59*, 183-194.

Hartwell, L.H. (1973). Three additional genes required for deoxyribonucleic acid synthesis in Saccharomyces cerevisiae. J Bacteriol *115*, 966-974.

Hopwood, B., and Dalton, S. (1996). Cdc45p assembles into a complex with Cdc46p/Mcm5p, is required for minichromosome maintenance, and is essential for chromosomal DNA replication. Proc Natl Acad Sci U S A *93*, 12309-12314.

Hua, X.H., and Newport, J. (1998). Identification of a preinitiation step in DNA replication that is independent of origin recognition complex and cdc6, but dependent on cdk2. J Cell Biol *140*, 271-281.

Jares, P., and Blow, J.J. (2000). Xenopus cdc7 function is dependent on licensing but not on XORC, XCdc6, or CDK activity and is required for XCdc45 loading. Genes Dev *14*, 1528-1540.

Johnston, L.H., and Thomas, A.P. (1982a). A further two mutants defective in initiation of the S phase in the yeast Saccharomyces cerevisiae. Mol Gen Genet *186*, 445-448.

Johnston, L.H., and Thomas, A.P. (1982b). The isolation of new DNA synthesis mutants in the yeast Saccharomyces cerevisiae. Mol Gen Genet *186*, 439-444.

Kamimura, Y., Masumoto, H., Sugino, A., and Araki, H. (1998). Sld2, which interacts with Dpb11 in Saccharomyces cerevisiae, is required for chromosomal DNA replication. Mol Cell Biol *18*, 6102-6109.

Kamimura, Y., Tak, Y.S., Sugino, A., and Araki, H. (2001). Sld3, which interacts with Cdc45 (Sld4), functions for chromosomal DNA replication in Saccharomyces cerevisiae. EMBO J *20*, 2097-2107.

Kanemaki, M., and Labib, K. (2006). Distinct roles for Sld3 and GINS during establishment and progression of eukaryotic DNA replication forks. EMBO J *25*, 1753-1763.

Kaplan, D.L., Davey, M.J., and O'Donnell, M. (2003). Mcm4,6,7 uses a "pump in ring" mechanism to unwind DNA by steric exclusion and actively translocate along a duplex. J Biol Chem *278*, 49171-49182.

Kaplan, D.L., and O'Donnell, M. (2004). Twin DNA pumps of a hexameric helicase provide power to simultaneously melt two duplexes. Mol Cell *15*, 453-465.

Kitada, K., Johnston, L.H., Sugino, T., and Sugino, A. (1992). Temperature-sensitive cdc7 mutations of Saccharomyces cerevisiae are suppressed by the DBF4 gene, which is required for the G1/S cell cycle transition. Genetics *131*, 21-29.

Klemm, R.D., Austin, R.J., and Bell, S.P. (1997). Coordinate binding of ATP and origin DNA regulates the ATPase activity of the origin recognition complex. Cell *88*, 493-502.

Kong, L., Ueno, M., Itoh, M., Yoshioka, K., and Takakura, N. (2006). Identification and characterization of mouse PSF1-binding protein, SLD5. Biochem Biophys Res Commun *339*, 1204-1207.

Kubota, Y., Takase, Y., Komori, Y., Hashimoto, Y., Arata, T., Kamimura, Y., Araki, H., and Takisawa, H. (2003). A novel ring-like complex of Xenopus proteins essential for the initiation of DNA replication. Genes Dev *17*, 1141-1152.

Labib, K., and Gambus, A. (2007). A key role for the GINS complex at DNA replication forks. Trends Cell Biol *17*, 271-278.

Labib, K., Tercero, J.A., and Diffley, J.F. (2000). Uninterrupted MCM2-7 function required for DNA replication fork progression. Science *288*, 1643-1647.

Lei, M., Kawasaki, Y., Young, M.R., Kihara, M., Sugino, A., and Tye, B.K. (1997). Mcm2 is a target of regulation by Cdc7-Dbf4 during the initiation of DNA synthesis. Genes Dev *11*, 3365-3374.

Lipford, J.R., and Bell, S.P. (2001). Nucleosomes positioned by ORC facilitate the initiation of DNA replication. Mol Cell *7*, 21-30.

Maiorano, D., Lutzmann, M., and Mechali, M. (2006). MCM proteins and DNA replication. Curr Opin Cell Biol *18*, 130-136.

Maiorano, D., Moreau, J., and Mechali, M. (2000). XCDT1 is required for the assembly of pre-replicative complexes in Xenopus laevis. Nature *404*, 622-625.

Manke, I.A., Lowery, D.M., Nguyen, A., and Yaffe, M.B. (2003). BRCT repeats as phosphopeptide-binding modules involved in protein targeting. Science *302*, 636-639.

Marahrens, Y., and Stillman, B. (1992). A yeast chromosomal origin of DNA replication defined by multiple functional elements. Science *255*, 817-823.

Marinsek, N., Barry, E.R., Makarova, K.S., Dionne, I., Koonin, E.V., and Bell, S.D. (2006). GINS, a central nexus in the archaeal DNA replication fork. EMBO Rep *7*, 539-545.

Masai, H., and Arai, K. (2002). Cdc7 kinase complex: a key regulator in the initiation of DNA replication. J Cell Physiol *190*, 287-296.

Masai, H., Matsui, E., You, Z., Ishimi, Y., Tamai, K., and Arai, K. (2000). Human Cdc7-related kinase complex. In vitro phosphorylation of MCM by concerted actions of Cdks and Cdc7 and that of a criticial threonine residue of Cdc7 bY Cdks. J Biol Chem *275*, 29042-29052.

Masai, H., Miyake, T., and Arai, K. (1995). hsk1+, a Schizosaccharomyces pombe gene related to Saccharomyces cerevisiae CDC7, is required for chromosomal replication. EMBO J *14*, 3094-3104.

Masai, H., Taniyama, C., Ogino, K., Matsui, E., Kakusho, N., Matsumoto, S., Kim, J.M., Ishii, A., Tanaka, T., Kobayashi, T., *et al.* (2006). Phosphorylation of MCM4 by Cdc7 kinase facilitates its interaction with Cdc45 on the chromatin. J Biol Chem *281*, 39249-39261.

Masumoto, H., Muramatsu, S., Kamimura, Y., and Araki, H. (2002). S-Cdk-dependent phosphorylation of Sld2 essential for chromosomal DNA replication in budding yeast. Nature *415*, 651-655.

Masumoto, H., Sugino, A., and Araki, H. (2000). Dpb11 controls the association between DNA polymerases alpha and epsilon and the autonomously replicating sequence region of budding yeast. Mol Cell Biol *20*, 2809-2817.

Mendez, J., and Stillman, B. (2003). Perpetuating the double helix: molecular machines at eukaryotic DNA replication origins. Bioessays *25*, 1158-1167.

Mizushima, T., Takahashi, N., and Stillman, B. (2000). Cdc6p modulates the structure and DNA binding activity of the origin recognition complex in vitro. Genes Dev *14*, 1631-1641.

Moir, D., Stewart, S.E., Osmond, B.C., and Botstein, D. (1982). Cold-sensitive cell-division-cycle mutants of yeast: isolation, properties, and pseudoreversion studies. Genetics *100*, 547-563.

Montagnoli, A., Valsasina, B., Brotherton, D., Troiani, S., Rainoldi, S., Tenca, P., Molinari, A., and Santocanale, C. (2006). Identification of Mcm2 phosphorylation sites by S-phase-regulating kinases. J Biol Chem *281*, 10281-10290.

Moyer, S.E., Lewis, P.W., and Botchan, M.R. (2006). Isolation of the Cdc45/Mcm2-7/GINS (CMG) complex, a candidate for the eukaryotic DNA replication fork helicase. Proc Natl Acad Sci U S A *103*, 10236-10241.

Nieduszynski, C.A., Hiraga, S., Ak, P., Benham, C.J., and Donaldson, A.D. (2007). OriDB: a DNA replication origin database. Nucleic Acids Res *35*, D40-46.

Nishitani, H., Lygerou, Z., Nishimoto, T., and Nurse, P. (2000). The Cdt1 protein is required to license DNA for replication in fission yeast. Nature *404*, 625-628.

Ogino, K., Takeda, T., Matsui, E., Iiyama, H., Taniyama, C., Arai, K., and Masai, H. (2001). Bipartite binding of a kinase activator activates Cdc7-related kinase essential for S phase. J Biol Chem *276*, 31376-31387.

Pape, T., Meka, H., Chen, S., Vicentini, G., van Heel, M., and Onesti, S. (2003). Hexameric ring structure of the full-length archaeal MCM protein complex. EMBO Rep *4*, 1079-1083.

Pasero, P., Duncker, B.P., Schwob, E., and Gasser, S.M. (1999). A role for the Cdc7 kinase regulatory subunit Dbf4p in the formation of initiation-competent origins of replication. Genes Dev *13*, 2159-2176.

Patterson, M., Sclafani, R.A., Fangman, W.L., and Rosamond, J. (1986). Molecular characterization of cell cycle gene CDC7 from Saccharomyces cerevisiae. Mol Cell Biol *6*, 1590-1598.

Perkins, G., and Diffley, J.F. (1998). Nucleotide-dependent prereplicative complex assembly by Cdc6p, a homolog of eukaryotic and prokaryotic clamp-loaders. Mol Cell *2*, 23-32.

Raghuraman, M.K., Winzeler, E.A., Collingwood, D., Hunt, S., Wodicka, L., Conway, A., Lockhart, D.J., Davis, R.W., Brewer, B.J., and Fangman, W.L. (2001). Replication dynamics of the yeast genome. Science *294*, 115-121.

Randell, J.C., Bowers, J.L., Rodriguez, H.K., and Bell, S.P. (2006). Sequential ATP hydrolysis by Cdc6 and ORC directs loading of the Mcm2-7 helicase. Mol Cell *21*, 29-39.

Rao, H., and Stillman, B. (1995). The origin recognition complex interacts with a bipartite DNA binding site within yeast replicators. Proc Natl Acad Sci U S A *92*, 2224-2228.

Ricke, R.M., and Bielinsky, A.K. (2004). Mcm10 regulates the stability and chromatin association of DNA polymerase-alpha. Mol Cell *16*, 173-185.

Ricke, R.M., and Bielinsky, A.K. (2006). A conserved Hsp10-like domain in Mcm10 is required to stabilize the catalytic subunit of DNA polymerase-alpha in budding yeast. J Biol Chem *281*, 18414-18425.

Rowles, A., Tada, S., and Blow, J.J. (1999). Changes in association of the Xenopus origin recognition complex with chromatin on licensing of replication origins. J Cell Sci *112 (Pt 12)*, 2011-2018.

Sato, M., Gotow, T., You, Z., Komamura-Kohno, Y., Uchiyama, Y., Yabuta, N., Nojima, H., and Ishimi, Y. (2000). Electron microscopic observation and single-stranded DNA binding activity of the Mcm4,6,7 complex. J Mol Biol *300*, 421-431.

Sawyer, S.L., Cheng, I.H., Chai, W., and Tye, B.K. (2004). Mcm10 and Cdc45 cooperate in origin activation in Saccharomyces cerevisiae. J Mol Biol *340*, 195-202.

Schwacha, A., and Bell, S.P. (2001). Interactions between two catalytically distinct MCM subgroups are essential for coordinated ATP hydrolysis and DNA replication. Mol Cell *8*, 1093-1104.

Sclafani, R.A. (2000). Cdc7p-Dbf4p becomes famous in the cell cycle. J Cell Sci *113 (Pt 12)*, 2111-2117.

Seki, T., Akita, M., Kamimura, Y., Muramatsu, S., Araki, H., and Sugino, A. (2006). GINS is a DNA polymerase epsilon accessory factor during chromosomal DNA replication in budding yeast. J Biol Chem *281*, 21422-21432.

Sheu, Y.J., and Stillman, B. (2006). Cdc7-Dbf4 phosphorylates MCM proteins via a docking site-mediated mechanism to promote S phase progression. Mol Cell *24*, 101-113.

Stinchcomb, D.T., Struhl, K., and Davis, R.W. (1979). Isolation and characterisation of a yeast chromosomal replicator. Nature *282*, 39-43.

Tada, S., Chong, J.P., Mahbubani, H.M., and Blow, J.J. (1999). The RLF-B component of the replication licensing system is distinct from Cdc6 and functions after Cdc6 binds to chromatin. Curr Biol *9*, 211-214.

Tak, Y.S., Tanaka, Y., Endo, S., Kamimura, Y., and Araki, H. (2006). A CDK-catalysed regulatory phosphorylation for formation of the DNA replication complex Sld2-Dpb11. EMBO J *25*, 1987-1996.

Takahashi, T.S., Wigley, D.B., and Walter, J.C. (2005). Pumps, paradoxes and ploughshares: mechanism of the MCM2-7 DNA helicase. Trends Biochem Sci *30*, 437-444.

Takayama, Y., Kamimura, Y., Okawa, M., Muramatsu, S., Sugino, A., and Araki, H. (2003). GINS, a novel multiprotein complex required for chromosomal DNA replication in budding yeast. Genes Dev *17*, 1153-1165.

Takeda, D.Y., and Dutta, A. (2005). DNA replication and progression through S phase. Oncogene *24*, 2827-2843.

Tanaka, S., and Diffley, J.F. (2002). Interdependent nuclear accumulation of budding yeast Cdt1 and Mcm2-7 during G1 phase. Nat Cell Biol *4*, 198-207.

Tanaka, S., Umemori, T., Hirai, K., Muramatsu, S., Kamimura, Y., and Araki, H. (2007). CDK-dependent phosphorylation of Sld2 and Sld3 initiates DNA replication in budding yeast. Nature *445*, 328-332.

Tsuji, T., Ficarro, S.B., and Jiang, W. (2006). Essential role of phosphorylation of MCM2 by Cdc7/Dbf4 in the initiation of DNA replication in mammalian cells. Mol Biol Cell *17*, 4459-4472.

Tsuyama, T., Tada, S., Watanabe, S., Seki, M., and Enomoto, T. (2005). Licensing for DNA replication requires a strict sequential assembly of Cdc6 and Cdt1 onto chromatin in Xenopus egg extracts. Nucleic Acids Res *33*, 765-775.

Tye, B.K. (1999). MCM proteins in DNA replication. Annu Rev Biochem *68*, 649-686.

Ueno, M., Itoh, M., Kong, L., Sugihara, K., Asano, M., and Takakura, N. (2005). PSF1 is essential for early embryogenesis in mice. Mol Cell Biol *25*, 10528-10532.

Varrin, A.E., Prasad, A.A., Scholz, R.P., Ramer, M.D., and Duncker, B.P. (2005). A mutation in Dbf4 motif M impairs interactions with DNA replication factors and confers increased resistance to genotoxic agents. Mol Cell Biol *25*, 7494-7504.

Walter, J.C. (2000). Evidence for sequential action of cdc7 and cdk2 protein kinases during initiation of DNA replication in Xenopus egg extracts. J Biol Chem *275*, 39773-39778.

Weinreich, M., and Stillman, B. (1999). Cdc7p-Dbf4p kinase binds to chromatin during S phase and is regulated by both the APC and the RAD53 checkpoint pathway. EMBO J *18*, 5334-5346.

Wilmes, G.M., and Bell, S.P. (2002). The B2 element of the Saccharomyces cerevisiae ARS1 origin of replication requires specific sequences to facilitate pre-RC formation. Proc Natl Acad Sci U S A *99*, 101-106.

Woodward, A.M., Gohler, T., Luciani, M.G., Oehlmann, M., Ge, X., Gartner, A., Jackson, D.A., and Blow, J.J. (2006). Excess Mcm2-7 license dormant origins of replication that can be used under conditions of replicative stress. J Cell Biol *173*, 673-683.

Wyrick, J.J., Aparicio, J.G., Chen, T., Barnett, J.D., Jennings, E.G., Young, R.A., Bell, S.P., and Aparicio, O.M. (2001). Genome-wide distribution of ORC and MCM proteins in S. cerevisiae: high-resolution mapping of replication origins. Science *294*, 2357-2360.

Yabuuchi, H., Yamada, Y., Uchida, T., Sunathvanichkul, T., Nakagawa, T., and Masukata, H. (2006). Ordered assembly of Sld3, GINS and Cdc45 is distinctly regulated by DDK and CDK for activation of replication origins. EMBO J *25*, 4663-4674.

Yoon, H.J., and Campbell, J.L. (1991). The CDC7 protein of Saccharomyces cerevisiae is a phosphoprotein that contains protein kinase activity. Proc Natl Acad Sci U S A *88*, 3574-3578.

Yoon, H.J., Loo, S., and Campbell, J.L. (1993). Regulation of Saccharomyces cerevisiae CDC7 function during the cell cycle. Mol Biol Cell *4*, 195-208.

Zegerman, P., and Diffley, J.F. (2007). Phosphorylation of Sld2 and Sld3 by cyclin-dependent kinases promotes DNA replication in budding yeast. Nature *445*, 281-285.

Zou, L., and Stillman, B. (1998). Formation of a preinitiation complex by S-phase cyclin CDK-dependent loading of Cdc45p onto chromatin. Science *280*, 593-596.

Zou, L., and Stillman, B. (2000). Assembly of a complex containing Cdc45p, replication protein A, and Mcm2p at replication origins controlled by S-phase cyclin-dependent kinases and Cdc7p-Dbf4p kinase. Mol Cell Biol *20*, 3086-3096.

Chapter II

The S-phase kinase, DDK, requires priming phosphorylation to bind and modify origin-linked Mcm2-7 complex

Summary

The essential S-phase kinase, Cdc7-Dbf4 or Dbf4-dependent kinase (DDK), acts at each origin of replication to trigger initiation of replication. Origins of replication are marked during G1 by formation of the pre-Replicative Complex (pre-RC), which results in the loading of the replicative DNA helicase, the Mcm2-7 complex, onto origin DNA. DDK targets the Mcm2-7 complex for modification with a preference for those MCM complexes that are associated with chromatin. Here we use *in vitro* assembled pre-RCs to address the mechanisms that lead to the targeting of DDK to the origin. In the context of the pre-RC, we find that DDK preferentially targets Mcm4 and Mcm6. Using assays that distinguish between different populations of Mcm2-7 complexes, we show that DDK preferentially targets MCM complexes that are most tightly linked to origin DNA and that DDK association with and phosphorylation of pre-RC-linked MCM complexes requires prior phosphorylation of the pre-RC. These findings support a model in which phosphorylation of Mcm2-7 subunits during pre-RC formation leads to the preferential targeting of origin-associated MCM complexes by DDK during S-phase.

Introduction

Eukaryotic DNA replication initiates from hundreds of different sites in the genome called origins of replication. These events are tightly controlled during the cell cycle to ensure that no origin initiates more than once in a given cell cycle, yet all chromosomal DNA is completely replicated (Arias and Walter, 2007). Central to this control is the strict temporal separation of origin selection and origin activation during the cell cycle. During origin selection, which occurs in late M- and G1-phase, the Origin Recognition Complex (ORC) binds to origin DNA, recruits Cdc6 and Cdt1 and together these proteins load the Mcm2-7 helicase onto the origin to form the pre-replicative complex (pre-RC) (Mendez and Stillman, 2003). This complex marks and licenses all potential origins of replication for replication initiation. As cells enter S-phase, activation of two S-phase-specific protein kinases, Clb5/Cdc28 (CDK) and the Dbf4-dependent kinase, Cdc7 (DDK), triggers initiation of replication at pre-RCs. CDK and DDK stimulate the stable association of downstream initiation factors with the origin, including Sld2, Sld3, Dpb11, Cdc45 and GINS (Labib and Gambus, 2007). Recruitment of these factors to the origin results in the formation of the pre-initiation complex (pre-IC) and the activation of the Mcm2-7 helicase. Once activated, Mcm2-7 unwinds origin DNA to provide the ssDNA template required to recruit the DNA synthesis machinery (Pacek and Walter, 2004).

The targets and consequences of CDK and DDK during replication initiation are only now being understood. Recent studies have demonstrated that Sld2 and Sld3 are the only replication proteins whose phosphorylation by CDK is essential for initiation of replication (Tanaka et al., 2007; Zegerman and Diffley, 2007). Interestingly, phosphorylation of each protein stimulates its association with a third replication factor, Dpb11, which is required for the recruitment of DNA pol ε to origin DNA (Masumoto et al., 2000).

The replication proteins targeted by DDK and the consequences of these modifications are less well understood. *In vitro* assays have shown that DDK phosphorylates subunits of the MCM complex (Masai and Arai, 2002), Cdc45 (Nougarede et al., 2000) and DNA polymerase α (Weinreich and Stillman, 1999). Of

these potential targets, both *in vivo* and *in vitro* studies support Mcm2, Mcm4 and Mcm6 as the most likely targets of phosphorylation by DDK (Masai and Arai, 2002; Masai et al., 2006; Montagnoli et al., 2006; Sheu and Stillman, 2006; Tsuji et al., 2006). The identification of a mutation in *MCM5* that allows cell growth in the absence of DDK activity further supports the hypothesis that the MCM complex is the essential target of DDK (Hardy et al., 1997). DDK activity is required for association of Cdc45 with chromatin (Walter, 2000; Zou and Stillman, 2000) and recent studies suggest that Cdc45 and the four protein GINS complex associates with and activates the helicase activity of the Mcm2-7 complex (Gambus et al., 2006; Moyer et al., 2006; Pacek et al., 2006). This has led to the hypothesis that DDK phosphorylation of the MCM complex stimulates the formation of this complex, although direct evidence for this model is lacking.

In addition to targeting replication initiation proteins, several lines of evidence indicate that DDK is recruited to the origin DNA. One-hybrid assays showed that Dbf4 associates with origins in an ORC-dependent manner (Dowell et al., 1994). Two-hybrid and co-immunoprecipitation studies have identified Mcm2, Orc2 and Orc3 as potential binding partners for Dbf4 (Duncker et al., 2002; Varrin et al., 2005), leading to the hypothesis that Dbf4 recruits Cdc7 to the origin. Two additional observations are consistent with the recruitment of DDK to the origin. First, *in vivo* studies suggest DDK preferentially phosphorylates chromatin bound MCM complexes (Sheu and Stillman, 2006). Second, DDK acts on individual origins as they initiate throughout S-phase rather than globally activating its target proteins (Bousset and Diffley, 1998; Donaldson et al., 1998). Despite these observations, how DDK is recruited to the origin and whether this event is regulated remains unclear.

The Cdc7/Dbf4 kinase is unlikely to be the only kinase that phosphorylates the MCM complex. Recent evidence suggests that the MCM complex needs to be previously phosphorylated to be a substrate for DDK. For example, Mcm2 purified from yeast is a good substrate of DDK, but dephosphorylating Mcm2 (by treatment with phosphatase) prior to incubation with DDK decreases the ability of DDK to phosphorylate Mcm2 (Kihara et al., 2000). Similarly, studies using *Xenopus* egg extracts showed that MCM proteins are phosphorylated by multiple kinases and that different MCM subunits are

phosphorylated or dephosphorylated depending on cell cycle stage (Pereverzeva et al., 2000). The identity of these other kinases and the mechanism by which the prior phosphorylation stimulates DDK action remain unknown.

We sought to gain a better mechanistic understanding of how DDK is targeted to the pre-RC and which pre-RC proteins are phosphorylated by DDK in the context of the pre-RC. To this end, we used an extract based pre-RC assembly assay to form pre-RCs, which were then used as a substrate in DDK kinase assays. In this context, we found that Mcm4 and Mcm6 are the major proteins phosphorylated by DDK. Importantly, DDK binds these pre-RCs and we find that the MCM complex is the only component of the pre-RC essential for this interaction. We also show that DDK preferentially targets a subpopulation of MCM complexes that are tightly associated with DNA after pre-RC formation. Lastly, we found that targeting of DDK to the MCM complex required prior phosphorylation of the pre-RC.

Results

Purification and Activity of Cdc7/Dbf4

To study the phosphorylation of the pre-RC by DDK, we purified DDK from asynchronous *S. cerevisiae* cells over-expressing epitope tagged versions of Cdc7 and Dbf4. We chose purification from yeast cells to ensure that Cdc7 and Dbf4 were appropriately modified as previous studies suggested that this kinase can be regulated not only by Dbf4 abundance but also by other modifications (Yoon et al., 1993). We purified both a wild-type DDK and a "kinase deficient" mutant (Cdc7-D163A) that contained a single mutation in the ATP binding site as a negative control (Ohtoshi et al., 1997). The final purified DDK preparation was composed of Cdc7 and Dbf4 in equimolar ratios (Fig. 1A).

We tested the kinase activity of the purified DDK using Mcm6 protein purified from yeast as a substrate. Mcm6 is phosphorylated by wild-type DDK but is not phosphorylated by the kinase deficient DDK (Fig. 1B, lanes 3 and 4), indicating that there was no contaminating Mcm6 kinase activity in the preparation. We observed that both Cdc7 and Dbf4 were also phosphorylated in the sample containing wild-type DDK, consistent with previous reports that DDK has autophosphorylation activity (Kihara et al., 2000). We also observed a small amount of phosphorylation (about 5% as compared to wild-type) of Dbf4 and Cdc7 in the samples containing kinase deficient DDK. It is possible that this is due to a contaminating kinase. Alternatively, this phosphorylation may be due to a residual amount of kinase activity in the kinase deficient mutant.

DDK phosphorylation of the pre-RC

Although it is thought that DDK phosphorylates the heterohexameric MCM complex *in vivo*, previous *in vitro* studies of DDK phosphorylation of MCM proteins have only investigated individual MCM subunits or MCM complex subassemblies as substrates (Masai and Arai, 2002). In the cell, however, it is thought that DDK modifies MCM complexes after they have been loaded onto origin DNA during pre-RC formation (Sheu and Stillman, 2006). To investigate DDK phosphorylation of MCM proteins in a more physiological situation, we tested the ability of DDK to phosphorylate Mcm2-7

Figure 1.

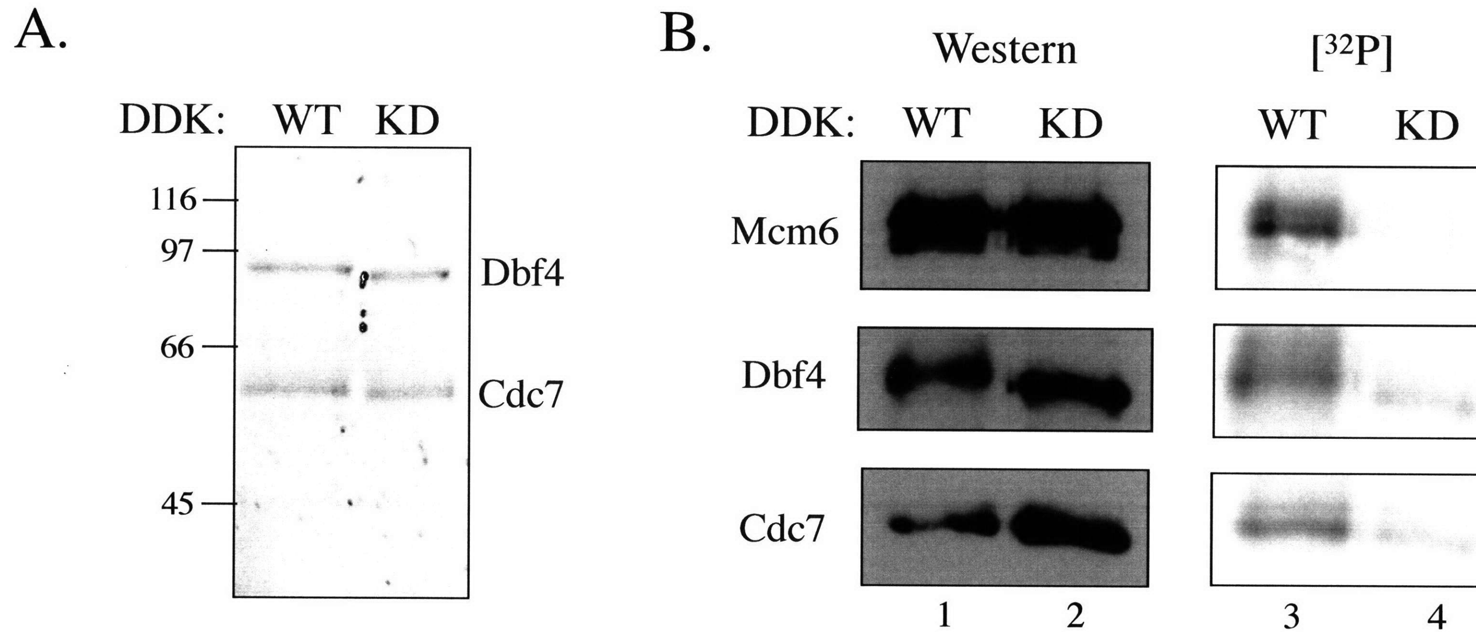


Fig 1: Purification and Activity of Cdc7/Dbf4 (DDK) (A) *S. cerevisiae* Cdc7/Dbf4 kinase was purified from asynchronous yeast cells overexpressing Cdc7-proA and Dbf4-CBP using calmodulin and heparin column chromatography (see Materials and Methods). Aliquots of heparin column fractions were analyzed by 8% SDS/PAGE separation and stained by Sypro Orange. Lanes: WT (wild-type); (KD) kinase deficient Cdc7-D163A. (B) Phosphorylation of Mcm6p by the Cdc7/Dbf4 kinase. 125 ng (700fmol) of both wild-type and kinase deficient Cdc7/Dbf4 were incubated with 400ng of Mcm6p at 25°C for 15 min in the presence of [γ-^{32}P]ATP. Samples were analyzed for the presence of Mcm6, Cdc7 and Dbf4 by immunoblotting and phosphorylation was detected by autoradiography.

complexes in the context of the pre-RC. To generate this substrate, we used an *in vitro* assay that assembles pre-RCs on origin DNA attached to magnetic beads (Bowers et al., 2004). The resulting pre-RCs can be readily purified and reflect the properties of *in vivo* assembled pre-RCs. To determine if DDK phosphorylated proteins were components of the pre-RC, we also tested DDK modification of proteins that assembled onto DNA lacking an ORC binding site and, therefore, lacked associated pre-RCs (Fig. 2A).

When pre-RCs were treated with DDK we observed four prominently phosphorylated proteins (Fig. 2B, lane 1). The two strongly phosphorylated and lower molecular weight proteins were the result of autophosphorylation of Cdc7 and Dbf4. These proteins were phosphorylated in both wild-type and mutant origin samples to which wild-type DDK was added but phosphorylation was greatly reduced in the samples containing kinase deficient DDK. The remaining two prominently phosphorylated proteins showed the properties of pre-RC components targeted by DDK: they were only present when wild-type origin DNA was used in the pre-RC assembly assay (Fig. 2B, compare lanes 1 and 2) and phosphorylation of these proteins was not observed when kinase deficient DDK was used in the assay (Fig. 2B, compare lanes 1 and 3). Finally, we observed lesser phosphorylation of two additional proteins. One of these proteins co-migrated with the lower of the two pre-RC specific bands and was detectable when mutant origin DNA was used during pre-RC assembly and thus was unlikely to be a pre-RC component. The second protein migrated just below the pre-RC specific proteins and was specific to complexes assembled on wild-type origin DNA. However, this protein was equivalently modified in the wild-type and mutant DDK reactions and we suspect that this phosphorylation is due to a second, unidentified kinase.

Identification of the pre-RC targets of DDK

We next determined which pre-RC components were targeted by DDK in the context of the pre-RC. Based on previous analysis of DDK function and because the two prominently phosphorylated proteins migrated in the region of the Mcm2-7 proteins, we focused our studies on the Mcm2-7 proteins. We assembled pre-RCs using extracts derived from five strains, each of which expressed a distinct BCCP (biotin carboxyl carrier protein) epitope-tagged Mcm subunit (the BCCP-tagged Mcm3 strain was not

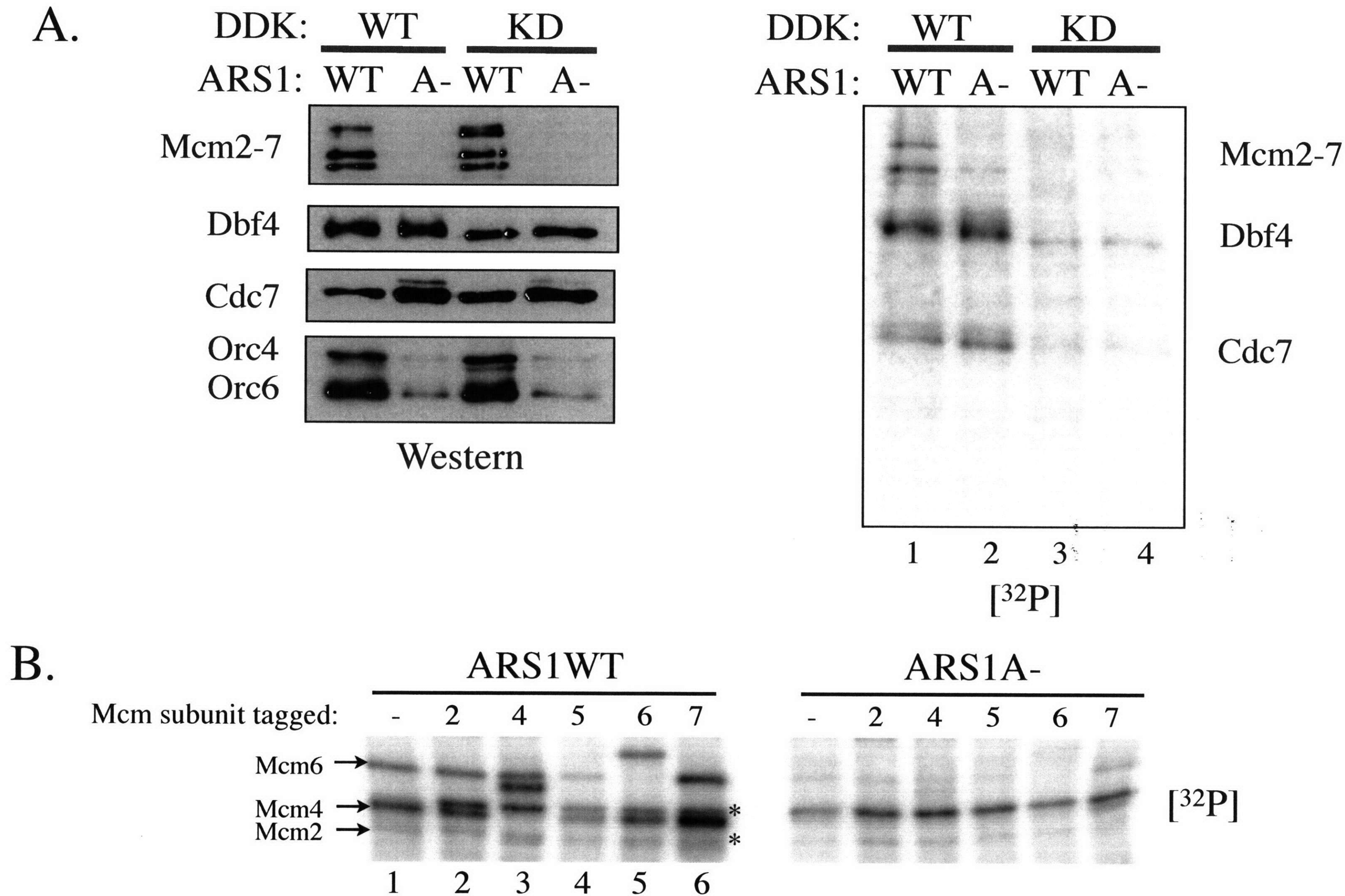


Fig 2: DDK phosphorylates Mcm4 and Mcm6 in the context of the pre-RC (A) DDK Phosphorylation of purified pre-RCs. Pre-RC assembly assays were performed with WT and mutant (A-) ARS1 containing DNAs. DNA associated proteins were then treated with wild-type and kinase deficient DDK in the presence of [γ-^{32}P]ATP for 15 minutes at 25°C. Samples were analyzed by SDS/PAGE, blotted and probed as described in Fig. 1B along with polyclonal anti-Orc1-6 antibody. Samples were also analyzed by autoradiography. (B) Mcm4 and Mcm6 are phosphorylated by Cdc7/Dbf4. Pre-RCs were assembled with wild-type and mutant ARS1 containing DNAs and extracts in which a single Mcm subunit was BCCP tagged (except Mcm3). Pre-RCs were then treated with wild-type Cdc7/Dbf4 kinase in the presence of [γ-^{32}P]ATP. Samples were analyzed by immunoblotting and autoradiography. Arrows indicate the bands that are shifted when tagged with BCCP. Asterisks indicate origin-independent phosphoproteins.

viable). We prepared G1-arrested extracts from each Mcm-BCCP-tagged strain as well as an untagged control strain. Using these extracts we assembled and purified pre-RCs and incubated them with DDK and radioactive ATP.

Consistent with Mcm4 and Mcm6 being DDK targets, pre-RCs containing BCCP-tagged Mcm4 and Mcm6 showed a shift in the location of one of the two proteins phosphorylated in a pre-RC-dependent manner (Fig. 2B lanes 3 and 5). A third radioactively labeled protein that migrated at a slightly lower mobility than untagged Mcm4 did not change mobility in any of the extracts derived from the BCCP-tagged strains. Although it was possible that this was Orc1 or Mcm3, both of which have a similar molecular weight, this band was also seen in control experiments using mutant origin DNA ("ARS1 A-"), indicating that it is not a pre-RC component and is probably the same contaminant seen in the figure 2B. We also observed a weakly phosphorylated pair of proteins that migrated slightly faster than Mcm4. The slower migrating protein of the doublet appears to be a contaminant as it is seen in the mutant origin control experiments. In contrast, the faster migrating protein of the doublet disappears when pre-RCs were prepared from the BCCP-tagged Mcm2 strain (Fig. 2B lane 2). Phosphorylated Mcm2-BCCP may co-migrate with phosphorylated Mcm4 and, thus, cannot be observed. Based on these findings, we conclude that, in the context of the pre-RC, Mcm4 and Mcm6 are the primary DDK targets in the context of the pre-RC and that Mcm2 is a minor phosphorylation product.

DDK binds to the pre-RC

Previous studies have shown that Dbf4 binds to Orc2, Orc3 and Mcm2 (Varrin et al., 2005) and that Dbf4 is recruited to an origin *in vivo* (Dowell et al., 1994). Therefore, we asked if purified DDK binds the pre-RC *in vitro*. To this end, we repeated the pre-RC phosphorylation experiment described above but after DDK treatment we re-isolated the origin DNA and any associated DDK. In addition to the pre-RC components, both Cdc7 and Dbf4 co-purified with the origin DNA (Fig. 3A). Consistent with the origin association of DDK being pre-RC dependent, Cdc7 and Dbf4 did not associate with DNA containing a mutant origin.

Figure 3.

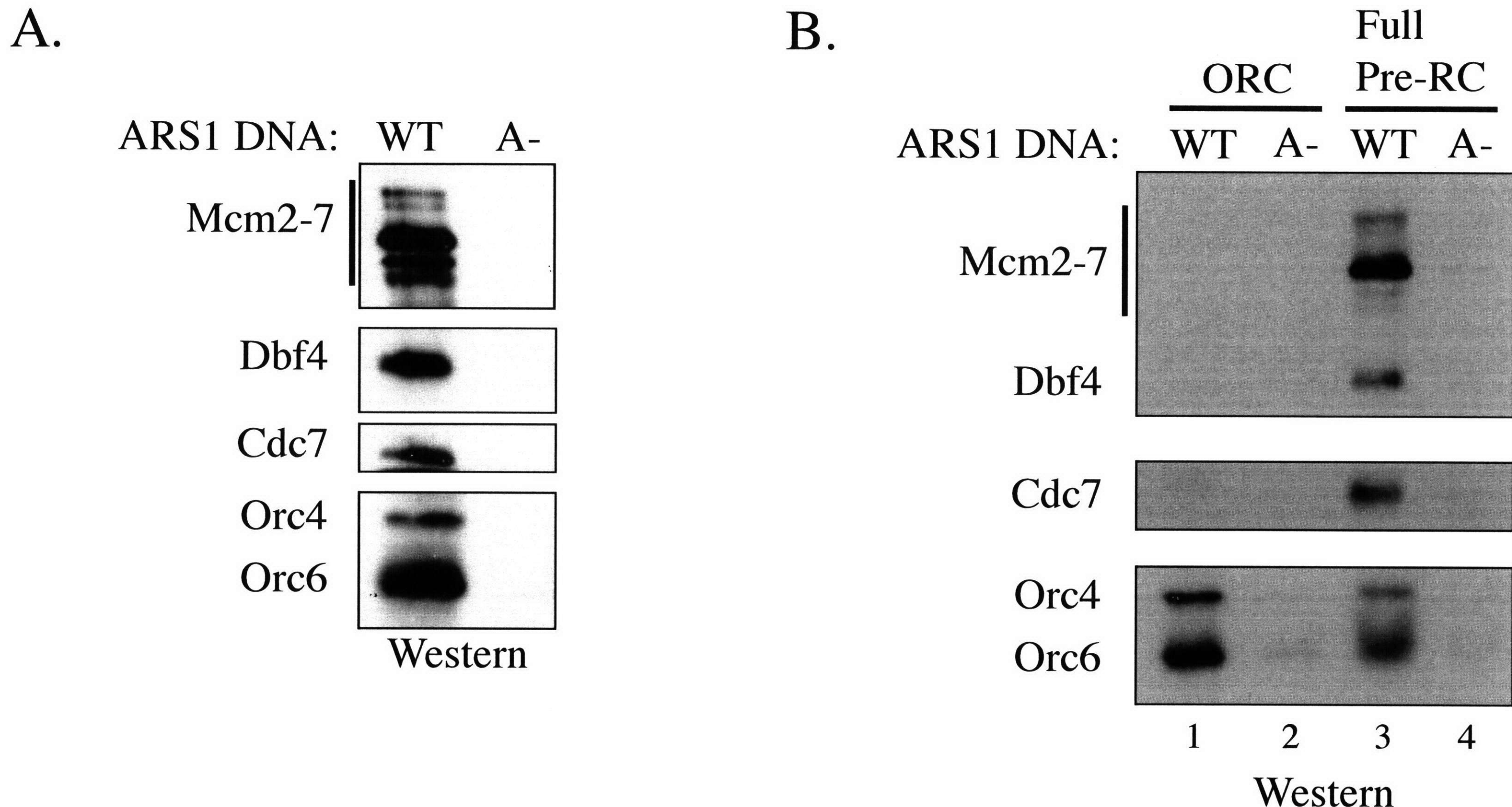


Fig 3: DDK associates with the pre-RC in a MCM-dependent manner (A) Cdc7/Dbf4 associates with the pre-RC. Pre-RC assembly assays were performed with wild-type and mutant ARS1 containing DNAs. DNA-associated proteins were then treated with wild-type Cdc7/Dbf4 and ATP. Upon conclusion of the kinase incubation, proteins associating with the DNA were isolated and analyzed by immunoblotting. (B) ORC is not sufficient to recruit Cdc7/Dbf4. DDK association with the pre-RC was measured as described in Fig3A except Cdc6 was not included in the reactions labeled "ORC". Without Cdc6, ORC binds to the DNA but cannot recruit Cdt1 or the MCM complex.

We next asked which pre-RC components were required to recruit DDK to origin DNA. We first addressed whether ORC could recruit DDK independently of other pre-RC components. Using extracts lacking Cdc6, we prepared DNA templates associated with ORC but not other pre-RC components (Fig. 3B). Under these conditions, ORC associates with origin DNA but the remaining pre-RC components do not (see Fig. 3B, lane 1). No DDK association was detectable with DNA templates that associated with ORC but not other pre-RC components (Fig. 3B, lanes 1 and 2). Thus, ORC was not sufficient to recruit DDK to origin DNA. To address the role of Mcm2-7 in recruiting DDK, we assembled pre-RCs and treated them with a high-salt wash. Previous studies showed that only a "loaded" subpopulation of Mcm2-7 complexes is retained on origin DNA after high-salt treatment of pre-RCs. ORC, Cdc6 and Cdt1 are quantitatively removed by this treatment (Bowers et al., 2004). After normalizing the amount of MCM proteins in each assay, we tested DDK association with low- and high-salt-washed pre-RCs. We found that DDK bound to both complexes with similar affinity (Fig. 3C, compare lanes 1 and 3). We conclude that DDK is recruited to the origin DNA primarily through interactions with the MCM proteins and not ORC.

Figure 3.

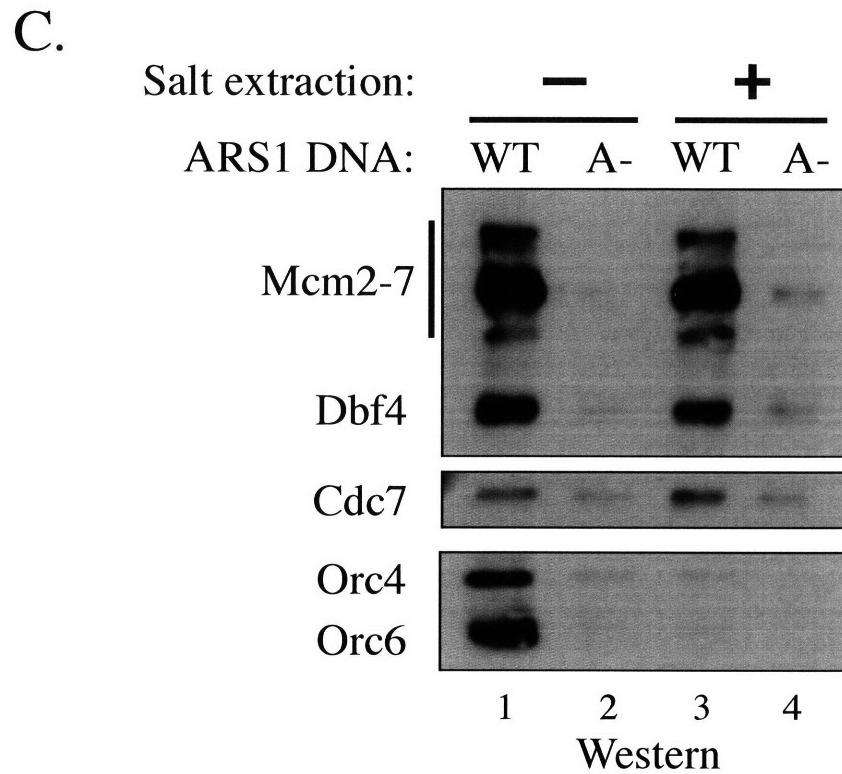


Fig 3: DDK associates with the pre-RC in a MCM-dependent manner (C) The MCM complex is essential for recruitment of Cdc7/Dbf4. Pre-RC assembly assays were performed as described in Fig3A with the exception that pre-RCs labeled "+" for salt were washed with H+/500mM NaCl before being incubated with DDK.

DDK preferentially modifies DNA-associated MCM complexes

In vivo studies suggest that DDK distinguishes between chromatin bound and free MCM complexes (Sheu and Stillman, 2006) and we sought to determine the mechanism of this distinction. To compare DDK modification of MCM proteins that were and were not part of a pre-RC, we took advantage of the limited half-life of MCM complex association with origin DNA to isolate different populations of MCM proteins. In the course of performing the pre-RC/DDK kinase assay, we observed that a portion of the MCM complexes (roughly 50%) that bound to the DNA in the pre-RC assay dissociated from the DNA during the kinase assay. This release of MCM complexes is not due to DDK phosphorylation, as neither DDK nor ATP is required to observe MCM dissociation (Supplementary Fig. 1). Importantly, the released proteins represent a pool of purified Mcm2-7 complexes with the same subunit stoichiometry as those associated with the origin DNA (see figure 4B). Thus, two subpopulations of DNA-linked Mcm2-7 complexes can be distinguished by their stability on DNA during extended incubation in buffer alone.

To compare DDK phosphorylation of the two populations of MCM complexes, pre-RCs were formed and incubated with kinase buffer lacking DDK. The supernatant, containing "free" MCM complexes that dissociated from the DNA after pre-RC formation, was separated from the MCM complexes retained on the DNA. After resuspending the DNA-bound MCM complexes in an equivalent volume of kinase buffer, we added DDK to both populations (Fig. 4A). Importantly, similar quantities of MCM complexes were present in the two kinase assays (as measured by immunoblotting). Although DDK robustly modified the origin associated MCM proteins, there was substantially less phosphorylation of the free MCM complexes (Fig. 4B). Thus, DNA bound MCM proteins were the preferred DDK substrate.

DDK preferentially modifies loaded MCM complexes in an ORC-independent manner

We considered several different hypotheses to explain the basis for the preferential phosphorylation of DNA-linked MCM complexes. It is possible that pre-RC-linked MCM complexes contain a mixed population of MCM complexes that have

Figure 4.

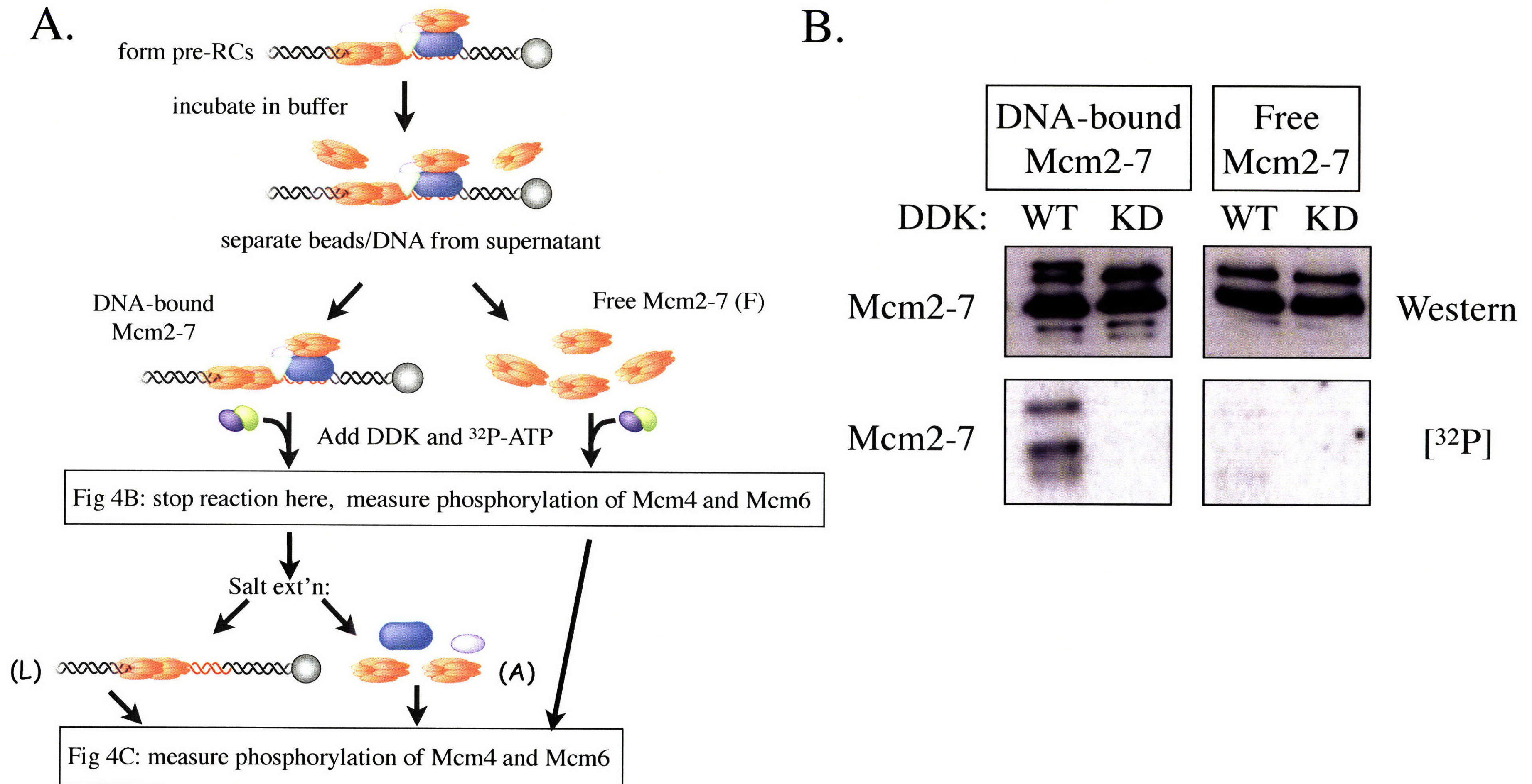


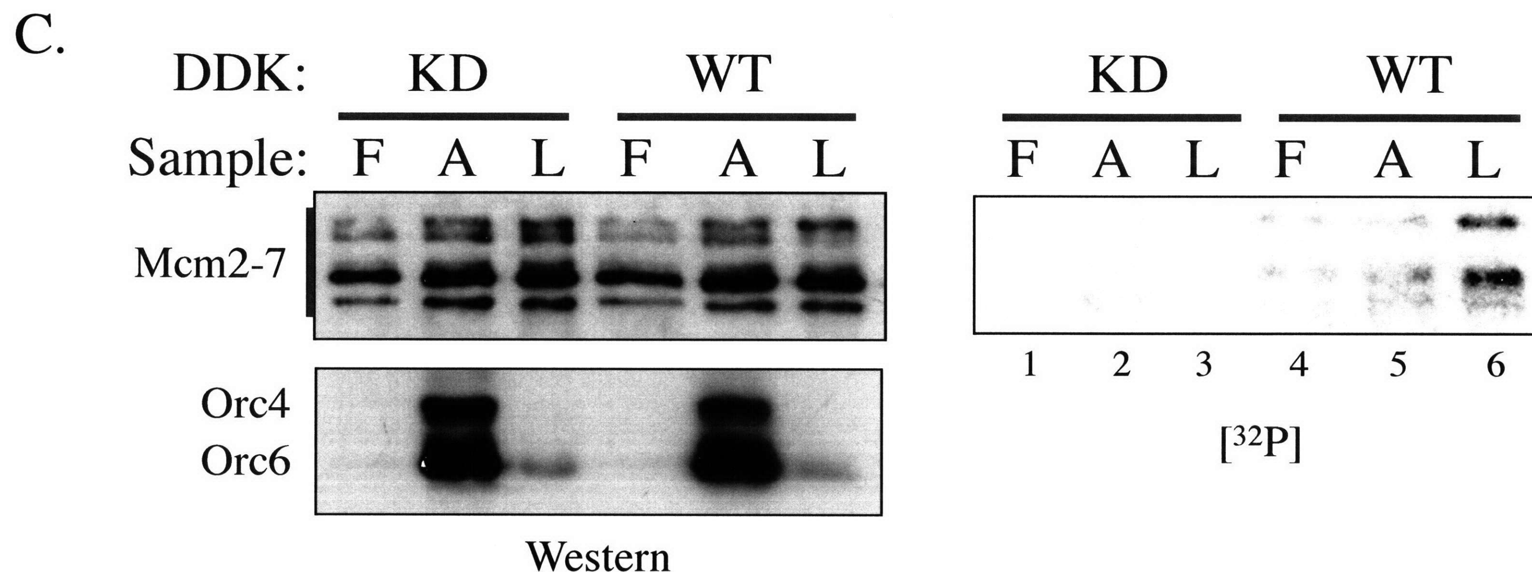
Fig 4: Cdc7/Dbf4 preferentially phosphorylates loaded MCM complexes (A) Experimental Outline for Figs 4B and 4C: Pre-RC assembly assays were performed. DNA-bound pre-RCs were incubated with H+/150mM KGlut for 10min at 25°C. The DNA was isolated and separated from the buffer supernatant. Either wild-type or mutant DDK was added to the proteins remaining bound to the DNA and to the proteins that dissociated from the DNA. In Fig 4B, the samples were analyzed after this step. In Fig 4C, the proteins remaining bound to the DNA were further treated with a high salt wash after treatment with DDK. The proteins remaining bound to the DNA were labeled (L), proteins dissociating from the DNA after salt wash were labeled (A), and the proteins dissociating from the DNA after the original buffer incubation were labeled (F). Samples were analyzed by immunoblotting and autoradiography. (B) Cdc7/Dbf4 preferentially phosphorylates DNA-bound MCM complexes. The experiment was done as described in Fig 4A.

different characteristics (such as conformation or modification state) that confer different dissociation rates from DNA. In this hypothesis, DDK would preferentially target those MCM complexes with the lower dissociation rate. Alternatively, because ORC remains on the DNA as part of the pre-RC, ORC could activate DDK phosphorylation of MCM complexes. Although ORC is not sufficient to recruit DDK to the origin, this observation does not eliminate the possibility that ORC stimulates DDK phosphorylation of pre-RC-linked MCM complexes.

To address these different possibilities, we asked whether DDK distinguished between different DNA-linked MCM complexes. We have demonstrated previously that MCM complexes associating with the origin DNA during the pre-RC assay fall into two distinct populations: *associated* MCM complexes whose origin association is ORC-dependent and salt-sensitive and *loaded* MCM complexes whose origin association is ORC-independent and salt-insensitive. To determine if DDK preferentially modified one of these populations of pre-RC-linked MCM proteins, we modified the experiment in Fig. 4B (see Fig. 4A). After incubation of DDK with DNA-bound pre-RCs, we extracted the pre-RCs with high-salt buffer, separating the loaded from associated MCM complexes. Interestingly, even though DDK was given equal access to the loaded and associated MCM complexes during the kinase assay, the loaded MCM complexes were more heavily phosphorylated than the associated or free MCM complexes (Fig. 4C, lanes 4, 5, and 6 of autorad). This data suggests that DDK preferentially modifies loaded MCM complexes relative to associated or free MCM complexes.

The experiment described above left open the possibility that the preferential phosphorylation of loaded MCM complexes is mediated by the presence of ORC at the origin. The previous experiment could not eliminate this possibility since the high-salt extraction that removes associated MCM complexes and ORC was performed after DDK treatment. To address the role of ORC during phosphorylation of MCM complexes in the pre-RC, we compared DDK phosphorylation of MCM complexes before and after high-salt extraction of pre-RCs. Interestingly, although only 50% of the MCM complexes remained on the DNA after salt-extraction (loaded MCM complexes), autoradiography

Figure 4.



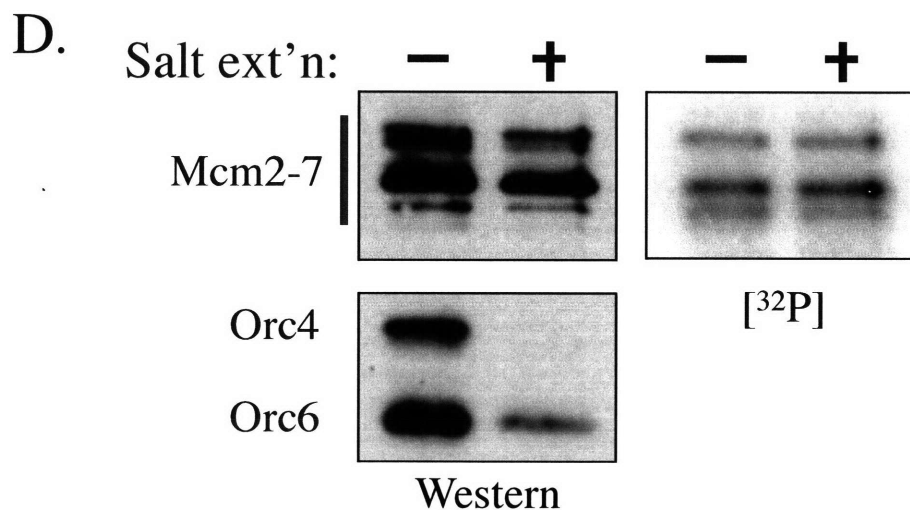


Fig 4: Cdc7/Dbf4 preferentially phosphorylates loaded MCM complexes (C) Cdc7/Db4 preferentially phosphorylates loaded MCM complexes. The experiment was performed as described in Fig 4A. (D) ORC is not required for Cdc7/Dbf4 phosphorylation of Mcm2-7. The experiment was performed as described in Fig3C with the exception that mutant ARS1 DNA was not used and [γ-^{32}P]ATP was added to the reaction.

showed a similar level of phosphorylation as MCM complexes that were not salt extracted (loaded and associated MCM complexes) (Fig. 4D). Because the salt-extracted MCM complexes lack ORC, we conclude that ORC did not contribute to the preferential phosphorylation of loaded MCM complexes.

Prior phosphorylation of the pre-RC is required for DDK to target MCM complexes

Since the presence of ORC did not explain the preferential phosphorylation of loaded MCM proteins by DDK, we considered two alternative (but not mutually exclusive) hypotheses: (1) MCM complexes that are loaded take on a new conformation that is targeted by DDK; or (2) loaded MCM complexes are differentially modified in a manner that stimulates phosphorylation by DDK. We were particularly interested in the latter possibility as previous studies suggested that DDK targets peptides that have been previously phosphorylated (Cho et al., 2006; Kihara et al., 2000; Montagnoli et al., 2006). To test this hypothesis, we prepared pre-RCs, treated them with λ phosphatase, washed away the phosphatase and then tested the ability of the de-phosphorylated pre-RCs to bind to and act as a substrate for DDK.

Dephosphorylation of the pre-RC had several important consequences. First, we found that dephosphorylated pre-RCs lost the ability to bind DDK (Fig. 5A, compare lanes 1 and 3). Immunoblotting showed that the change in DDK association was not due to the displacement of MCM proteins (or ORC) from the DNA after phosphatase treatment. Second, prior phosphatase treatment dramatically reduced the ability of DDK to phosphorylate Mcm4 and Mcm6 but did not affect phosphorylation of the background band that co-migrates with Mcm4 (Fig. 5B). Importantly, we continued to see robust autophosphorylation of Cdc7 and Dbf4 after phosphatase treatment (Supplementary Fig. 2), indicating that the defect in Mcm4 and Mcm6 phosphorylation was not due to incomplete phosphatase removal. We also observed a new phosphoprotein after phosphatase treatment but only in lanes in which pre-RC formation occurred (Fig. 5B, lanes 3 and 7). Importantly, this modification is not affected by mutation of DDK suggesting that another kinase present in the DDK preparation or the pre-RC is responsible. Overall, these data provide strong evidence that preferential targeting of

pre-RC-linked MCM complexes by DDK is regulated by prior phosphorylation and suggests that loaded MCM proteins are selectively phosphorylated.

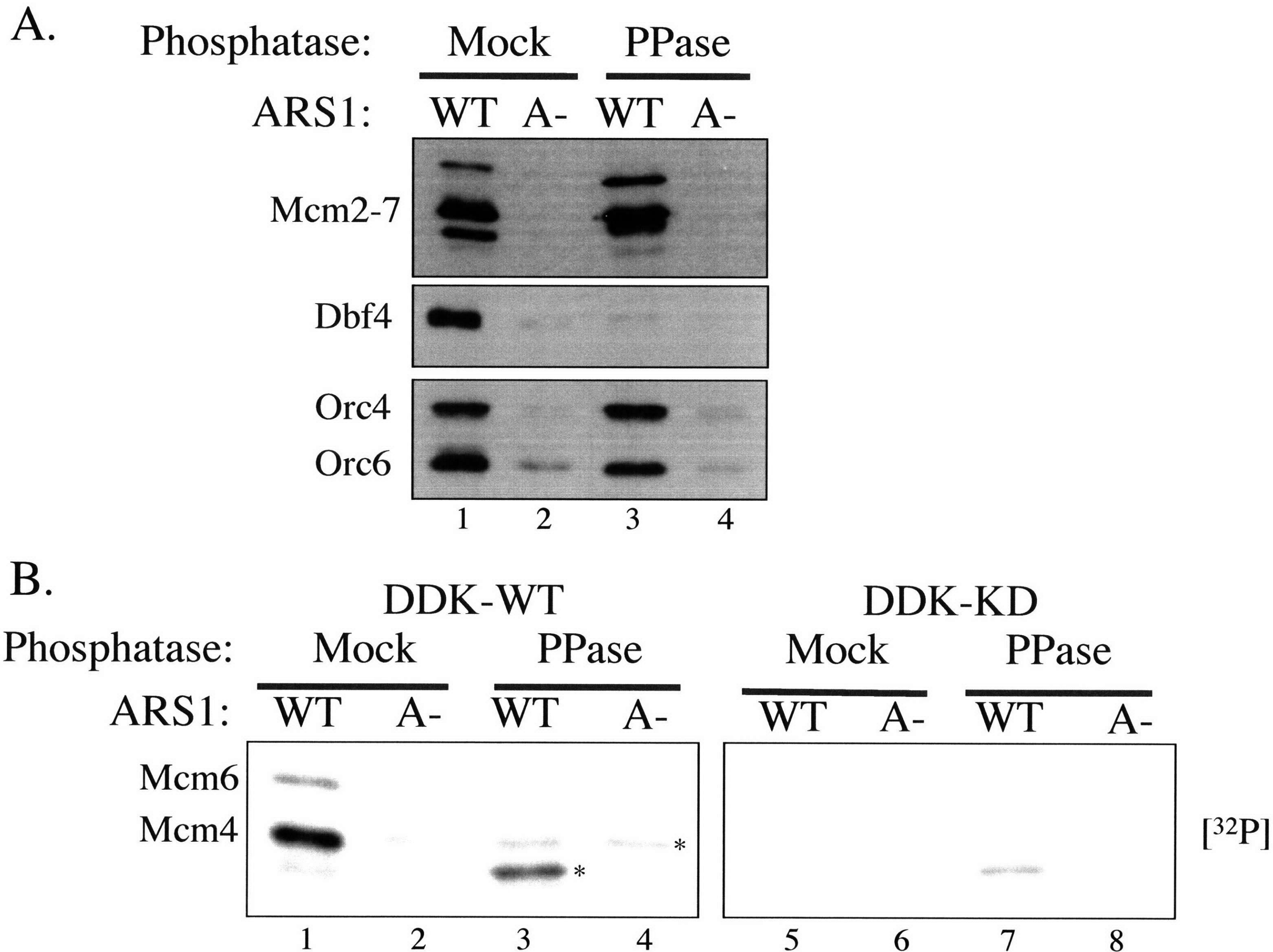


Fig 5: Prior phosphorylation of the pre-RC is required for DDK association and phosphorylation of the MCM complex (A) DDK does not bind to dephosphorylated pre-RCs. Assembled pre-RCs were either treated or mock-treated with λ phosphatase for 5 min at 25°C. Treated pre-RCs were then washed and incubated with wild-type and mutant DDK and [γ-^{32}P]ATP. The proteins remaining bound to the DNA were isolated following the DDK incubation and analyzed by immunoblotting and autoradiography. (B) DDK requires prior phosphorylation of the pre-RC to modify Mcm4 and Mcm6. Autoradiography results of experiment performed in Fig5A. Asterisk indicate nonspecific phosphorylation products.

Discussion

In vivo, only a fraction of total MCM complexes in the nucleus are loaded onto chromatin and used in DNA replication. Recent studies indicate that this subset of MCM complexes is preferentially targeted by DDK (Sheu and Stillman, 2006). Although it is possible that ORC is involved in recruiting DDK to pre-RCs, our data indicates that ORC is neither sufficient to recruit DDK nor required for DDK phosphorylation of MCM complexes loaded onto DNA. Instead, we show that MCM complexes are sufficient to recruit DDK to origins and that DDK preferentially phosphorylates the most tightly origin-linked MCM complexes. Finally, we provide evidence that DDK requires previous phosphorylation of the pre-RC to bind to and modify pre-RC-linked MCM complexes. Taken together, our studies support a model in which preferential loading of phosphorylated Mcm2-7 complexes (or phosphorylation of loaded MCM complexes during G1) leads to the selective recruitment and further phosphorylation of loaded MCM complexes by DDK in late G1/early S-phase.

Our finding that the Mcm2-7 complex and not ORC is central to recruit DDK to the pre-RC is consistent with a number of previous observations. Studies in *Xenopus* egg extracts found that the chromatin association of Cdc7 is dependent on pre-RC formation but does not require either ORC or Cdc6 association with chromatin (Jares and Blow, 2000; Walter, 2000). Studies of the chromatin association of Dbf4 in *S. cerevisiae* cells found that Dbf4 is gradually lost from chromatin during passage through S-phase in a manner that closely parallels the loss of MCM proteins from the chromatin (Weinreich and Stillman, 1999). In addition, two-hybrid studies indicate an interaction between Dbf4 and Mcm2 (Varrin et al., 2005).

Other studies in *S. cerevisiae* support an interaction between ORC and Dbf4 that we did not observe in our studies (Duncker et al., 2002; Varrin et al., 2005). Since all our studies are performed with ORC derived from G1-phase cells, it is possible that the ORC-Dbf4 interactions observed previously occur at other times in the cell cycle when ORC is differentially modified. It is also possible that previous observations of DDK recruitment by ORC reflect a transient interaction that is not detected in our assay. Consistent with ORC not being important for DDK action at the origin, studies in *S. cerevisiae* cells and *Xenopus* cell extracts indicate that ORC is not required for

replication initiation after pre-RC assembly has occurred (Jares and Blow, 2000; Shimada et al., 2002).

The precise interaction that is responsible for recruiting DDK to the pre-RC remains to be determined. Previous one hybrid data suggested that it is Dbf4 rather than Cdc7 that is responsible for DDK recruitment (Dowell et al., 1994). Intriguingly, Dbf4 contains a region called "motif N" that is related to the BRCA1 C-terminal (BRCT) motif (Varrin et al., 2005). Because other BRCT motifs are known to act as phosphopeptide binding domains (Manke et al., 2003) it is tempting to speculate that this domain of Dbf4 mediates the phosphorylation-dependent association of DDK with origin-associated MCM complexes. Studies of the phosphorylation of purified Mcm4 by DDK identified a region in the Mcm4 N-terminus that interacts with DDK (Sheu and Stillman, 2006) and it will be interesting to determine whether this region of Mcm4 is phosphorylated *in vivo*. Given that DDK phosphorylates multiple MCM subunits, it is likely that there will be multiple binding sites for DDK in the intact complex. Alternatively, it is possible that one or more binding sites on a single MCM subunit are responsible for acting in cis and trans to direct phosphorylation of all of the DDK targeted subunits.

In our model, we suggest that priming phosphorylation dictates preferential phosphorylation of origin-linked MCM complex. Our results are consistent with two possible roles for this priming phosphorylation: (1) creating a binding site for DDK and/or (2) creating a target peptide for DDK phosphorylation. In support of the latter possibility, several studies suggest that DDK targets serines or threonines adjacent to an acidic amino acid (glutamate or aspartate) or adjacent to a phosphoserine or phosphothreonine (Cho et al., 2006; Montagnoli et al., 2006). However, our data strongly support a role for phosphorylation in recruiting DDK to the origin/MCM complex. Although most of our results can be explained by phosphorylation creating a binding site for DDK, we cannot eliminate for the possibility that the priming phosphorylation event creates target peptides for DDK, and that phosphorylation of Mcm2-7 by DDK leads in turn to stable DDK binding to the pre-RC. The kinase responsible for the priming phosphorylation of Mcm2-7 proteins in the pre-RC remains unknown. Although our studies suggest that prior phosphorylation and not conformational differences control DDK targeting of chromatin associated MCM

proteins, this observation suggests that the kinase responsible for the priming phosphorylation will distinguish between origin-linked versus free MCM complexes.

The use of priming phosphorylation to direct DDK to certain Mcm2-7 complexes could also play a regulatory role. If the extent or timing of such phosphorylation was not uniform from origin to origin, priming phosphorylation of the MCM complex could also provide a mechanism to control origin efficiency or origin timing. As sites targeted by this priming phosphorylation are identified it will be intriguing to determine how the level of modification varies across the genome.

Experimental Procedures

Yeast strains and plasmids

yLF52 and yLF53 were W303 transformed with plasmids pLF8 (Wt Cdc7) and pLF9 ($Cdc7^{D163A}$) respectively. pLF8 and pLF9 were made using the pESC split-Tap-URA plasmid. Cdc7 and Dbf4 were integrated into the plasmid using FseI/AsiSI and NotI/AscI respectively.

Protein Purification

Asynchronous yeast cells were grown in 1% raffinose and expression of Dbf4-CBP/Cdc7-proA was induced with addition of 2% galactose for 3 hours. Extracts were made by grinding frozen cell pellets in a motorized mortar/pestle followed by centrifugation at 21,000rpm for one hour. Purification was performed as described (Puig et al., 2001) with some modifications: KCl was used rather than NaCl as the salt in the buffer solutions. The calmodulin column was used first, the IgG column was not used. The eluate from the calmodulin column was bound to a heparin column at a low salt concentration (calmodulin elution buffer contains 150mM KCl). The proA tag was cleaved from Cdc7 (while bound to the heparin column) by incubation with TEV cleavage buffer and 20units of TEV (Puig et al, 1999). The protein was eluted from the heparin column with high salt (H/600mM KCl). Mcm6-TAP was purified as previously described (Puig et al. 1999).

Preparation of whole cell extracts

The WCEs used in the pre-RC assembly assays were made as previously described (Bowers et al, 2004).

Pre-RC assembly assay

Pre-RC assembly assays were performed as previously described (Randell et al, 2006).

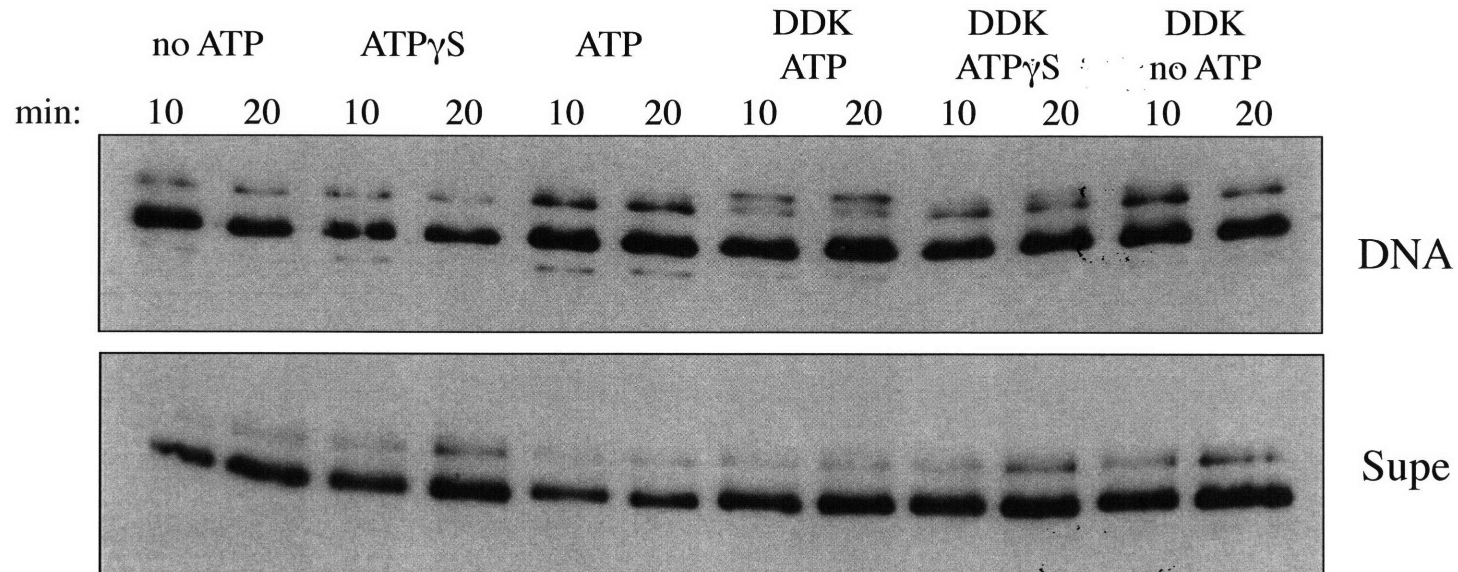
Kinase assays

Pre-RCs assembled from a 40ul pre-RC assembly reaction were incubated in a 30ul kinase reaction with 125ng of either wild-type or kinase dead DDK, 0.1 mM ATP, 3 uCi [γ-^{32}P]ATP and kinase buffer (50mM HEPES [pH7.6], 5mM Mg-acetate, 1mM EDTA, 1mM EGTA, 10% glycerol and 0.01% NP-40) containing 150mM KGlut, for 15 min at 25°C. After the reaction was over, the reaction was either (1) stopped by the addition of

12ul of 5X SDS-PAGE sample buffer or (2) the beads and supernatant were separated and 30ul 1X SDS-PAGE sample buffer was added to the beads and 12ul of 5X SDS sample buffer was added to the supernatant. Kinase assays with purified Mcm6 were similarly performed, except that 400ng of purified Mcm6-CBP was used in the reaction.

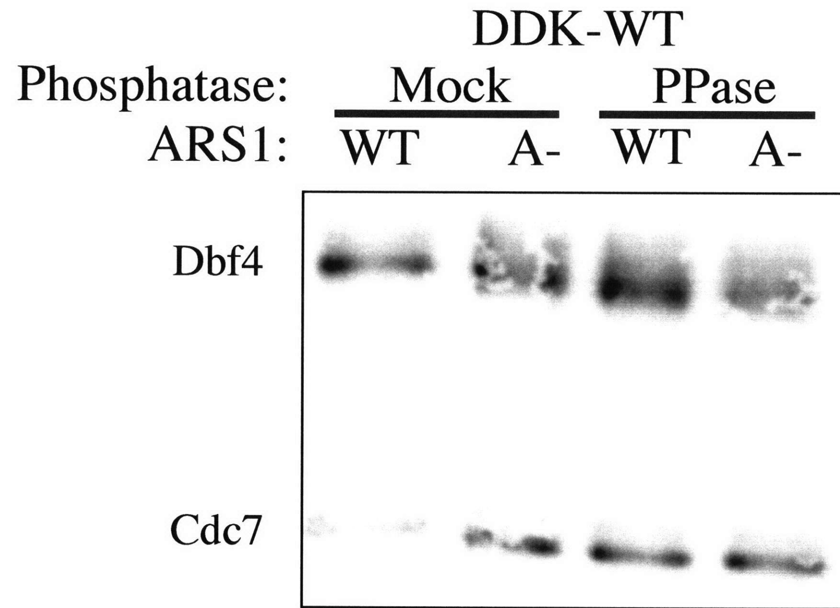
Supplementary Figures

Supplementary Figure 1.



Supplementary Figure 1: The presence of ATP, ATPγS, or DDK does not significantly affect the rate of dissociation of the MCM complex from the DNA. Pre-RC assembly assays were performed with WT ARS1 containing DNAs. DNA associated proteins were then treated with H buffer with 150mMKCl and with or without 1mM ATP, 1mM ATPγS, and 200 fmol of wild-type DDK for 15 minutes at 25°C. Samples were analyzed by SDS/PAGE, blotted and probed polyclonal anti-Mcm2-7 antibody.

Supplementary Figure 2.



Supplementary Figure 2: Prior phosphatase treatment of pre-RC does not affect DDK autophosphorylation. Autoradiography results of experiment performed in Fig5A. Data shown is DDK that did not bind to the pre-RC during the kinase reaction.

References

Arias, E.E., and Walter, J.C. (2007). Strength in numbers: preventing rereplication via multiple mechanisms in eukaryotic cells. Genes & development *21*, 497-518.

Bousset, K., and Diffley, J.F. (1998). The Cdc7 protein kinase is required for origin firing during S phase. Genes & development *12*, 480-490.

Bowers, J.L., Randell, J.C., Chen, S., and Bell, S.P. (2004). ATP hydrolysis by ORC catalyzes reiterative Mcm2-7 assembly at a defined origin of replication. Molecular cell *16*, 967-978.

Cho, W.H., Lee, Y.J., Kong, S.I., Hurwitz, J., and Lee, J.K. (2006). CDC7 kinase phosphorylates serine residues adjacent to acidic amino acids in the minichromosome maintenance 2 protein. Proc Natl Acad Sci U S A *103*, 11521-11526.

Donaldson, A.D., Fangman, W.L., and Brewer, B.J. (1998). Cdc7 is required throughout the yeast S phase to activate replication origins. Genes & development *12*, 491-501.

Dowell, S.J., Romanowski, P., and Diffley, J.F. (1994). Interaction of Dbf4, the Cdc7 protein kinase regulatory subunit, with yeast replication origins in vivo. Science (New York, N.Y *265*, 1243-1246.

Duncker, B.P., Shimada, K., Tsai-Pflugfelder, M., Pasero, P., and Gasser, S.M. (2002). An N-terminal domain of Dbf4p mediates interaction with both origin recognition complex (ORC) and Rad53p and can deregulate late origin firing. Proc Natl Acad Sci U S A *99*, 16087-16092.

Gambus, A., Jones, R.C., Sanchez-Diaz, A., Kanemaki, M., van Deursen, F., Edmondson, R.D., and Labib, K. (2006). GINS maintains association of Cdc45 with MCM in replisome progression complexes at eukaryotic DNA replication forks. Nature cell biology *8*, 358-366.

Hardy, C.F., Dryga, O., Seematter, S., Pahl, P.M., and Sclafani, R.A. (1997). mcm5/cdc46-bob1 bypasses the requirement for the S phase activator Cdc7p. Proc Natl Acad Sci U S A *94*, 3151-3155.

Jares, P., and Blow, J.J. (2000). Xenopus cdc7 function is dependent on licensing but not on XORC, XCdc6, or CDK activity and is required for XCdc45 loading. Genes & development *14*, 1528-1540.

Kihara, M., Nakai, W., Asano, S., Suzuki, A., Kitada, K., Kawasaki, Y., Johnston, L.H., and Sugino, A. (2000). Characterization of the yeast Cdc7p/Dbf4p complex purified from insect cells. Its protein kinase activity is regulated by Rad53p. J Biol Chem *275*, 35051-35062.

Labib, K., and Gambus, A. (2007). A key role for the GINS complex at DNA replication forks. Trends Cell Biol *17*, 271-278.

Manke, I.A., Lowery, D.M., Nguyen, A., and Yaffe, M.B. (2003). BRCT repeats as phosphopeptide-binding modules involved in protein targeting. Science (New York, N.Y *302*, 636-639.

Masai, H., and Arai, K. (2002). Cdc7 kinase complex: a key regulator in the initiation of DNA replication. J Cell Physiol *190*, 287-296.

Masai, H., Taniyama, C., Ogino, K., Matsui, E., Kakusho, N., Matsumoto, S., Kim, J.M., Ishii, A., Tanaka, T., Kobayashi, T., *et al.* (2006). Phosphorylation of MCM4 by Cdc7 kinase facilitates its interaction with Cdc45 on the chromatin. J Biol Chem *281*, 39249-39261.

Masumoto, H., Sugino, A., and Araki, H. (2000). Dpb11 controls the association between DNA polymerases alpha and epsilon and the autonomously replicating sequence region of budding yeast. Molecular and cellular biology *20*, 2809-2817.

Mendez, J., and Stillman, B. (2003). Perpetuating the double helix: molecular machines at eukaryotic DNA replication origins. Bioessays *25*, 1158-1167.

Montagnoli, A., Valsasina, B., Brotherton, D., Troiani, S., Rainoldi, S., Tenca, P., Molinari, A., and Santocanale, C. (2006). Identification of Mcm2 phosphorylation sites by S-phase-regulating kinases. J Biol Chem *281*, 10281-10290.

Moyer, S.E., Lewis, P.W., and Botchan, M.R. (2006). Isolation of the Cdc45/Mcm2-7/GINS (CMG) complex, a candidate for the eukaryotic DNA replication fork helicase. Proc Natl Acad Sci U S A *103*, 10236-10241.

Nougarede, R., Della Seta, F., Zarzov, P., and Schwob, E. (2000). Hierarchy of S-phase-promoting factors: yeast Dbf4-Cdc7 kinase requires prior S-phase cyclin-dependent kinase activation. Molecular and cellular biology *20*, 3795-3806.

Ohtoshi, A., Miyake, T., Arai, K., and Masai, H. (1997). Analyses of Saccharomyces cerevisiae Cdc7 kinase point mutants: dominant-negative inhibition of DNA replication on overexpression of kinase-negative Cdc7 proteins. Mol Gen Genet *254*, 562-570.

Pacek, M., Tutter, A.V., Kubota, Y., Takisawa, H., and Walter, J.C. (2006). Localization of MCM2-7, Cdc45, and GINS to the site of DNA unwinding during eukaryotic DNA replication. Molecular cell *21*, 581-587.

Pacek, M., and Walter, J.C. (2004). A requirement for MCM7 and Cdc45 in chromosome unwinding during eukaryotic DNA replication. EMBO J *23*, 3667-3676.

Pereverzeva, I., Whitmire, E., Khan, B., and Coue, M. (2000). Distinct phosphoisoforms of the Xenopus Mcm4 protein regulate the function of the Mcm complex. Molecular and cellular biology *20*, 3667-3676.

Puig, O., Caspary, F., Rigaut, G., Rutz, B., Bouveret, E., Bragado-Nilsson, E., Wilm, M., and Seraphin, B. (2001). The tandem affinity purification (TAP) method: a general procedure of protein complex purification. Methods *24*, 218-229.

Sheu, Y.J., and Stillman, B. (2006). Cdc7-Dbf4 phosphorylates MCM proteins via a docking site-mediated mechanism to promote S phase progression. Molecular cell *24*, 101-113.

Shimada, K., Pasero, P., and Gasser, S.M. (2002). ORC and the intra-S-phase checkpoint: a threshold regulates Rad53p activation in S phase. Genes & development *16*, 3236-3252.

Tanaka, S., Umemori, T., Hirai, K., Muramatsu, S., Kamimura, Y., and Araki, H. (2007). CDK-dependent phosphorylation of Sld2 and Sld3 initiates DNA replication in budding yeast. Nature *445*, 328-332.

Tsuji, T., Ficarro, S.B., and Jiang, W. (2006). Essential role of phosphorylation of MCM2 by Cdc7/Dbf4 in the initiation of DNA replication in mammalian cells. Mol Biol Cell *17*, 4459-4472.

Varrin, A.E., Prasad, A.A., Scholz, R.P., Ramer, M.D., and Duncker, B.P. (2005). A mutation in Dbf4 motif M impairs interactions with DNA replication factors and confers increased resistance to genotoxic agents. Molecular and cellular biology *25*, 7494-7504.

Walter, J.C. (2000). Evidence for sequential action of cdc7 and cdk2 protein kinases during initiation of DNA replication in Xenopus egg extracts. J Biol Chem *275*, 39773-39778.

Weinreich, M., and Stillman, B. (1999). Cdc7p-Dbf4p kinase binds to chromatin during S phase and is regulated by both the APC and the RAD53 checkpoint pathway. EMBO J *18*, 5334-5346.

Yoon, H.J., Loo, S., and Campbell, J.L. (1993). Regulation of Saccharomyces cerevisiae CDC7 function during the cell cycle. Mol Biol Cell *4*, 195-208.

Zegerman, P., and Diffley, J.F. (2007). Phosphorylation of Sld2 and Sld3 by cyclin-dependent kinases promotes DNA replication in budding yeast. Nature *445*, 281-285.

Zou, L., and Stillman, B. (2000). Assembly of a complex containing Cdc45p, replication protein A, and Mcm2p at replication origins controlled by S-phase cyclin-dependent kinases and Cdc7p-Dbf4p kinase. Molecular and cellular biology *20*, 3086-3096.

Chapter III

Phosphorylation Site Mapping of Pre-RC-Linked MCM Complexes

This work was done in collaboration with John C.W. Randell. J.C.W.R. assembled the pre-RCs used in the study. L.I.F. performed the DDK assay. Both J.C.W.R. and L.I.F. analyzed the results. Mass Spectrometry analysis was performed by the Taplin Facility at Harvard Medical School.

Summary

Initiation of DNA replication is controlled by two S-phase kinases, the Cyclin-dependent kinase, Clb5/6-Cdc28 (CDK) and Dbf4-dependent kinase, Dbf4-Cdc7 (DDK). DDK phosphorylation of the Mcm2-7 replicative helicase is a critical trigger that leads to the activation of this helicase. The mechanism by which DDK phosphorylation of the MCM complex leads to helicase activation is not known, nor the sites of MCM subunit phosphorylation. To address these and other issues, we have mapped the sites in the MCM subunits that are phosphorylated during *in vitro* pre-RC assembly and after these complexes are treated with DDK. We observed both DDK-dependent and DDK-independent phosphorylation sites on all MCM subunits except Mcm5. Most sites found in Mcm2, Mcm4 and Mcm6 were in their S/T-rich N-termini. These data not only provide information about overall phosphorylation patterns of G1-phase pre-RC-linked MCM complexes, but also provide critical information that will be useful for phospho-mutant analysis and creation of phospho-specific antibodies to address how these modifications function *in vivo*.

Introduction

Initiation of DNA replication is tightly controlled by protein phosphorylation. The key events of eukaryotic DNA replication are temporally segregated in the cell cycle. Origin selection is restricted to the late M- and early G1-phases of the cell cycle and origin activation only occurs after cells enter S-phase. Phosphorylation by two kinases, Cyclin-Dependent Kinase (CDK) and Dbf4-Dependent Kinase, Dbf4-Cdc7 (DDK), is critical to maintain these temporal distinctions. Outside of G1-phase, CDK phosphorylation of the DNA replication factors that participate in origin selection inhibits their function, ensuring that cells cannot re-initiate DNA replication until after the following G1-phase of the cell cycle. During S-phase, phosphorylation of a different subset of DNA replication factors by CDK and DDK is essential to trigger the events of replication initiation.

Initiation of eukaryotic DNA replication begins with the selection of potential origins during late M- and early G1-phase (Bell and Dutta, 2002). This process is called pre-Replicative Complex (pre-RC) formation and results in loading of the replicative DNA helicase onto origin DNA. The Origin Recognition Complex (ORC) is constitutively bound to origin-DNA throughout the cell cycle in *S. cerevisiae*. As cells exit M-phase, ORC recruits two proteins required for helicase loading, Cdc6 and Cdt1, to the DNA. ORC, Cdc6 and Cdt1 together act as a helicase-loader, recruiting then assembling the Mcm2-7 replicative helicase onto origin DNA, thus, completing pre-RC formation (Mendez and Stillman, 2003). Pre-RC formation marks all potential origins of DNA replication.

The two S-phase kinases, CDK and DDK, are both activated as cells enter S-phase and are required to trigger the initiation of replication from sites of pre-RC formation. These kinases modify a variety of other replication factors, including the MCM complex, Sld2 and Sld3 (Masai and Arai, 2002; Tanaka et al., 2007; Zegerman and Diffley, 2007). Although the mechanistic consequences of these modifications are not understood in all cases, in many instances phosphorylation leads to recruitment of the modified replication factors and other downstream factors, such as Cdc45 and Dpb11, to sites of pre-RC formation. These events, and other consequences of CDK and DDK activity, result in the

activation of the MCM helicase, DNA unwinding, recruitment of DNA synthesis machinery and formation of a pair of bi-direction replication forks.

A detailed mechanistic understanding of events downstream of pre-RC formation is lacking; however, at least some of the targets of CDK and DDK are known (Labib and Gambus, 2007). DDK phosphorylates the MCM complex, which is thought to be important in recruitment of MCM helicase-activating protein Cdc45 to the origin. CDK phosphorylates Sld2 and Sld3 leading to binding of both proteins to Dbp11. CDK may phosphorylate other proteins involved in initiation; however, recent evidence indicates that Sld2 and Sld3 are the only CDK substrates that *must* be phosphorylated prior to the initiation of replication (Tak et al., 2006; Tanaka et al., 2007; Zegerman and Diffley, 2007). Moreover, binding of Sld3 and Cdc45 at the origin is mutually dependent, suggesting that CDK and DDK must both function to recruit the full complement of downstream initiation factors.

Although both *in vivo* and *in vitro* studies indicate that DDK phosphorylates multiple MCM subunits, there is conflicting data as to which MCM subunits are phosphorylated. *In vitro* studies with individual subunits have shown that all MCM subunits except Mcm5 are DDK targets (Weinreich and Stillman, 1999). In contrast, *in vivo* studies have identified Mcm2, Mcm4 and possibly Mcm6 as the primary DDK substrates (Masai and Arai, 2002; Montagnoli et al., 2006; Sheu and Stillman, 2006; Tsuji et al., 2006).

Previous mass spectrometry studies, performed with human DDK and MCM complex, have shown that DDK targets Mcm2 and Mcm4 in their N-termini and also targets a particular consensus sequence (Cho et al., 2006; Montagnoli et al., 2006). DDK preferentially phosphorylates serines and threonines adjacent to an acidic residue (S/T-D/E) or a phosphorylated serine/threonine (S/T-S/T where second S/T site is phosphorylated). Not only does DDK specifically target the Mcm2 and Mcm4 N-termini, but other kinases do as well, though the consequences of these phosphorylation events are not well understood. Interestingly although the precise sequences of the N-termini of the MCM subunits are not well conserved, the N-termini of Mcm2 and Mcm4 from many different organisms consistently include an unusually high incidence of serines and threonines, often clustered together. Moreover, *in vivo* studies with budding yeast have

shown that the deletion of the Mcm4 N-terminus is lethal, but growth can be restored fusing the Mcm2 N-terminus to the Mcm4 N-terminal mutant (Sheu and Stillman, 2006). Similar observations have been made in *S. pombe* (Masai et al., 2006). These findings have led to the hypothesis that multiple phosphorylation events on each N-terminus rather than at specific sites on the N-terminus are critical for Mcm2-7 activation.

Global mass spectrometry studies in *S. cerevisiae* have been performed, both in asynchronous cells and in G1-phase arrested cells. In the study with asynchronous cells, six phosphorylated residues were identified on the MCM complex: four in the C-terminus of Mcm3 and two in the C-terminus of Mcm7 (Ficarro et al., 2002). In the study with G1-arrested cells, the same two Mcm7 phosphorylated residues were identified (Li et al., 2007). In Mcm3, one phosphorylated residue was found in the C-terminus that did not overlap with the asynchronous study and one phosphorylated residue was found in Mcm6. The biological significance of these phosphorylation events was not investigated, although as described in the next paragraph, another group has shown that the Mcm3 C-terminal CDK phosphorylation sites play a role in MCM complex nuclear localization.

Other labs have shown that other kinases besides DDK phosphorylate the MCM complex (Liku et al., 2005; Montagnoli et al., 2006). For example, minimal CDK consensus sites can be found in all of the MCM subunits, and CDK was shown to target the MCM complex (Liku et al., 2005). This phosphorylation is thought to be important for exporting MCM complexes not engaged in DNA replication out of the nucleus in S-phase, thus preventing new pre-RC formation outside of G1-phase. There are seven minimal CDK consensus sequences in the C-terminus of Mcm3 in a region of Mcm3 that contains a weak nuclear localization signal (NLS) and a weak nuclear export signal (NES). These data suggest that CDK phosphorylation of Mcm3 is important in MCM nuclear localization, although CDK phosphorylation of other subunits may regulate MCM complex nuclear localization as well. CDK phosphorylation of the MCM complex may also play a role in initiation of replication. If this is the case, however, this function is not essential, as Sld2 and Sld3 have been shown to be the only CDK targets required for initiation of DNA replication.

In Chapter II of this thesis, I describe results that suggest that prior phosphorylation of G1-phase pre-RC-linked MCM complexes is required for both DDK binding and

modification of the MCM complex. Because neither CDK nor DDK are active in G1, this suggests that a different kinase is involved in priming the MCM complex for binding to and phosphorylation by DDK. The identity of the kinase or kinases responsible for this priming event remains unclear and the location of the sites responsible remains unknown.

To begin more mechanistic studies of the consequences of MCM complex phosphorylation, we undertook mass spectrometry analysis of MCM complexes assembled into pre-RCs from G1-phase yeast extracts, both before and after treating with DDK. We have identified both DDK-dependent and DDK-independent phosphorylation sites on all of the MCM subunits except for Mcm5, for which no phosphorylation sites were found. Most of the identified phosphorylated residues were part of known kinase phosphorylation consensus sequences, which may be helpful in understanding which kinases target the MCM complex. In agreement with previously published literature, most phosphorylated sites (both DDK-dependent and DDK–independent) found in Mcm2, Mcm4 and Mcm6, were clustered in the N-terminus. Furthermore, our experiments detected seven of the ten phosphorylated residues identified in the two *S. cerevisiae* global mass spectrometry studies.

Results

Pre-RC reaction used in mass spectrometry analysis

To identify phosphorylated residues on pre-RC-linked MCM complexes, we performed *in vitro* pre-RC assembly reactions in which origin DNA linked to magnetic resin was incubated with G1-arrested yeast extracts. After assembling pre-RCs, they were either left untreated, treated with wild-type DDK and ATP, or treated with mutant "kinase dead" DDK and ATP. The proteins associated with the DNA were separated on a protein gel and were visualized by Coomassie staining (Fig. 1). To determine which bands were Mcm2-7 proteins, we compared the samples containing full pre-RCs to a sample without Cdc6 (no Mcm2-7 association with DNA) (Fig. 1, compare lane 1 to lanes 2 and 3). Because phosphorylated residues are easier to identify in more pure samples, the region of the gel containing the Mcm2-7 proteins was excised, divided into six regions (approximately containing one subunit per region), and submitted for mass spectrometry analysis.

Each gel region submitted for analysis contained many identified proteins and often more than one MCM subunit. Therefore, for each gel region submitted (six per sample) the two MCM subunits with the highest numbers of identified peptides (in each case coverage was greater than 70%) were analyzed for phosphorylated residues. Table 1 provides an overview of the results for each individual MCM subunit, including the number of DDK sites identified, the phosphorylation sequence of the identified DDK sites, the types of DDK-independent sites identified and the number of each type found.

Compilation of results of phosphorylation mapping of pre-RC-linked MCM complex

At least one DDK-dependent phosphorylation site was identified for each MCM subunit except for Mcm5 (Table 1). In accordance with previously published studies, many of these sites had an acidic amino acid adjacent to the phosphorylated serine or threonine residue. Interestingly, although Mcm2, 4, and 6 (or some combination therein) are thought to be the targets of DDK *in vivo* (Masai and Arai, 2002; Montagnoli et al., 2006; Sheu and Stillman, 2006; Tsuji et al., 2006), we observed DDK-phosphorylated residues on Mcm3 and Mcm7 as well. It is possible that these identified sites are false positives, particularly since two of the three residues identified were non-S/T-acidic sites.

Figure 1.

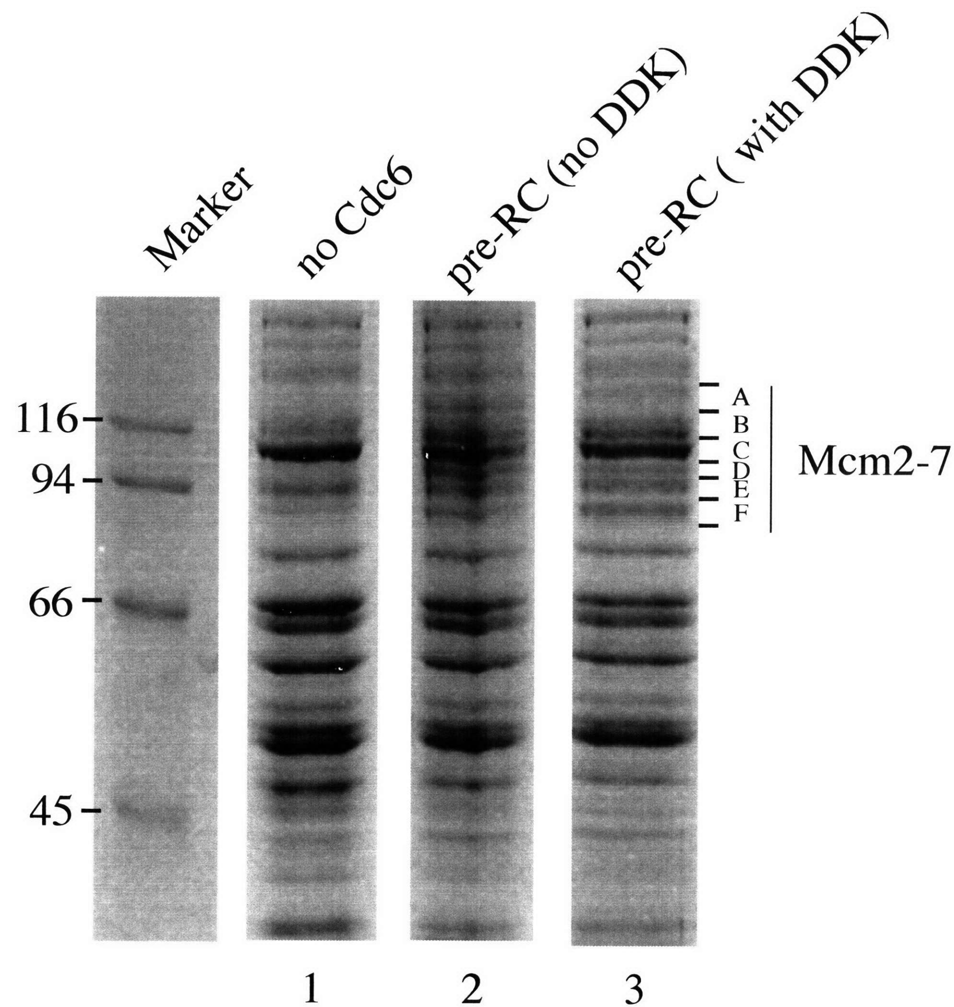


Figure 1. Pre-RC reactions used in mass spectrometry analysis. Pre-RC assembly reactions were performed and either visualized by Coomassie Blue staining(no DDK) or treated with DDK and visualized by Coomassie Blue staining (DDK). The "No Cdc6" sample was used solely as a control to determine the protein bands corresponding to the Mcm2-7 subunits. The region containing the Mcm2-7 proteins was cut into six sub-regions and the gel containing these regions (A-F) were excised and submitted for mass spectometry analysis.

On the other hand, it is possible that these are true phosphorylation events that are not easily detected *in vitro* or with individual MCM subunits as a substrate. Further analysis will be required to determine whether these are bone fide DDK phosphorylation sites.

Numerous phosphorylated residues that were independent of DDK were identified. As with the DDK-dependent sites, DDK-independent phosphorylation sites were identified for all MCM subunits except Mcm5. Interestingly, these phosphorylated residues fell into several groups according to their adjacent residues (see Table 1 and Fig. 2). For example, S/T-P sites were identified in all of the phosphorylated MCM subunits. S/T followed by a proline is the minimal phosphorylation motif for CDK. We also identified a number of S/T-Q sites, the phosphorylation motif for the ATM/ATR family of kinases. Four such sites were identified in Mcm4 and Mcm6 and one site in Mcm7. S/T-acidic-X-acidic sites, the casein kinase II phosphorylation motif, were identified in Mcm2 (4), Mcm3 (1) and Mcm7 (1). Finally, most of the remaining phosphorylated residues had aliphatic animo acids following the phosphorylated serine or threonine. S/T-aliphatic phosphorylation motifs are used by several kinases such as PKA (Ubersax and Ferrell, 2007) and Slk1 (Songyang et al., 1994). The functional relevance of these phosphorylation sites is currently unknown, but because they are found prior to DDK phosphorylation, one or any combination of these modifications could be responsible for the priming phosphorylation required for DDK binding and phosphorylation described in Chapter II.

The majority of the detected Mcm2, 4, and 6 phosphorylation sites were clustered in the N-terminal region (Fig. 2 A, C and D; Fig. 3). 89% (24/27) of the phosphorylated residues in Mcm2, 4 and 6 were in the N-termini prior to the core conserved OB-fold-like domain. The distribution of minimal kinase consensus sequences of the phosphorylated residues was different between the subunits. For example, Mcm2 and Mcm6 had one DDK consensus site phosphorylated (S/T-D/E) and Mcm4 had three such sites. All three subunits had one CDK consensus site phosphorylated and either one or two phosphorylated S/T-aliphatic site. Mcm2 had four phosphorylated putative CK II sites, but Mcm4 and 6 had none. On the other hand, Mcm4 and Mcm6 had four phosphorylated putative ATM/ATR kinase sites and Mcm2 did not have any.

The distribution of phosphorylation sites is markedly different in Mcm3 and Mcm7 as compared to Mcm2, 4 and 6 (Fig 2 and Fig. 3). We did not identify any phosphorylated residues in the Mcm3 and Mcm7 unconserved N-terminal regions. In Mcm3, eight of the nine phosphorylated residues detected are in the last 200 amino acids of the C-terminus of the protein. Of the seven sites that are DDK-independent, four are putative CDK sites, one is a putative CK II site and the other two are S/T-aliphatic sites. The single phosphorylation site not found in the C-terminus of Mcm3 is one of the two DDK-dependent sites identified. This phosphorylation site is found in the OB-fold-like domain of Mcm3. Mcm7 has six phosphorylation sites interspersed throughout its sequence, though three of the six are in the last 35 C-terminal amino acids. The other three sites are all within the OB-fold-like domain, including the single DDK-dependent phosphorylation site. Of the five DDK-independent phosphorylated residues, two are putative CDK sites, one is a putative CK II site, one is a putative ATM/ATR sites and one is a S-aliphatic site.

Table 1.

MCM subunit	DDK sites [a]	Sequence of DDK site phosphorylated[b]	Other sites [c]	Other putative kinase sites identified[d]
Mcm2	1	SE	6	CDK: 1 CK II: 4 S/T-aliphatic: 1
Mcm3	2	TD, TA	7	CDK: 4 CK II: 1 S/T-aliphatic: 1 Other: 1
Mcm4	3	SD, TSD (both T and S phosphorylated	10	CDK: 1 ATM/ATR: 4 S/T-aliphatic: 2 S/T-acidic: 3
Mcm5	none		none	
Mcm6	1	TD	7	CDK: 1 ATM/ATR: 4 S/T-aliphatic: 2
Mcm7	1	SM	5	CDK: 2 CK II: 1 S/T-aliphatic: 1 ATM/ATR: 1

[a] "DDK sites" are those sites only found in the samples treated with wild-type DDK

[b] For DDK sites, we show the residue phosphorylated and the adjacent amino acid (the +1 position).

[c] "Other sites" were sites found in any sample analysis not solely identified in the wild-type DDK samples

[d] For other sites phosphorylated, we categorize the sites according to known minimal consensus sequences for other kinases (see text) and the number of these sites in each subunit are listed.

Figure 2A.

Mcm2

```
  1 MSDNRRRRRE EDDSDSENEL PPSSPQQHFR GGMNPVSSPI GSPDMINPEG
 51 DDNEVDDVPD IDEVEEQMNE VDLMDDNMYE DYAADHNRDR YDPDQVDDRE
101 QQELSLSERR RIDAQLNERD RLLRNVAYID DEDEEQEGAA QLDEMGLPVQ
151 RRRRRRQYED LENSDDDLLS DMDIDPLREE LTLESLSNVK ANSYSEWITQ
201 PNVSRTIARE LKSFLLEYTD ETGRSVYGAR IRTLGEMNSE SLEVNYRHLA
251 ESKAILALFL AKCPEEMLKI FDLVAMEATE LHYPDYARIH SEIHVRISDF
301 PTIYSLRELR ESNLSSLVRV TGVVTRRTGV FPQLKYVKFN CLKCGSILGP
351 FFQDSNEEIR ISFCTNCKSK GPFRVNGEKT VYRNYQRVTL QEAPGTVPPG
401 RLPRHREVIL LADLVDVSKP GEEVEVTGIY KNNYDGNLNA KNGFPVFATI
451 IEANSIKRRE GNTANEGEEG LDVFSWTEEE EREFRKISRD RGIIDKIISS
501 MAPSIYGHRD IKTAVACSLF GGVPKNVNGK HSIRGDINVL LLGDPGTAKS
551 QILKYVEKTA HRAVFATGQG ASAVGLTASV RKDPITKEWT LEGGALVLAD
601 KGVCLIDEFD KMNDQDRTSI HEAMEQQSIS ISKAGIVTTL QARCSIIAAA
651 NPNGGRYNST LPLAQNVSLT EPILSRFDIL CVVRDLVDEE ADERLATFVV
701 DSHVRSHPEN DEDREGEELK NNGESAIEQG EDEINEQLNA RQRRLQRQRK
751 KEEEISPIPQ ELLMKYIHYA RTKIYPKLHQ MDMDKVSRVY ADLRRESIST
801 GSFPITVRHL ESILRIAESF AKMRLSEFVS SYDLDRAIKV VVDSFVDAQK
851 VSVRRQLRRS FAIYTLG
```

Figure 2. Compiled results of phosphorylation site mapping of pre-RC linked MCM complexes, part 1. (A) Sites of phosphorylation on Mcm2 (B) Sites of phosphorylation on Mcm3. The putative NLS (residues 766-772) and NES (residues 834-842) are highlighted by italics (C) Sites of phosphorylation on Mcm4 (D) Sites of phosphorylation on Mcm6 (E) Sites of phosphorylation on Mcm7; All sites of phosphorylation are highlighted by 18 pt font and bold lettering. Green = DDK-dependent; Red = DDK-independent

Figure 2B.

Mcm3

1 MEGSTGFDGD ATTFFAPDAV FGDRVRRFQE FLDTFTSYRD SVRSIQVYNS

51 NNAANYNDDQ DDADERDLLG DDDGDDLEKE KKAASSTSLN ILPHRIIISL

101 DDLREFDRSF WSGILVEPAY FIPPAEKALT DLADSMDDVP HPNASAVSSR

151 HPWKLSFKGS FGAHALSPRT LTAQHLNKLV SVEGIVTKTS LVRPKLIRSV

201 HYAAKTGRFH YRDY**T**DATTT LTTRIPTPAI YPTEDTEGNK LTTEYGYSTF

251 IDHQRITVQE MPEMAPAGQL PRSIDVILDD DLVDKTKPGD RVNVVGVFKS

301 LGAGGMNQSN SNTLIGFKTL ILGNTVYPLH ARSTGVAARQ MLTDFDIRNI

351 NKLSKKKDIF DILSQSLAPS IYGHDHIKKA ILLMLMGGVE KNLENGSHLR

401 GDINILMVGD PSTAKSQLLR FVLNTASLAI ATTGRGSSGV GLTAAVTTDR

451 ETGERRLEAG AMVLADRGVV CIDEFDKMTD VDRVAIHEVM EQQTVTIAKA

501 GIHTTLNARC SVIAAANPVF GQYDVNRDPH QNIALPDSLL SRFDLLFVVT

551 DDINEIRDRS ISEHVLRTHR YLPPGYLEGE PVRERLNLSL AVGEDADINP

601 EEHSNSGAGV ENEGEDDEDH VFEKFNPLLQ AGAKLAKNKG NYNGTEIPKL

651 VTIPFLRKYV QYAKERVIPQ LTQEAINVIV KNYTDLRNDD NTKKSPITAR

701 TLETLIRLAT AHAKVRLSKT VNKVDAKVAA NLLRFALLGE DIGNDIDEEE

751 **S**EYEEALSKR SPQK**S***PKKRQ* *RV*RQPA**S**NSG **S**PIKSTPRRS **T**AS**S**VNATPS

801 SARRILRFQD DEQNAGEDDN DIM**S**PLPADE EAE*LQRRLQL* *GL*RV**S**PRRRE

851 HLHAPEEGSS GPLTEVGTPR LPNVSSAGQD DEQQQSVISF DNVEPGTIST

901 GRLSLISGII ARLMQTEIFE EESYPVASLF ERINEELPEE EKFSAQEYLA

951 GLKIMSDRNN LMVADDKVWR

Figure 2C.

Mcm4

1 MSQQSSSPTK EDNNSSSPVV PNPDSVPPQL SSPALFYSSS S**S**QGDIYGRN

51 N**S**QNLSQGEG NIRAAIGS**S**P LNFPSS**S**QRQ N**S**DVFQSQGR QGRIRSSASA

101 SGRSRYH**S**DL R**S**DRALPTSS S**S**LGRNGQNR VHMRRNDIH**T** **S**DLSSPRRIV

151 DFDTRSGVNT LDTSSS**S**APP **S**EASEPLRII WGTNVSIQEC TTNFRNFLMS

201 FKYKFRKILD EREEFINNTT DEELYYIKQL NEMRELGTSN LNLDARNLLA

251 YKQTEDLYHQ LLNYPQEVIS IMDQTIKDCM VSLIVDNNLD YDLDEIETKF

301 YKVRPYNVGS CKGMRELNPN DIDKLINLKG LVLRSTPVIP DMKVAFFKCN

351 VCDHTMAVEI DRGVIQEPAR CERIDCNEPN SMSLIHNRCS FADKQVIKLQ

401 ETPDFVPDGQ TPHSISLCVY DELVDSCRAG DRIEVTGTFR SIPIRANSRQ

451 RVLKSLYKTY VDVVHVKKVS DKRLDVDTST IEQELMQNKV DHNEVEEVRQ

501 ITDQDLAKIR EVAAREDLYS LLARSIAPSI YELEDVKKGI LLQLFGGTNK

551 TFTKGGRYRG DINILLCGDP STSKSQILQY VHKITPRGVY TSGKGSSAVG

601 LTAYITRDVD TKQLVLESGA LVLSDGGVCC IDEFDKMSDS TRSVLHEVME

651 QQTISIAKAG IITTLNARSS ILASANPIGS RYNPNLPVTE NIDLPPPLLS

701 RFDLVYLVLD KVDEKNDREL AKHLTNLYLE DKPEHISQDD VLPVEFLTMY

751 ISYAKEHIHP IITEAAKTEL VRAYVGMRKM GDDSRSDEKR ITATTRQLES

801 MIRLAEAHAK MKLKNVVELE DVQEAVRLIR SAIKDYATDP KTGKIDMNLV

851 QTGKSVIQRK LQEDLSREIM NVLKDQASDS MSFNELIKQI NEHSQDRVES

901 SDIQEALSRL QQEDKVIVLG EGVRRSVRLN NRV

Figure 2D.

Mcm6

```
   1  MSSPFPADTP SSNRPSNSSP PPSSIGAGFG SSSGLDSQIG SRLHFPSSSQ
  51  PHVSNSQTGP FVNDSTQFSS QRLQTDGSAT NDMEGNEPAR SFKSRALNHV
 101  KKVDDVTGEK VREAFEQFLE DFSVQSTDTG EVEKVYRAQI EFMKIYDLNT
 151  IYIDYQHLSM RENGALAMAI SEQYYRFLPF LQKGLRRVVR KYAPELLNTS
 201  DSLKRSEGDE GQADEDEQQD DDMNGSSLPR DSGSSAAPGN GTSAMATRSI
 251  TTSTSPEQTE RVFQISFFNL PTVHRIRDIR SEKIGSLLSI SGTVTRTSEV
 301  RPELYKASFT CDMCRAIVDN VEQSFKYTEP TFCPNPSCEN RAFWTLNVTR
 351  SRFLDWQKVR IQENANEIPT GSMPRTLDVI LRGDSVERAK PGDRCKFTGV
 401  EIVVPDVTQL GLPGVKPSST LDTRGISKTT EGLNSGVTGL RSLGVRDLTY
 451  KISFLACHVI SIGSNIGASS PDANSNNRET ELQMAANLQA NNVYQDNERD
 501  QEVFLNSLSS DEINELKEMV KDEHIYDKLV RSIAPAVFGH EAVKKGILLQ
 551  MLGGVHKSTV EGIKLRGDIN ICVVGDPSTS KSQFLKYVVG FAPRSVYTSG
 601  KASSAAGLTA AVVRDEEGGD YTIEAGALML ADNGICCIDE FDKMDISDQV
 651  AIHEAMEQQT ISIAKAGIHA TLNARTSILA AANPVGGRYN RKLSLRGNLN
 701  MTAPIMSRFD LFFVILDDCN EKIDTELASH IVDLHMKRDE AIEPPFSAEQ
 751  LRRYIKYART FKPILTKEAR SYLVEKYKEL RKDDAQGFSR SSYRITVRQL
 801  ESMIRLSEAI ARANCVDEIT PSFIAEAYDL LRQSIIRVDV DDVEMDEEFD
 851  NIESQSHAAS GNNDDNDDGT GSGVITSEPP ADIEEGQSEA TARPGTSEKK
 901  KTTVTYDKYV SMMNMIVRKI AEVDREGAEE LTAVDIVDWY LLQKENDLGS
 951  LAEYWEERRL AFKVIKRLVK DRILMEIHGT RHNLRDLENE ENENNKTVYV
1001  IHPNCEVLDQ LEPQDSS
```

Figure 2E.

Mcm7

```
  1  MSAALPSIQL PVDYNNLFNE ITDFLVTFKQ DTLSSDATRN ENEDENLDAE
 51  NIEQHLLEKG PKYMAMLQKV ANRELNSVII DLDDILQYQN EKFLQGTQAD
101  DLVSAIQQNA NHFTELFCRA IDNNMPLPTK EIDYKDDVLD VILNQRRLRN
151  ERMLSDRTNE IRSENLMDTT MDPPSSMNDA LREVVEDETE LFPPNLTRRY
201  FLYFKPLSQN CARRYRKKAI SSKPLSVRQI KGDFLGQLIT VRGIITRVSD
251  VKPAVEVIAY TCDQCGYEVF QEVNSRTFTP LSECTSEECS QNQTKGQLFM
301  STRASKFSAF QECKIQELSQ QVPVGHIPRS LNIHVNGTLV RSLSPGDIVD
351  VTGIFLPAPY TGFKALKAGL LTETYLEAQF VRQHKKKFAS FSLTSDVEER
401  VMELITSGDV YNRLAKSIAP EIYGNLDVKK ALLLLLVGGV DKRVGDGMKI
451  RGDINVCLMG DPGVAKSQLL KAICKISPRG VYTTGKGSSG VGLTAAVMKD
501  PVTDEMILEG GALVLADNGI CCIDEFDKMD ESDRTAIHEV MEQQTISISK
551  AVINTNPGAR TSILAAANPL YGRINPRLSP LDNINLPAAL LSRFDILFLM
601  LDIPSRDDDE KLAEHVTYVH MHNKQPDLDF TPVEPSKMRE YIAYAKTKRP
651  VMSEAVNDYV VQAYIRLRQD SKREMDSKFS FGQATPRTLL GIIRLSQALA
701  KLRLADMVDI DDVEEALRLV RVSKESLYQE TNKSKEDESP TTKIFTIIKK
751  MLQETGKNTL SYENIVKTVR LRGFTMLQLS NCIQEYSYLN VWHLINEGNT
801  LKFVDDGTMD TDQEDSLVST PKLAPQTTAS ANVSAQDSDI DLQDA
```

Figure 3.

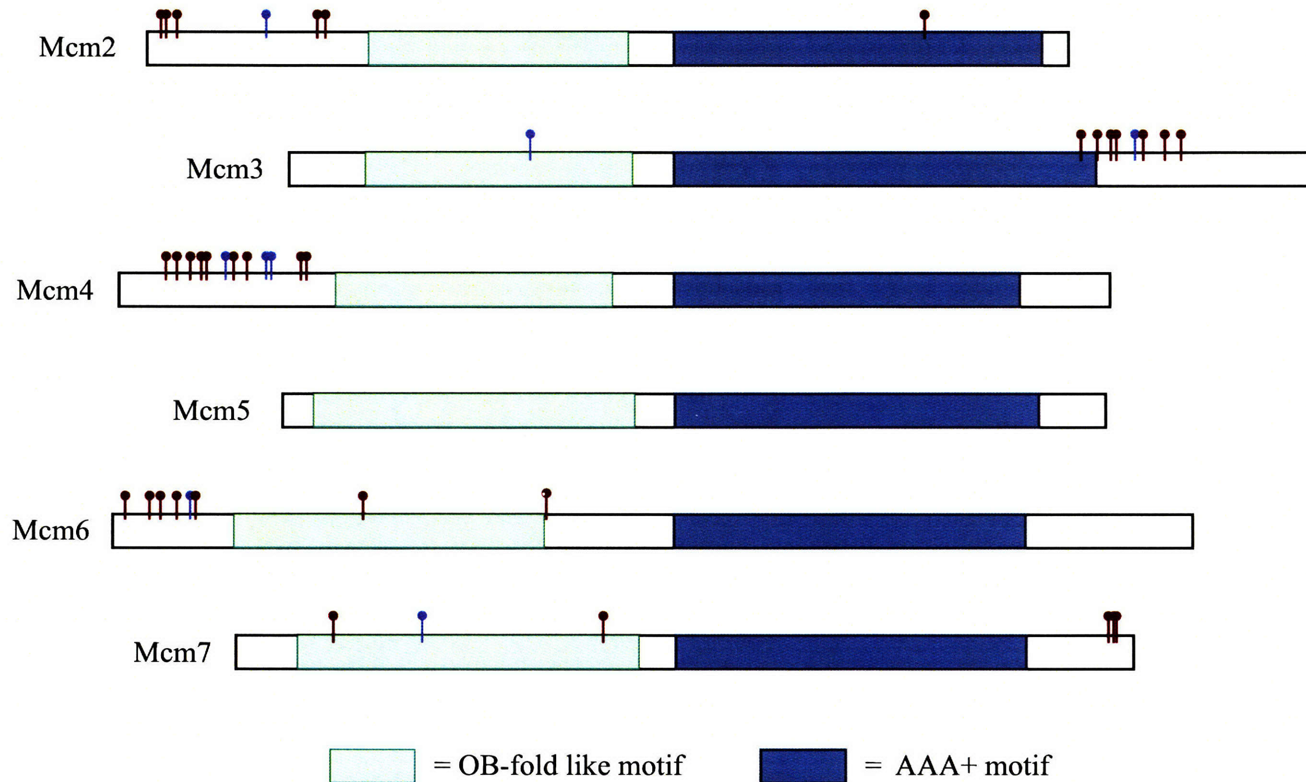


Figure 3. Compiled results of phosphorylation site mapping of pre-RC linked MCM complexes, part 2. Overall view of location of mapped phosphorylated residues on Mcm2-7 proteins. MCM subunits are aligned by the N-terminal end of the conserved AAA+ motif. Each subunit is to scale. Blue = DDK dependent sites; Red = non-DDK dependent sites

Discussion

Identification of the sites of protein phosphorylation is critical to understand how the target protein is regulated by phosphorylation. This is particularly true if there is a high incidence of S/T (or in some cases Y) residues in the proteins of interest. The N-termini of Mcm2, Mcm4 and Mcm6 are very S/T rich and previous data studying DDK phosphorylation of Mcm4 suggests that the specific residues phosphorylated are not as important as the number of phosphates attached to this subunit. Our own data indicates that prior phosphorylation of the MCM complex is important in controlling DDK binding to and phosphorylation of pre-RC-linked MCM complexes (Ch. II). As the first step to understand how phosphorylation of the MCM complex by DDK and other kinases controls its function, we mapped the phosphorylation sites of pre-RC-linked MCM complexes both before and after treatment with DDK.

There are both advantages and disadvantages to mapping phosphorylation sites from purified or extract-based proteins or protein complexes. The advantage is that high concentrations of particular proteins can be used in the analysis, leading to better coverage and more comprehensive results. However, important factors could be missing from the analysis. On the other hand, *in vivo* phosphorylation mapping is useful for ensuring physiological relevance of the results, but it is difficult to get comprehensive data for many proteins. In our DDK phosphorylation analysis, it is possible that we're missing other important factors (such as CDK) therefore, as we go forward with these studies, we will begin to add additional factors to the reaction and observe how this changes the patterns of phosphorylation detected. We can also incorporate immuno-precipitation (IP) of MCM complexes into our analysis. For example, we could IP MCM complexes out of G1-arrested extracts after pre-RC formation and determine whether there are differences in phosphorylation state of the MCM complexes that were not incorporated into the pre-RC.

The majority of phosphorylated residues detected in Mcm2, 4 and 6 in our study were found in the N-termini of these proteins. Interestingly, domain and sequence analysis of Mcm2, 4 and 6 shows an S/T-rich N-terminal region prior to the conserved MCM domains (Ch I, Fig 2). The N-terminal regions of the MCM subunits are not well conserved, but there is a striking conservation of the S/T richness of Mcm2 and Mcm4.

Moreover, Mcm2, 4 and 6 have larger N-terminal regions prior to the OB-fold-like domain as compared to the other MCM subunits. This is especially true in the case of Mcm2 and Mcm4, which have about 200 amino acids in this region. Mcm6 contains a 110 amino acid region prior to the ATPase domain. In contrast, the other three subunits, Mcm3 (70aa), Mcm5 (20aa) and Mcm7 (50aa), have smaller N-terminal region (Ch I, Fig 2 and this chapter, Fig 3.). These data suggest that although the N-terminal regions of the MCM subunits are not conserved, the function associated with phosphorylation of Mcm2, 4 and 6 may be well conserved.

A total of eight DDK-specific phosphorylation sites were identified in this study, distributed across all of the phosphorylated MCM subunits. The DDK sites identified in Mcm2, 4 and 6 were in the N-terminal portions of the proteins, in agreement with previous *in vivo* and *in vitro* studies. These data suggest that although we may not have identified all of the possible DDK sites, the residues we found to be phosphorylated are likely to be physiologically relevant. Data from other labs, however, suggests that the specific sites phosphorylated by DDK are not as important as the presence of a number of phosphorylation sites (i.e. having enough serines and threonines in the sequence) (Masai et al., 2006; Sheu and Stillman, 2006). In Mcm3 and Mcm7, two of the DDK-dependent sites were located in the OB-fold-like regions of these subunits. We do not typically see phosphorylation of these subunits in a kinase assay, however, they may be phosphorylated *in vivo* and the locations of these residues suggest that DDK could play some role in controlling the MCM complex association with DNA. Creating phospho-site mutants in the identified DDK phosphorylation sites should be helpful in understanding whether those particular residues are important or if DDK can target other sites even if the "first choice" phosphorylation sites are mutated.

Our *in vitro* assay for pre-RC formation in combination with phospho-site mutants should be helpful in gaining a better understanding of the consequences of DDK phosphorylation. For example, it is possible that DDK phosphorylation causes a conformational change in the Mcm2-7 complex. This could lead to direct activation of the helicase. Alternatively, because studies in Drosophila suggest that downstream initiation factors such as GINS and Cdc45 are important in activating the helicase (Moyer et al., 2006), a conformational change could facilitate recruitment of these proteins to the

Mcm2-7 complex. It is also possible that DDK phosphorylation of the MCM complex could create a phospho-peptide binding site for either Cdc45 or the GINS, although Cdc45 is more likely as this protein has been shown to bind to the MCM complex directly. We also found two DDK-dependent phosphorylation sites in the OB-fold-like domains of two MCM subunits, Mcm3 and Mcm7. OB-fold-like domains are often involved in binding single-stranded DNA, therefore, it is possible that DDK plays some role in regulating the activity of the MCM complex as well as binding of downstream initiation factors.

In this study, we mapped non-DDK-specific phosphorylation sites to all of the MCM subunits except for Mcm5. We are particularly interested in DDK-independent phosphorylation sites given that we have recently shown that DDK binding and activity depends on other MCM phosphorylation events (Chapter II). The majority of the sites phosphorylated were part of known minimal consensus sequences for a variety of kinases. Many of these kinases are well characterized, however, other than a role for CDK in MCM nuclear export, little is known about the regulatory role that other kinases may play in controlling MCM function. Below, we will discuss these kinases and potential roles that they could have in MCM function.

We identified at least one phosphorylated CDK consensus site in each of the modified MCM subunits. As mentioned above, CDK phosphorylation promotes nuclear export of the MCM complex during S-phase as a means to prevent re-replication (Liku et al., 2005; Nguyen et al., 2000; Nguyen et al., 2001). The C-terminus of Mcm3 contains an NLS, an NES, and seven minimal CDK consensus sites that are important in controlling export from the nucleus. In our mapping studies, of the seven S/T's that these studies suggested are important for nuclear localization, four were identified as phosphorylation sites. The MCM complexes analyzed in our study, however, were isolated from G1-arrested yeast extracts. At the time of this G1-arrest, overall CDK activity is low (including Cln-CDK). Therefore, phosphorylation of Mcm3 at these residues is either constitutive or is mediated by a kinase besides CDK. We don't understand why these residues would be phosphorylated in G1, however, it is possible that all of the CDK consensus sites must be phosphorylated for nuclear export. Thus, S-phase CDK would be important for phosphorylating the other residues, and possibly

CDK sites in other subunits, to bring about export in S-phase. Many S/T-P sites in other regions of other subunits are not phosphorylated (especially in the N-terminus of Mcm4), suggesting that CDK could target the MCM complex for activation at the G1 to S-transition.

We mapped potential Mec1 or Tel 1 sites (ATM/ATR kinase family; S/T-Q) on Mcm4, Mcm6, and Mcm7. Mec and Tel1 are the only budding yeast homologs of the ATM/ATR family of kinases. They are highly related, somewhat redundant kinases best known for their role in response to DNA damage. However, Mec1 is an essential protein in yeast (under non-DNA damage conditions) and studies in *ATM-/-* mice have shown that mouse embryonic fibroblasts (MEFs) from these mice passage slowly through the G1- to S-phase transition after serum stimulation (Xu and Baltimore, 1996). The majority of phosphorylated S/T-Q sites were found in Mcm4 and Mcm6 and these are the subunits that DDK primarily targets in the pre-RC/DDK assay. Therefore, Mec1 and/or Tel1 are viable candidates for the priming kinase required for DDK phosphorylation of the MCM complex. It is worth noting that these studies were done using a linear DNA fragment and it is possible that this phosphorylation is due to adjacent DNA ends being recognized by these DNA damage checkpoint kinases. However, another kinase, Rad53 (Chk1) is activated by Mec1/Tel1/DNA damage function. There is evidence, however, that Rad53 is not activated in the whole cell extracts used for *in vitro* pre-RC formation (J. Randell pers. comm.), suggesting that Mec1 nor Tel1 are not being activated in the DNA damage response pathway.

We also mapped potential CK II sites (S/T-acidic-X-acidic) to Mcm2, Mcm3 and Mcm7. CK II is an essential kinase, conserved from yeast through humans, which targets over one hundred different proteins involved in many cellular processes. In *S. cerevisiae*, CK II has a role in cell morphology, cell polarity, and cell size (Canton and Litchfield, 2006). Because CK II has many different roles in cellular processes, mutating specific potential CK II sites in the MCM subunits would be especially helpful to ascertain the role of CK II in MCM complex regulation, although this approach will be helpful for all of the different potential kinase sites we identified.

In general, our data agree with other available mass spectrometry studies performed in *S. cerevisiae*. Phosphorylation site mapping has not been performed specifically on

MCM complexes, however global phosphorylation site mapping of both G1-arrested and asynchronous yeast cells has been performed. In our study, we detected five of the six residues identified in the asynchronous study and two of the four sites identified in the G1-arrested yeast cell study. It is possible that we did not identify the other three sites due to lack of coverage or protein, however it is more likely that the three residues found to be phosphorylated in other studies are due to phosphorylation events that do not occur on pre-RC-linked MCM complexes. Interestingly, we mapped many more sites in the MCM complex than were found in the global phosphorylation studies probably due to the more focused nature of our analysis and the lack of complete coverage in the global studies.

Identifying the sites of phosphorylation of G1-phase pre-RC-linked MCM complexes will be useful in many different ways. We are particularly interested in understanding how DDK phosphorylation of the MCM complex is controlled by prior phosphorylation of the MCM complex. Similarly, we would like to determine how DDK phosphorylation controls binding of other replication factors and activation of the helicase activity. Our data suggest that the pre-RC-linked MCM complexes are modified by several other kinases. Although we do not know whether the DDK-independent phosphorylation events are constitutive or cell cycle regulated, it will be interesting to discover whether they are important in initiation of DNA replication or for other cell cycle events. Construction of phospho-specific mutants will be useful in analyzing the consequences of particular phosphorylation events. Moreover, the information gained in this study will be useful in the construction of phospho-specific antibodies to gain a better understanding of where and when these phosphorylation events are occurring.

Experimental Procedures

Preparation of whole cell extracts

The WCEs used in the pre-RC assembly assays were made as described previously (Bowers et al, 2004).

Pre-RC assembly assay

Pre-RC assembly assays were performed as previously described (Randell et al, 2006) except that a 25 fold scale-up reaction was performed. The samples were analyzed on an 8% SDS-PAGE protein gel and stained with Coomassie Blue.

DDK Kinase assays

Kinase assays with wild-type DDK and kinase dead DDK (Dbf4-Cdc7D163A) was performed as described in Chapter II except that a 25 fold scale-up reaction was performed with each DDK kinase.

Mass Spectrometry Analysis

Six gel slices from the region of migration of the MCM proteins were excised from the protein gel and sent to the Taplin Facility at Harvard Medical School for mass spectrometry analysis. For each gel slice, the two MCM subunits with the highest peptide representation were analyzed for evidence of phosphorylation sites. Any phosphorylation site that appeared solely in the sample with wild-type DDK was considered to be DDK-dependent. All other sites found were considered non-DDK-dependent sites.

References

Bell, S.P., and Dutta, A. (2002). DNA replication in eukaryotic cells. Annu Rev Biochem *71*, 333-374.

Canton, D.A., and Litchfield, D.W. (2006). The shape of things to come: an emerging role for protein kinase CK2 in the regulation of cell morphology and the cytoskeleton. Cell Signal *18*, 267-275.

Cho, W.H., Lee, Y.J., Kong, S.I., Hurwitz, J., and Lee, J.K. (2006). CDC7 kinase phosphorylates serine residues adjacent to acidic amino acids in the minichromosome maintenance 2 protein. Proc Natl Acad Sci U S A *103*, 11521-11526.

Ficarro, S.B., McCleland, M.L., Stukenberg, P.T., Burke, D.J., Ross, M.M., Shabanowitz, J., Hunt, D.F., and White, F.M. (2002). Phosphoproteome analysis by mass spectrometry and its application to Saccharomyces cerevisiae. Nat Biotechnol *20*, 301-305.

Labib, K., and Gambus, A. (2007). A key role for the GINS complex at DNA replication forks. Trends Cell Biol *17*, 271-278.

Li, X., Gerber, S.A., Rudner, A.D., Beausoleil, S.A., Haas, W., Villen, J., Elias, J.E., and Gygi, S.P. (2007). Large-scale phosphorylation analysis of alpha-factor-arrested Saccharomyces cerevisiae. J Proteome Res *6*, 1190-1197.

Liku, M.E., Nguyen, V.Q., Rosales, A.W., Irie, K., and Li, J.J. (2005). CDK phosphorylation of a novel NLS-NES module distributed between two subunits of the Mcm2-7 complex prevents chromosomal rereplication. Mol Biol Cell *16*, 5026-5039.

Masai, H., and Arai, K. (2002). Cdc7 kinase complex: a key regulator in the initiation of DNA replication. J Cell Physiol *190*, 287-296.

Masai, H., Taniyama, C., Ogino, K., Matsui, E., Kakusho, N., Matsumoto, S., Kim, J.M., Ishii, A., Tanaka, T., Kobayashi, T., *et al.* (2006). Phosphorylation of MCM4 by Cdc7 kinase facilitates its interaction with Cdc45 on the chromatin. J Biol Chem *281*, 39249-39261.

Mendez, J., and Stillman, B. (2003). Perpetuating the double helix: molecular machines at eukaryotic DNA replication origins. Bioessays *25*, 1158-1167.

Montagnoli, A., Valsasina, B., Brotherton, D., Troiani, S., Rainoldi, S., Tenca, P., Molinari, A., and Santocanale, C. (2006). Identification of Mcm2 phosphorylation sites by S-phase-regulating kinases. J Biol Chem *281*, 10281-10290.

Moyer, S.E., Lewis, P.W., and Botchan, M.R. (2006). Isolation of the Cdc45/Mcm2-7/GINS (CMG) complex, a candidate for the eukaryotic DNA replication fork helicase. Proc Natl Acad Sci U S A *103*, 10236-10241.

Nguyen, V.Q., Co, C., Irie, K., and Li, J.J. (2000). Clb/Cdc28 kinases promote nuclear export of the replication initiator proteins Mcm2-7. Curr Biol *10*, 195-205.

Nguyen, V.Q., Co, C., and Li, J.J. (2001). Cyclin-dependent kinases prevent DNA re-replication through multiple mechanisms. Nature *411*, 1068-1073.

Sheu, Y.J., and Stillman, B. (2006). Cdc7-Dbf4 phosphorylates MCM proteins via a docking site-mediated mechanism to promote S phase progression. Mol Cell *24*, 101-113.

Songyang, Z., Blechner, S., Hoagland, N., Hoekstra, M.F., Piwnica-Worms, H., and Cantley, L.C. (1994). Use of an oriented peptide library to determine the optimal substrates of protein kinases. Curr Biol *4*, 973-982.

Tak, Y.S., Tanaka, Y., Endo, S., Kamimura, Y., and Araki, H. (2006). A CDK-catalysed regulatory phosphorylation for formation of the DNA replication complex Sld2-Dpb11. EMBO J *25*, 1987-1996.

Tanaka, S., Umemori, T., Hirai, K., Muramatsu, S., Kamimura, Y., and Araki, H. (2007). CDK-dependent phosphorylation of Sld2 and Sld3 initiates DNA replication in budding yeast. Nature *445*, 328-332.

Tsuji, T., Ficarro, S.B., and Jiang, W. (2006). Essential role of phosphorylation of MCM2 by Cdc7/Dbf4 in the initiation of DNA replication in mammalian cells. Mol Biol Cell *17*, 4459-4472.

Ubersax, J.A., and Ferrell, J.E., Jr. (2007). Mechanisms of specificity in protein phosphorylation. Nat Rev Mol Cell Biol *8*, 530-541.

Weinreich, M., and Stillman, B. (1999). Cdc7p-Dbf4p kinase binds to chromatin during S phase and is regulated by both the APC and the RAD53 checkpoint pathway. EMBO J *18*, 5334-5346.

Xu, Y., and Baltimore, D. (1996). Dual roles of ATM in the cellular response to radiation and in cell growth control. Genes Dev *10*, 2401-2410.

Zegerman, P., and Diffley, J.F. (2007). Phosphorylation of Sld2 and Sld3 by cyclin-dependent kinases promotes DNA replication in budding yeast. Nature *445*, 281-285.

Chapter IV

Future Experiments

Summary of Results

To initiate DNA replication in early S-phase of each cell cycle, the replicative helicase, Mcm2-7 is first loaded onto DNA as part of the pre-RC at selected origins of replication in G1-phase. At the G1- to S-phase transition, two kinases, DDK and CDK, activate the selected origins. The process of activating origins for initiation requires an active helicase to unwind DNA, and recruitment of the DNA synthesis machinery to replicate the DNA. My work has focused on gaining a more mechanistic understanding of how DDK binds to and targets its substrate, the Mcm2-7 replicative helicase. We have also begun studies, including mapping the phosphorylated residues on the pre-RC linked MCM complexes, to better understand events that occur downstream of DDK phosphorylation.

In Chapter II of this thesis, I discussed biochemical experiments examining phosphorylation of the Mcm2-7 complex by DDK in the context of pre-RCs purified from *S. cerevisiae* G1-arrested whole cell extracts. We discovered that the Mcm4 and Mcm6 subunits are the principal phosphorylation targets of DDK and that the MCM complex itself is the only pre-RC component necessary to recruit DDK to the origin. Observations made by other labs suggest that DDK specifically targets chromatin associated MCM complexes, even though these MCM complexes are a fraction of total MCM proteins in the nucleus. We present evidence that DDK can differentiate between different populations of MCM complexes, even if they are both DNA-bound. Our results suggest that something beyond recruitment of the MCM complex to the origin, such as modification state or conformation, is important for targeting of DDK. In support of this idea, we present evidence that prior phosphorylation of the pre-RC is required for targeting the of MCM complex for DDK phosphorylation.

In Chapter III, I describe a collaborative effort to map the phosphorylation sites on purified pre-RCs, both before and after treatment with DDK. Our initial results demonstrate that MCM complexes may be targeted for phosphorylation by multiple kinases (including DDK). Moreover, different MCM subunits have distinct patterns of phosphorylation. Mcm2, 4 and 6 N-termini are targeted for phosphorylation both by DDK and by other kinases. Mcm3 and Mcm7 are also targeted both by DDK and other

kinases, however, Mcm3 is primarily targeted at its C-terminus and Mcm7 is targeted throughout its sequence. No phosphorylation sites were found in Mcm5. These results give us a general understanding of phosphorylation patterns of pre-RC linked MCM complexes. Moreover, these data provide critical information to address the importance of Mcm2-7 phosphorylation, at least some of which will be described here.

Mapping the domains in Dbf4 and the MCM complex responsible for recruitment of DDK to the pre-RC

Our data shows that the MCM complex is both necessary and sufficient to recruit DDK to the origin. Moreover, data from other labs suggest that Dbf4 interacts with Mcm2 in a two-hybrid assay (Varrin et al., 2005) and that a region of Mcm4 binds Dbf4 in GST-pulldown experiments (Sheu and Stillman, 2006). Nevertheless, the precise nature of this interaction is not well understood. For example, it is possible that more than one MCM subunit is involved in the interaction of DDK with the MCM complex. If this is true, it would be interesting to determine whether interactions with multiple subunits are redundant for DDK association or whether the interactions are additive. As a starting point, I will address whether a small domain in Mcm4 that has been shown to be required for binding of Dbf4 by GST-pulldown experiments disrupts binding of DDK in our pre-RC/DDK kinase assay. If not, then other mutations in other MCM subunits, such as Mcm2, would be created to understand which subunits are required for association of DDK with the MCM complex. The region in Mcm4 determined to be important for binding Dbf4 is just C-terminal to the S/T rich N-terminal phosphorylated region. We could make an analogous mutation in the Mcm2 subunit and determine whether this region is also involved in binding Dbf4.

Our results demonstrate that prior phosphorylation of the MCM complex is required for DDK binding to the MCM complex. This data suggests that Dbf4 might be a phospho-peptide binding protein. Intriguingly, Dbf4 has a conserved motif, part of which is homologous to a portion of the canonical BRCT domain. BRCT domains are often involved in phospho-peptide interactions. The other part of this conserved motif in Dbf4 is homologous in other Dbf4 species but not to the BRCT domain, thus this domain was termed the *BR*CT and *Db*f4p similarity (BRDF) motif (Gabrielse et al., 2006). There is

conflicting *in vivo* data whether the BRDF domain in Dbf4 is required for normal cell cycle progression or whether it might primarily be involved in response to DNA damage. For example, one group reported that the entire BRDF motif could be deleted with normal growth under non-DNA damaging conditions (Gabrielse et al., 2006). However, another group made a smaller deletion within the BRDF domain and saw very slow growth (Varrin et al., 2005). Therefore it is possible that this domain is important in binding MCM complexes and we can test this directly in our pre-RC/DDK assay. We could make a variety of mutations in this region of Dbf4, not only in the BRDF domain, but in other conserved domains as well (see Ch I, Fig. 5). We can also directly ask whether Dbf4 is a phospho-peptide binding protein. In collaboration with the Yaffe Lab, which has phospho-peptide libraries, we can determine whether Dbf4 (or DDK) binds phospho-peptides, and if so, whether it binds specific phospho-peptides. Moreover, if Dbf4 is a phospho-peptide binding protein, we can delete the BRDF domain and directly test whether this domain is involved in phospho-peptide binding using the phospho-peptide binding library.

We do not yet understand how binding of DDK to the MCM complex correlates with kinase activity. For example, we describe results in Ch II which demonstrate that dephosphorylation of the pre-RC eliminates DDK binding to and phosphorylation of the Mcm2-7 complex. This data suggests that binding and activity are correlated, but to what extent? We would like to understand whether the ability to phosphorylate the MCM complex is absolutely dependent on the ability of DDK to bind the MCM complex, and whether the opposite is true as well. We can address these questions in a variety of ways. We can create deletion mutations in Dbf4 that are outside the regions involved in binding Cdc7 (so that Dbf4 can still bind and activate Cdc7), and test for their effect on DDK binding in the pre-RC/DDK kinase assay. If there are mutants in Dbf4 that activate Cdc7 for kinase activity but do not bind the pre-RC, then we could ask how binding affects phosphorylation of the MCM complex. We also have a kinase deficient DDK mutant. If DDK binding to the MCM complex were dependent on kinase activity, then we would expect that this mutant would not bind to the MCM complex. We have tested this in our pre-RC/DDK assay (Supp Fig. 1) and while this mutant did not bind the MCM complex as well as wild-type DDK, we did see non-specific binding that was not wild-type origin-

dependent, therefore further studies with this mutant will need to be performed. Creating phospho-site mutants in the MCM subunits that abolish the ability of DDK to phosphorylate the MCM complex would be another approach to asking this question.

DDK phosphorylation of the MCM complex: what are the crucial targets and downstream consequences of this phosphorylation event?

It is well established that DDK phosphorylates the MCM complex and that this phosphorylation event is important for downstream events in initiation of DNA replication. However, identifying specific mutations in the MCM complex that eliminate DDK phosphorylation has been difficult. Data from other labs suggests that specific phosphorylation sites targeted for phosphorylation by DDK are not as important as the ability to phosphorylate a particular region of the MCM subunit (Masai et al., 2006; Sheu and Stillman, 2006). For example, the N-terminal 174 amino acids of Mcm4 are phosphorylated by DDK and are essential for cell growth. However, this N-terminal region can be replaced either by a S/T rich N-terminal region of another MCM subunit (such as Mcm2) or by a random protein epitope (such as HA) with serines or threonines included throughout the sequence (Sheu and Stillman, 2006). Moreover, there is data that DDK phosphorylation of multiple MCM subunits is redundant for cell growth such that deleting a DDK targeted region of one subunit is somewhat deleterious, but deleting DDK targeted regions in multiple subunits is lethal to the cell (Masai et al., 2006).

In Chapter III, we describe results of an effort to map the DDK phosphorylation sites in MCM complexes associated with the pre-RC. Interestingly, we did not find many DDK-specific sites, suggesting that either DDK phosphorylates the MCM complex on a few specific residues or possibly that CDK activity is required for full DDK phosphorylation. It will be interesting to determine through phospho-mutant analysis whether the sites we found are the important targets for phosphorylation or whether other sites can be phosphorylated by DDK as well. Moreover, we can make phospho-mutants in multiple MCM subunits if deleting phosphorylated residues in one MCM subunit does not have an affect *in vivo.*

In vivo studies by other labs have correlated hyper-phosphorylation of Mcm4 (as seen by western blot protein gel mobility shift) with the ability of Cdc45 to be recruited

to chromatin (Masai et al., 2006; Sheu and Stillman, 2006). Mcm4 hyper-phosphorylation was dependent on DDK activity and was only detected on MCM complexes associating with chromatin. Consistent with Cdc45 binding to the Mcm2-7 complex, both Mcm2 and Mcm5 have been shown to co-IP with Cdc45 in S-phase (Zou and Stillman, 1998). These data suggest that phosphorylation of chromatin associated Mcm4 by DDK leads to the recruitment of Cdc45 to the chromatin. However, a direct link between these two events has not been shown. We could approach this question in several ways. For example, we could purify pre-RC's, treat with DDK (or not) and add purified Cdc45 to determine whether Cdc45 can interact with DNA-bound MCM complexes. If other factors were involved in this recruitment, such as Sld3, we would not see association in this relatively purified system. In this case, we could add yeast extracts that are made from cells arrested with a *cdc7ts* allele and determine whether extract derived Cdc45 (and any other proteins) binds to the pre-RC.

Do other kinases control the function of the MCM complex?

As described in Chapters II and III, our work strongly suggests that other kinases besides DDK target the MCM complex for phosphorylation. Others have also observed phosphorylation by other kinases and it is clear that CDK phosphorylation of the Mcm2-7 complex drives its nuclear export (Liku et al., 2005). On the other hand, whether these phosphorylation events influence the function of the MCM during initiation of replication is unknown. Interestingly, most of the residues in the MCM complex that we found to be phosphorylated were part of the minimal consensus sequences of many kinases known. For example, we found S/T-Q sites, which are typical of the ATM/ATR family of kinases. We found phosphorylated casein kinase II consensus sites (S/T-acidic-X-acidic), and phosphorylated CDK consensus sites (S/T-P). Most of the other phosphorylated residues were part of a larger class of kinase consensus sites (S/T-aliphatic), which can be targeted by many kinases and, therefore, is less informative.

Although the role of these kinases in MCM complex function is not clear, there is information as to other purposes they have in the cell. For example, in *S. cerevisiae*, the only known ATM/ATR family kinases are Mec1 and Tel1. The role of these two kinases in response to DNA damage is most well understood, however, at least Mec1 is also

required for normal cell cycle progression. This essential role is generally thought to be involved in nucleotide biosynthesis (the lethality of the deletion can be bypassed by mutations that stimulate ribonucleotide reductase synthesis)(Zhao et al., 2000), however, there could be other roles that have not been uncovered. There is evidence for a role for CDK-mediated phosphorylation of the MCM complex in targeting the MCM complexes for export from the nucleus after DNA replication is finished. We found phosphorylated residues at typical CDK-consensus sites in all the MCM subunits except for Mcm5. However, these MCM complexes were isolated from cells in which S-phase CDKs are inactive. This may mean that these phosphorylated residues to do not play a role in MCM complex export from the nucleus or that other phosphorylated residues are required for export. Alternatively, these MCM complexes may be protected from export due to their association with the DNA.

The information we gained by mapping the phosphorylated residues on pre-RC-linked MCM complexes may be very useful in uncovering the functions of different kinases in MCM complex regulation. To determine the roles of the different putative kinase sites that we identified, we could mutate each of the different classes of consensus kinase sites we found to determine whether they are important in initiation of DNA replication or for normal cell cycle progression. CDK phosphorylation of the MCM complex may play a non-essential role in initiation of DNA replication. Although phosphorylation of Sld2 and Sld3 are the minimal essential targets of CDK, it is clear that replication is much less efficient when these pathways are bypassed and CDK is eliminated. The existence of multiple S-S-P sites in the N-termini of Mcm2, Mcm4 and Mcm6 (see Ch III, Fig 2) suggest the intriguing possibility that S-phase CDKs phosphorylate the second serine, which creates a phosphorylation site for DDK at the first serine. Moreover, both Mcm4 and Mcm6 have multiple S/T-S/T-Q sites in their N-termini suggesting that Mec1 could function in the same way. Therefore, it is possible that phosphorylation by CDK and/or Mec1 is one of several avenues to activate the Mcm2-7 complex and that phosphorylation of the MCM complexes could integrate several signaling pathways (Mec1, CDK, DDK) and lead to corresponding levels of initiation.

Preferential targeting by DDK of different populations of MCM complexes

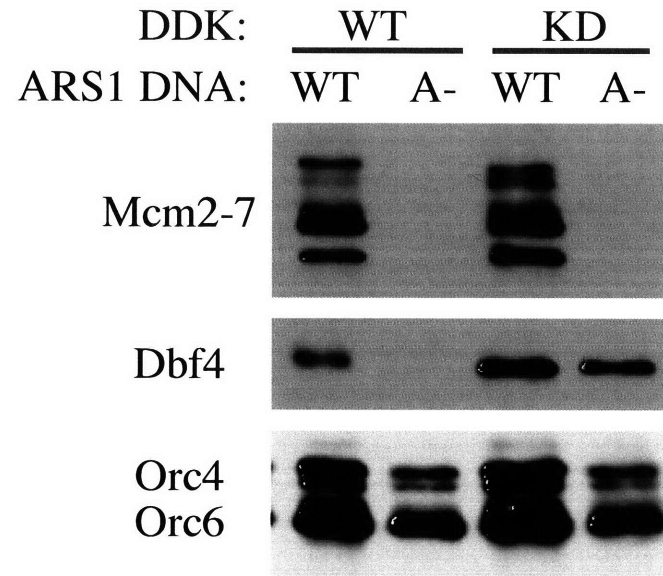
In S-phase, only a small portion of total nuclear MCM complexes associates with the chromatin and participates in DNA replication. Studies from other labs suggest that DDK has the ability to specifically target these chromatin associated MCM complexes and presumably these are the MCM complexes taking part in DNA replication. A simple explanation as to how this might come about is that DDK is recruited to the origin specifically by ORC. Because most of the ORC in the cell is at origins of replication, DDK would be specifically targeted to those MCM complexes loaded onto chromatin at origins and destined to take part in DNA replication. However, we show that DDK is recruited to the pre-RC by the MCM complex, rather than ORC. This is consistent with studies that strongly indicate that ORC is not required for events after pre-RC formation (Jares and Blow, 2000; Walter, 2000). Moreover, we show that not only can DDK distinguish between MCM complexes on and off the DNA in the context of our assay, but also that DDK distinguishes between two DNA-bound populations of MCM complexes (loaded and associated). These results suggest that DDK is distinguishing between particular characteristics or features of the different populations of MCM complexes.

One possibility for how these MCM complexes may be different is in the amount of phosphorylation by other kinases. For example, if MCM complexes that are loaded onto the DNA are preferentially targeted for phosphorylation by another kinase, then this may direct the preferential targeting of DDK of loaded MCM complexes. The phospho-mutant analysis described above may be useful in determining whether this hypothesis is correct. For example, we may identify a set of phospho-site mutations in the MCM proteins that cannot be distinguished as associated and loaded MCM complexes by DDK.

Another possibility is that a conformational change dictates preferential phosphorylation of loaded MCM complexes. We believe that the association of the MCM complex with the DNA is different between associated and loaded MCM complexes; therefore, it is quite possible that these complexes are in a different conformation. The possibility of phosphorylation and conformation dictating preferential phosphorylation by DDK are not mutually exclusive ideas, as it is also possible that a prior phosphorylation event of loaded MCM complexes causes a conformational change that leads to preferential targeting by DDK. We may be able to distinguish between these

different possibilities using phospho-mutant analysis in combination with partial-tryptic digestion of the protein (to see if we can distinguish between different conformations of the MCM complex).

Supplemental Figure 1.



Supplemental Figure 1: Association of kinase dead DDK to the pre-RC as compared to wild-type DDK. Experiment was performed as described in Ch. II, Fig 3A.

References

Gabrielse, C., Miller, C.T., McConnell, K.H., DeWard, A., Fox, C.A., and Weinreich, M. (2006). A Dbf4p BRCA1 C-terminal-like domain required for the response to replication fork arrest in budding yeast. Genetics *173*, 541-555.

Jares, P., and Blow, J.J. (2000). Xenopus cdc7 function is dependent on licensing but not on XORC, XCdc6, or CDK activity and is required for XCdc45 loading. Genes Dev *14*, 1528-1540.

Liku, M.E., Nguyen, V.Q., Rosales, A.W., Irie, K., and Li, J.J. (2005). CDK phosphorylation of a novel NLS-NES module distributed between two subunits of the Mcm2-7 complex prevents chromosomal rereplication. Mol Biol Cell *16*, 5026-5039.

Masai, H., Taniyama, C., Ogino, K., Matsui, E., Kakusho, N., Matsumoto, S., Kim, J.M., Ishii, A., Tanaka, T., Kobayashi, T., *et al.* (2006). Phosphorylation of MCM4 by Cdc7 kinase facilitates its interaction with Cdc45 on the chromatin. J Biol Chem *281*, 39249-39261.

Sheu, Y.J., and Stillman, B. (2006). Cdc7-Dbf4 phosphorylates MCM proteins via a docking site-mediated mechanism to promote S phase progression. Mol Cell *24*, 101-113.

Varrin, A.E., Prasad, A.A., Scholz, R.P., Ramer, M.D., and Duncker, B.P. (2005). A mutation in Dbf4 motif M impairs interactions with DNA replication factors and confers increased resistance to genotoxic agents. Mol Cell Biol *25*, 7494-7504.

Walter, J.C. (2000). Evidence for sequential action of cdc7 and cdk2 protein kinases during initiation of DNA replication in Xenopus egg extracts. J Biol Chem *275*, 39773-39778.

Zhao, X., Georgieva, B., Chabes, A., Domkin, V., Ippel, J.H., Schleucher, J., Wijmenga, S., Thelander, L., and Rothstein, R. (2000). Mutational and structural analyses of the ribonucleotide reductase inhibitor Sml1 define its Rnr1 interaction domain whose inactivation allows suppression of mec1 and rad53 lethality. Mol Cell Biol *20*, 9076-9083.

Zou, L., and Stillman, B. (1998). Formation of a preinitiation complex by S-phase cyclin CDK-dependent loading of Cdc45p onto chromatin. Science *280*, 593-596.

Appendix I

Stability of MCM Complex Association with Circular and Linear DNA Templates

Introduction

The Mcm2-7 complex plays an essential role in DNA replication as the replicative helicase (Takahashi et al., 2005). This complex associates with the DNA during pre-Replicative Complex (pre-RC) formation in G1-phase of the cell cycle (Dutta and Bell, 1997) and its continued association with the DNA is required for DNA replication fork progression in S-phase (Labib et al., 2000). Crystallographic and EM analysis of the archaeal MCM complex indicates that the protein forms a double-hexameric, ring-shaped structure (Chong et al., 2000; Fletcher et al., 2003; Pape et al., 2003) with the two hexamers in a head-to-head orientation. The method by which the MCM complex unwinds DNA is a mystery including whether it encircles single-stranded or double-stranded DNA during this process (Takahashi et al., 2005). Moreover, studies suggest that the MCM complex is initially loaded onto DNA in an inactive state (as part of the pre-RC) and is not activated for helicase activity until downstream protein factors bind to the complex (Moyer et al., 2006; Zou and Stillman, 2000). The state of association of the MCM complex with the DNA in the inactive state is not known, nor if this changes upon activation of the helicase.

Both ORC and Cdc6 are members of the AAA+ family of ATPases that are required to assemble the Mcm2-7 complex onto origin of replication DNA (Bell and Dutta, 2002). Studies of the effects of ATP hydrolysis mutants and non-hydrolyzable ATP analogs in an *in vitro* pre-RC assembly system has elucidated the role of these proteins in loading the MCM complex onto the DNA (Ch I, Fig 1) (Bowers et al., 2004; Randell et al., 2006). The initial association of the Mcm2-7 complex with origin DNA does not depend on ATP hydrolysis by ORC or Cdc6, however, ATP hydrolysis by Cdc6 is required to change the manner of MCM complex association with DNA (Randell et al., 2006). Before Cdc6 hydrolyzes its ATP, Mcm2-7 association with DNA is dependent on ORC. If ORC is extracted from the DNA using a high-salt wash, this "associated" form of the MCM complex dissociates from the DNA as well. In contrast, after Cdc6 hydrolyzes ATP, the MCM complex is stabilized on DNA and no longer requires ORC or Cdc6 to remain associated with the origin DNA. This "loaded" form of the MCM

complex is resistant to extraction from the DNA by a high-salt buffer. Given the stability of these MCM complexes on DNA and the fact that MCM complexes are thought to form a ring-like structure around DNA (Fletcher et al., 2003), we hypothesized that salt-stable MCM complexes are encircling the DNA (Randell et al., 2006).

To begin to determine whether loaded MCM complexes encircle origin DNA, we studied the stability of the MCM complexes on circular versus linear origin-containing DNA templates. If loaded MCM complexes are encircling and sliding along the DNA, we hypothesized that they would be more stable on circular DNA templates as compared to linear DNA templates. Here I present preliminary evidence that loaded MCM complexes associate more stably with circular DNA templates as compared to linear forms of the same DNA. I also find that the stability of loaded MCM complexes on a linear template differs depending on which side of the origin is closest to the free DNA end.

Results

To assess the stability of different populations of MCM complexes on circular and linear DNA templates, we formed pre-RCs and either washed the pre-RCs with high-salt buffer, to isolate the loaded Mcm2-7 population, or washed the pre-RCs with low-salt buffer, leaving both loaded and associated MCM complexes on the DNA (Fig 1A). These *in vitro* assembled pre-RCs contain anywhere from 30-50% loaded MCM complexes, therefore salt extraction of the pre-RC removes 50-70% of the MCM complexes associated with the DNA (Bowers et al., 2004). To normalize the amount of MCM complexes on the DNA after high-salt wash to the amount of MCM complexes on the DNA after the low-salt wash, we initially started with three times the amount of pre-RCs for the high-salt washed samples. After pre-RCs were washed, they were returned to a low-salt buffer and their dissociation from the DNA measured (Fig 1A).

The different DNA templates (Fig 1B) had different amounts of pre-RC formation (Fig 2). At this time, it is not clear whether this is due to differences in the amount of DNA used or whether different templates have different efficiencies of pre-RC formation (or both). Because each sample had different amounts of MCM complexes binding to the DNA, we determined the percentage of MCM complexes dissociating from the DNA for each individual sample. We determined the percentage of MCM complexes dissociating into the supernatant by dividing the amount of MCM complexes in the supernatant by the total amount of MCM complexes in the sample (supe/(supe + DNA)).

Only small changes were observed in the association of the mixed population of MCM complexes (no salt extraction) with the different DNA templates used (Table 1). For example, ~26% of MCM complexes dissociated from the circular DNA template whereas ~29% dissociated from SacII linearized template and ~20% from the XhoI linear template. Similar small differences were observed in both experiments performed, however, further reiterations of the experiments will be required to determine the significance of the different stability of the mixed population of MCM complexes.

More significant changes in dissociation were observed with the different DNA templates when only loaded MCM complexes were bound to the DNA. The lowest amount of MCM dissociation was observed in the sample containing loaded MCM complexes on circular DNA (Table 1 and Fig 2) in which ~13% of the MCM complexes

dissociated from the DNA. Interestingly, about 2.5 times that amount of loaded MCM molecules dissociated from the linear^{XhoI} DNA template (~34%). The results of the linear^{SacII} DNA template were intermediate with ~24% of the MCM complexes dissociating into the supernatant. These data suggests that loaded MCM complexes are more stable on circular than linear DNA templates, however, their stability on linear DNA templates is dependent on which side of the origin is closest to the free end of the DNA.

We can also compare the amount of MCM complexes dissociating from the same template, but in non-salt- versus salt-extracted samples (Table 1). More MCM complexes dissociated from the circular DNA in the non-salt washed as compared to the salt-washed samples (26% vs 13%) suggesting that both loaded and associated MCM complexes dissociate from the DNA in the non-salt washed samples. The same was true for linear^{SacII} DNA template except only about 5% more MCM complexes dissociated from the DNA in the non-salt extracted samples (29% vs 24%). This result was not the same when the experiment was performed with linear^{XhoI} template. In this case, less MCM complexes dissociated from the non-salt-washed pre-RCs (20% vs 34%). Although we do not know the percentage of loaded MCMs dissociating from the DNA in the non-salt-washed sample, it must be less than in the case where only loaded MCM complexes are associating with DNA. These results suggests that loaded MCM complexes associate more stably on the linear^{XhoI} DNA template when the pre-RCs are not salt-extracted.

Figure 1.

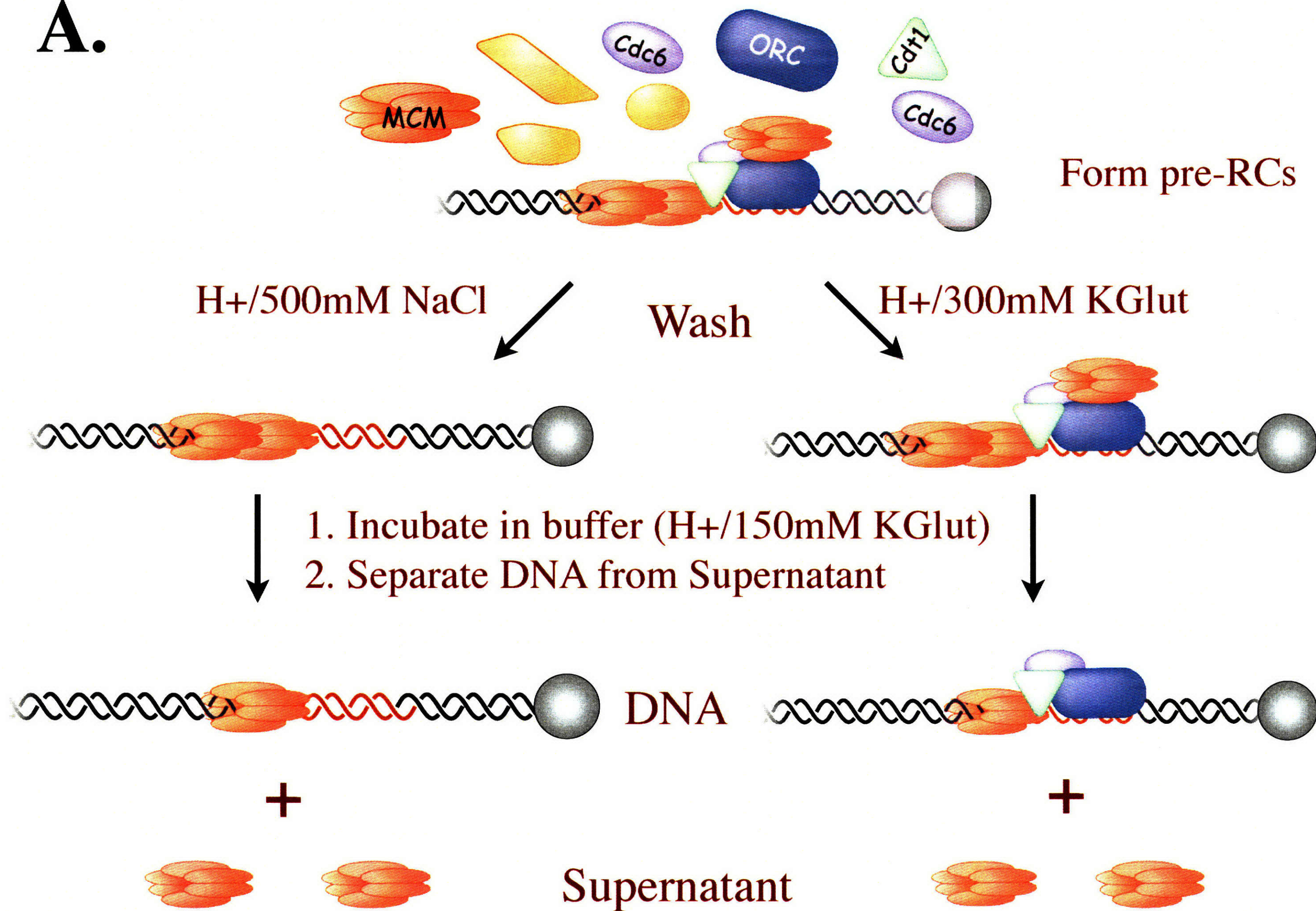
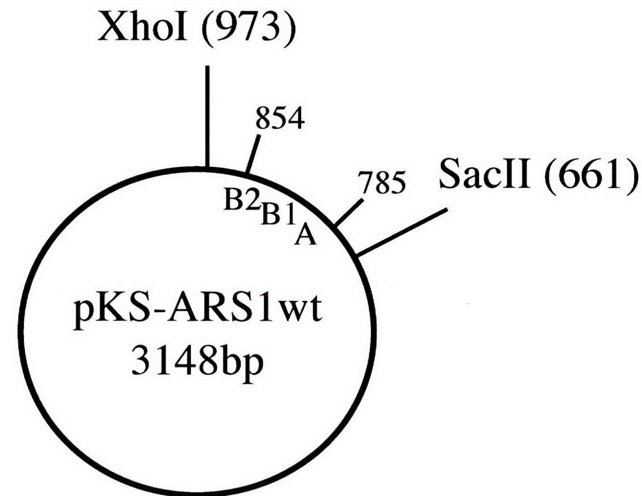


Figure 1.

B.



C.

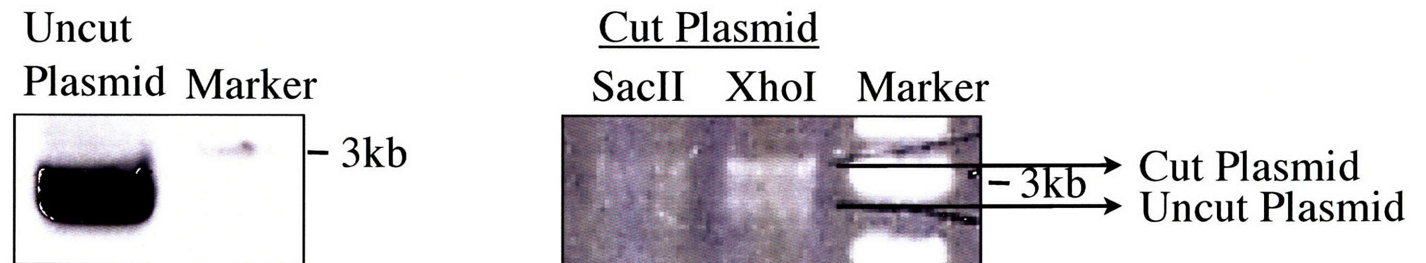


Figure 1. Experimental outline and template used in experiments. (A) An outline of the experiment performed in Fig 2. Pre-RCs were assembled and washed in either low-salt buffer (300mM KGlut) or high salt buffer (500mM NaCl). The proteins remaining associated with the DNA were then incubated in buffer containing 150mM KGlut. The DNA and supernatant were separated and analyzed by western blot with Mcm2-7 antibody. (B) The pKS-ARS1wt plasmid showing the sites of linearization in relation to the ARS1 origin (785-854) with the orientation of the A, B1 and B2 elements shown. (C) Cut and uncut plasmid separated on 1% agarose gel. The amounts shown are not equivalent to the amounts used in the described experiments.

Figure 2.

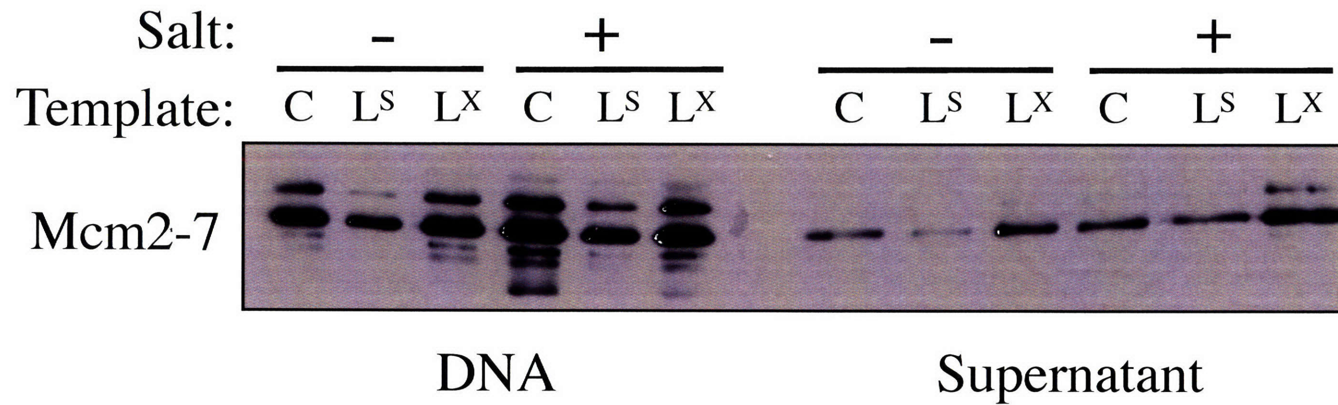


Figure 2. Raw data of Experiment described in Fig 1. Experient was performed as described in Fig 1A. C = circular DNA; L^S = circular DNA linearized with SacII restriction enzyme; L^X = circular DNA linearized with XhoI restriction enzyme.

Table 1.

Sample	% MCM protein in Supernatant (Expt 1)[a]	% MCM protein in Supernatant (Expt 2)[a]
No Salt Extraction		
Circular	27	26
Linear^{SacII}	30	29
Linear^{XhoI}	24	20
Salt Extraction		
Circular	15	13
Linear^{SacII}	21	24
Linear^{XhoI}	36	34

[a] The results of two independent experiments are shown

Discussion

Our preliminary investigation into the stability of pre-RC-linked MCM complex association with different DNA templates yielded some potentially interesting results. We found that binding of high salt-washed (loaded) MCM complexes to the DNA was more stable on circular DNA than on linear DNA. Moreover, more loaded and associated MCM complexes (no-salt-extraction) dissociated from circular DNA than just loaded MCM complexes (salt-extraction), suggesting that associated MCM complexes dissociated from the DNA as well during the buffer incubation. The same result occurred with linear^{SacII} DNA template, although only slightly more loaded and associated MCM complexes dissociated from the DNA as compared to just loaded MCM complexes. These data are consistent with previous findings showing that the high-salt extraction causes the dissociation of loosely associated DNA binding proteins (including ORC and associated MCM complexes). Interestingly, the opposite result occurred using the linear^{XhoI} template. The amount of dissociation of the loaded and associated MCM complexes from linear^{XhoI} DNA template was less than the amount of loaded MCM complexes dissociating from the same DNA template. Below we propose a model to explain these preliminary results.

We have previously hypothesized that loaded MCM complexes encircle duplex DNA (Randell et al., 2006), resulting in relative resistance to DNA dissociation during high salt-extraction. One explanation for our results is that loaded MCM complexes are encircling duplex DNA and are sliding off the ends of the linear DNA molecule. This hypothesis could also explain why, in the case of linear^{XhoI} DNA, less loaded MCM complexes dissociate from non-salt-extracted pre-RCs. The DNA-binding proteins, which are stably bound to the DNA in the non-salt extracted samples, may be hindering the movement of the loaded MCM complexes along the DNA molecule. However, washing the DNA with high-salt buffer disrupts the interaction of most proteins with DNA, therefore, the absence of DNA-bound proteins (besides MCM complexes) in the salt-extracted samples could afford loaded MCM complexes greater movement along the DNA. It is not clear why this result is not the same for the linear^{SacII} DNA template, however, only 5% more MCM complexes are dissociating from the DNA in the samples containing both loaded and associated MCM complexes as compared to loaded MCM

complexes. Therefore, it is possible that some stabilization of loaded MCM complexes on DNA is occurring, just not to the same extent.

The data described here suggests there are differences in MCM dissociation from the DNA depending which side of the origin is closest to the free end of the DNA. For example, when the DNA was linearized on the side closest to the A element (SacII), loaded MCM complexes were more stable than if the DNA was linearized on the side closest to the B2 element (XhoII). This difference is puzzling but could be very interesting. One of the unanswered questions in the literature is whether ORC directs loading of MCM complexes onto both sides of the origin or only one side. ORC is an asymmetrical protein complex, but it must somehow direct the formation of a symmetrical, bidirectional replication forks. If our hypothesis is correct and loaded MCM complexes are destabilized from linear DNA by sliding off the ends, then the differences in dissociation from linear^{SacII} and linear^{XhoI} DNA templates may suggest that more loaded MCM complexes are closer to XhoI (B2 side) than SacII (A side). Interestingly, this idea is supported by other studies that have shown that mutations in the B2 element of ARS1 interfere with MCM association with the origin (Lipford and Bell, 2001; Zou and Stillman, 1998).

As discussed in the introduction of this Appendix, it is thought that Mcm2-7 helicase function is not activated until the later stages of replisome formation, therefore, it is not clear whether the MCM complex has helicase activity or moves along DNA at the early stage of pre-RC formation. In the experiments described here, no ATP was included in the incubating buffer, and other experiments suggest that, after initial pre-RC formation, the presence or absence of ATP in the incubating buffer does not affect MCM association with the DNA (Ch II, Supp Fig. 1). DNA helicase activity requires ATP hydrolysis, therefore, it is likely that if MCM complexes are moving along DNA, they are not doing so by activating their helicase activity in these experiments. A relatively small percentage of MCM complexes are dissociating from the DNA during the buffer incubation, therefore, if MCM complexes are moving along the DNA, they may only be traveling small distances.

The experiments described here are preliminary, however, they may lead to interesting discoveries about the nature of the MCM complex binding to DNA and to the

origin. The differences in the amount of MCM complexes dissociated from each template are not striking in some cases; however, the results were similar over two different experiments with independently prepared template. These experiments will continue to be repeated to determine how the results deviate.

In future studies, we will address some minor experimental issues. For example, we observed that the circularized template might have been incompletely linearized (see Fig 1C). The restriction enzyme digest was performed while the plasmids were attached to the magnetic resin. We hypothesize that, due to the size of the resin, the restriction enzyme may have been partially excluded from the sites of DNA cutting. To address this potential weakness, we will perform experiments in which the DNA is digested before attachment to the magnetic resin to try to improve the extent of DNA cleavage. This could significantly increase the amount of MCM complexes dissociating from linear templates. We will also address the differences in the amount of pre-RC formation on the different templates and try to determine whether we can normalize these to a greater extent and whether there are inherent differences in pre-RC formation on the different DNA templates.

Once the experimental procedure is perfected, we plan to continue pursuing this line of experiments. To determine actual dissociation rates, we will perform a time course of incubation of DNA-bound MCM complexes in low-salt buffer. Preliminary experiments were not performed in this way as the experiment requires large amounts of reagents; however, since the preliminary data appears encouraging, we will perform these experiments. We will also determine whether the same results are achieved with an origin that is oriented in the opposite direction (i.e. XhoI site near the A element and the SacII site near the B2 element). This would suggest that more loaded MCM complexes are being placed on the B2 side of the origin than the side next to the A element. We will determine the stability of loaded MCM complexes when the free end of the DNA is further away from the origin. We would expect that if the MCM complexes are sliding off the free DNA end but are not moving very freely along DNA, then the rate of dissociation would decrease with increasing distance of the free end from the origin. It is also possible to cut the plasmid closer to the origin, however, there is data that suggests this may compromise overall MCM loading (W. Lam, unpublished results). Finally, we

will perform these experiments using a Cdc6 ATP hydrolysis mutant (Cdc6EG) in the pre-RC assembly assay, as this mutant has been shown to recruit only associated MCM complexes to DNA (Randell et al., 2006). This will allow us to compare associated MCM populations to loaded MCM populations rather than comparing loaded MCM complexes to a mixed population of loaded and associated MCM complexes.

Experimental Procedures

Preparation of whole cell extracts

The WCEs used in the pre-RC assembly assays were made as described previously (Bowers et al, 2004).

Preparation of pKS-ARS1wt DNA-coupled beads

pKS-ARS1wt plasmid was randomly crosslinked to biotin using biotin photoprobe reagent (Vector Laboratories). Biotinylated pKS-ARS1wt was coupled to streptavidin-coated magnetic beads (Dynal) by incubation overnight at room temperature. Unbound DNA was washed away by two washes with buffer H/300 (see Bowers et al, 2004). A portion of the bead bound plasmid was linearized using either XhoI or SacII restriction endonucleases to generate linear derivatives of the circular templates.

Pre-RC assembly assay

Pre-RC assembly assays were performed as previously described (Randell et al, 2006) except 100 fmol of DNA was used per reaction.

MCM Dissociation assay

A 30ul pre-RC assembly reaction was performed. The assembled pre-RC were either washed with three times with H/300mM KGlut (no salt extraction) or once with H/300mM KGlut, once with H/500mM NaCl, and once with H/300mM KGlut (see Bowers et al., 2004). After these washes, the proteins remaining bound to the DNA were incubated by rotation in H/150mM KGlut for 45 min at room temp. The beads were precipitated and the supernatant was collected. 20ul of 1X SDS sample buffer was added to the beads/DNA and 12ul of 5X SDS PAGE buffer was added to the supernatant fraction and the samples were incubated at 95C for 5 min. The samples were analyzed on a 7% SDS-PAGE protein gel. Western Blots were performed as previously described (Bowers et al, 2004).

References

Bell, S.P., and Dutta, A. (2002). DNA replication in eukaryotic cells. Annu Rev Biochem *71*, 333-374.

Bowers, J.L., Randell, J.C., Chen, S., and Bell, S.P. (2004). ATP hydrolysis by ORC catalyzes reiterative Mcm2-7 assembly at a defined origin of replication. Mol Cell *16*, 967-978.

Chong, J.P., Hayashi, M.K., Simon, M.N., Xu, R.M., and Stillman, B. (2000). A double-hexamer archaeal minichromosome maintenance protein is an ATP-dependent DNA helicase. Proc Natl Acad Sci U S A *97*, 1530-1535.

Dutta, A., and Bell, S.P. (1997). Initiation of DNA replication in eukaryotic cells. Annu Rev Cell Dev Biol *13*, 293-332.

Fletcher, R.J., Bishop, B.E., Leon, R.P., Sclafani, R.A., Ogata, C.M., and Chen, X.S. (2003). The structure and function of MCM from archaeal M. Thermoautotrophicum. Nat Struct Biol *10*, 160-167.

Labib, K., Tercero, J.A., and Diffley, J.F. (2000). Uninterrupted MCM2-7 function required for DNA replication fork progression. Science *288*, 1643-1647.

Lipford, J.R., and Bell, S.P. (2001). Nucleosomes positioned by ORC facilitate the initiation of DNA replication. Mol Cell *7*, 21-30.

Moyer, S.E., Lewis, P.W., and Botchan, M.R. (2006). Isolation of the Cdc45/Mcm2-7/GINS (CMG) complex, a candidate for the eukaryotic DNA replication fork helicase. Proc Natl Acad Sci U S A *103*, 10236-10241.

Pape, T., Meka, H., Chen, S., Vicentini, G., van Heel, M., and Onesti, S. (2003). Hexameric ring structure of the full-length archaeal MCM protein complex. EMBO Rep *4*, 1079-1083.

Randell, J.C., Bowers, J.L., Rodriguez, H.K., and Bell, S.P. (2006). Sequential ATP hydrolysis by Cdc6 and ORC directs loading of the Mcm2-7 helicase. Mol Cell *21*, 29-39.

Takahashi, T.S., Wigley, D.B., and Walter, J.C. (2005). Pumps, paradoxes and ploughshares: mechanism of the MCM2-7 DNA helicase. Trends Biochem Sci *30*, 437-444.

Zou, L., and Stillman, B. (1998). Formation of a preinitiation complex by S-phase cyclin CDK-dependent loading of Cdc45p onto chromatin. Science *280*, 593-596.

Zou, L., and Stillman, B. (2000). Assembly of a complex containing Cdc45p, replication protein A, and Mcm2p at replication origins controlled by S-phase cyclin-dependent kinases and Cdc7p-Dbf4p kinase. Mol Cell Biol *20*, 3086-3096.