Cell Cycle Regulation of Complex Formation at Origins of DNA Replication

by

Gwendolyn M. Wilmes

B.A Biology
Amherst College, 1997

Submitted to the Department of Biology
in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy in Biology
at the
Massachusetts Institute of Technology

February, 2004

Signature of Author: _____
Department of Biology
November 18, 2003

Certified by: _____
Stephen P. Bell
Associate Professor of Biology
Thesis Supervisor

Accepted by: _____
Alan D. Grossman
Professor of Biology
Chair, Committee for Graduate Students

Cell Cycle Regulation of Complex Formation at Origins of DNA Replication

By
Gwendolyn M. Wilmes

Submitted to the Department of Biology on November 19, 2003 in partial fulfillment of the requirements for the Degree of Doctor of Philosophy in Biology.

ABSTRACT

Eukaryotic DNA replication is regulated by the formation of protein complexes over the initiator protein, the Origin Recognition Complex (ORC). In the G1 phase of the cell cycle, the pre-Replicative Complex (pre-RC) associates with ORC at the origin, priming the origin for initiation of DNA replication. Upon entry into S phase, the pre-RC is activated by the combined activities of two protein kinases, the S phase specific Dbf4 Dependent Kinase (DDK) Cdc7 and the Cyclin Dependent Kinase (CDK) Cdc28. After the origin initiates or is replicated over in S phase, a complex called the post-replicative Complex (post-RC) is left at the origin until CDK levels drop and pre-RCs reform in the next G1. In this work, both the *cis* acting sequences necessary at the origin to form pre-RCs and the *trans* acting proteins that regulate pre-RC formation were studied.

Along with an essential ORC binding site, the well characterized yeast origin *ARS1* requires specific sequences at an auxillary sequence element, the B2 element, to load pre-RCs. These sequences resemble an extra ORC binding site, but do not bind a second ORC *in vitro*. Mutations in these sequences are rescued by overexpression of the pre-RC component Cdc6. Together, these results suggest that one of the proteins in the pre-RC interacts with this DNA sequence to facilitate complex formation.

An interaction between the smallest subunit of ORC, Orc6, and the S phase cyclin Clb5 was documented. The molecular domains responsible for this interaction are a hydrophobic patch on Clb5 and an RXL motif and CDK phosphorylation sites on Orc6. Clb5 associates stably with origins after they replicate, forming part of the post-RC. This interaction aids in prevention of re-replication within a single cell cycle. This suggests that the S phase cyclin, which first activates DNA replication, then acts locally at the origin to prevent pre-RC formation.

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

Dedicated with love to the women who have inspired me,
my mother and my grandmother.

Acknowledgements

My time in graduate school has benefited enormously from stimulating and enriching interactions with a great number of friends and colleagues. I would like to acknowledge in particular the members of the Bell lab for all of their help over the years. They have made my graduate career both enjoyable and productive, and this work would not have been possible without such a great and supporting environment to work in. In addition, I would like to thank Francis Trainor for showing me, at an early age, the joy that a career in science could hold.

In the last two years of graduate school I have enjoyed an engaging and fruitful collaboration with Fred Cross's laboratory. Fred has also been a great mentor to me and has provided a unique and enriching perspective to our work together.

I would like to thank the members of my thesis committee, Angelika Amon, Tania Baker, Anindya Dutta, and Terry Orr-Weaver. Their thoughtful advice and suggestions have been particularly helpful.

Finally, I am indebted to my thesis advisor, Steve Bell, for creating such a stimulating research and learning environment. His encouragement and support have been invaluable to this work.

Table of Contents

Chapter I

Introduction

Overview

The replicon model of genome duplication, proposed 40 years ago, hypothesizes that the initiation of chromosomal replication is controlled by the binding of a protein (the initiator) to a specific genomic sequence (the replicator) (Jacob et al. 1963). This theory has since been confirmed and elucidated in prokaryotes and in eukaryotic viruses. In eukaryotes, however, this paradigm must be expanded to account for larger genomes and the multiple replicons required to ensure faithful completion of DNA replication each cell cycle.

Budding and fission yeasts are the only eukaryotes in which both the initiator protein, the six subunit Origin Recognition Complex (ORC), and the replicator sequences are well-characterized. The replicator sequences coincide with the sites of replication initiation, called origins of replication (Deshpande and Newlon 1992). In *S. cerevisiae* the origins are short, only approximately 100 base pairs, and can be identified based on their ability to confer autonomous replication on plasmids (Marahrens and Stillman 1992). In higher eukaryotes, replicator sequences have been more difficult to identify and may or may not be determined by specific sequences. Despite the relative simplicity of replication origins in *S. cerevisiae*, almost all of the proteins involved in replication initiation are conserved throughout eukaryotes. Therefore *S. cerevisiae* is an ideal organism in which to establish the basic mechanisms of eukaryotic replication initiation.

To ensure genomic stability, every origin in the cell must replicate only once each cell cycle. This control is managed by large protein complexes assembled at each origin that are required both for an origin to initiate and to monitor whether that origin has

replicated. The formation and activation of these protein complexes is regulated by oscillating kinase activities throughout the cell cycle.

To initiate replication from a particular origin, a pre-Replicative Complex (pre-RC) must first be assembled at the origin. This complex, originally defined by a nuclease protection of the origin *in vivo* (Diffley et al. 1994), consists minimally of ORC, the MCM2-7 complex, which is thought to be the eukaryotic replicative DNA helicase, and Cdc6 and Cdt1, two proteins that are required for recruitment of the MCM2-7 complex to the origin (Bell and Dutta 2002). The precise architecture of these proteins within the pre-RC is unknown.

Pre-RC activation is triggered by two protein kinases, the Cyclin Dependent Kinase (CDK) Cdc28, and the Dbf4 Dependent Kinase (DDK) Cdc7 (Bell and Dutta 2002). The activating subunits of both kinases are synthesized in late G1. DDK activity is necessary throughout S phase to fire individual origins (Donaldson et al. 1998a) and probably associates directly with the origin to trigger initiation locally (Bousset and Diffley 1998; Pasero et al. 1999). In contrast, CDK activity probably triggers initiation at a global level, as it functions prior to S phase entry in yeast (Nougarede et al. 2000), and is probably not associated with the chromatin (Pasero et al. 1999).

After an origin has initiated replication, it is critical that it does not reinitiate before the cell has undergone mitosis, so that each daughter cell inherits exactly one complement of chromosomal DNA. To prevent re-initiation during a single cell cycle, the same CDK activity that activates pre-RCs also prevents reformation of the pre-RC. The pre-RC is dismantled as replication initiates, leaving ORC at the origin forming a complex known as the post-RC (Diffley et al. 1994). The post-RC persists at the origin

after initiation until the next G1 phase. When the cells enter G1, CDK activity is lowered, allowing the formation but not the activation of the pre-RC (Dahmann et al. 1995).

CDK activity prevents pre-RC formation by phosphorylating almost all of the components of the pre-RC. Orc2 and Orc6 are phosphorylated, although the mechanism by which this prevents re-initiation is unknown (Nguyen et al. 2001). Cdc6 phosphorylation leads to its degradation (Calzada et al. 2000; Drury et al. 2000; Perkins et al. 2001). Phosphorylation of the MCM2-7 complex leads to its export from the nucleus once released from the chromatin (Labib et al. 1999; Nguyen et al. 2000). Although Cdt1 itself may not be phosphorylated, Cdt1 is exported with the MCMs (Tanaka and Diffley 2002b). Although the same proteins are regulated by CDK phosphorylation in all organisms, the details are different in different organisms.

Clearly, the regulation of complex formation at origins is critical for ensuring that large eukaryotic genomes are faithfully duplicated. During graduate school I have characterized the cis-acting sequences that allow the pre-RC to form. I have also characterized the proteins in the post-RC and the mechanisms by which protein kinases prevent reformation of the pre-RC and re-replication. In this introduction I will review the structure of origins in eukaryotes, the proteins in the complexes that associate with origins, and the kinases that regulate the formation of these complexes.

Model Origins of DNA replication

To duplicate a large genome in a small amount of time, yeast cells initiate replication from defined origins spread along the chromosome that initiate at defined times throughout S phase. To understand how these sites are chosen, and how initiation is regulated, we must understand the sequences necessary to recruit the protein complexes involved in initiation. We must also understand the structural elements that allow DNA unwinding. In this section I will review the structure of known yeast origins, and how the various components of the pre-RC interact with each other and with the origin DNA.

E. coli- A paradigm for replication control

Studies in *E. coli* have provided a general model for origin function that has established a foundation for understanding origin function in more complex organisms. (reviewed in (Kornberg and Baker 1992)). The single *E. coli* origin, OriC, contains multiple 9mer repeats of the binding site for the initiator protein, DnaA (reviewed in (Messer et al. 2001)). DnaA binds cooperatively to these multiple sites. The origin also contains three AT-rich 13mer repeats, which serve as a DNA Unwinding Element (DUE) (Bramhill and Kornberg 1988a; Kowalski and Eddy 1989). These DNA elements are structurally unstable and are the first site of DNA unwinding. After DnaA multimerizes, ATP-bound DnaA interacts with specific sequences within the 13mers and unwinds the DNA to allow loading of the helicase (DnaB) by the helicase loader (DnaC) (reviewed in (Bramhill and Kornberg 1988b)). Helicase loading is followed by the association of the

primase and polymerases that allow replication forks to begin polymerizing bi-directionally. After initiation, DnaA dissociates from the origin, preventing re-initiation.

S. cerevisiae-Model eukaryotic origins

Despite growing amounts of information, we still do not understand all of the sequence requirements at *S. cerevisiae* origins. The approximate locations of the majority of the 350 or so chromosomal origins have been mapped by two complementary whole-genome DNA microarray analyses (Raghuraman et al. 2001; Wyrick et al. 2001). However, only four of these origins have been dissected in detail (Marahrens and Stillman 1992; Miller and Kowalski 1993; Rao et al. 1994; Huang and Kowalski 1996; Lin and Kowalski 1997). These characterized yeast origins are short, only about 100-150 bp long, and consist of a variable number of modular elements described below (also see Figure 1).

As predicted by the replicon model, the only essential element found at an origin is a binding site for the initiator protein, the Origin Recognition Complex (Bell and Stillman 1992). The ORC binding site matches the ARS Consensus Sequence (ACS), an 11 bp degenerate sequence that forms the essential A element at all yeast origins. Many origins have multiple matches to the ACS, and the actual ORC binding site is not always the best match to the consensus (Theis and Newlon 1997). Therefore, the essential site must be determined by mutational analysis, which has only been done at fifteen origins. One reason that the A element cannot be determined by identifying the best match to the ACS is that additional poorly characterized sequences may contribute to the ORC binding site. ORC protects a much larger region than the A element from nuclease digestion both

in vitro and *in vivo* (Bell and Stillman 1992; Diffley et al. 1994). Other sequences that may aid in ORC binding include a 3' box next to the ACS at some origins, a longer consensus, and a B1 element (described below) that interacts with Orc5 *in vitro* (Rao et al. 1994; Huang and Kowalski 1996; Lee and Bell 1997). It is also possible that other factors besides the DNA sequence help to determine which site ORC binds to *in vivo*, such as local chromatin structure or other replication proteins (Mizushima et al. 2000; Lipford and Bell 2001).

An ORC binding site is not sufficient to define an origin. ORC also binds to other sites in the genome that have additional functions, such as silencers (Bell et al. 1993; Wyrick et al. 2001). The additional sequences that define an origin are collectively known as B elements. These elements are not highly conserved at the sequence level, but are named based on their ability to be functionally interchanged for one another (Rao et al. 1994; Lin and Kowalski 1997). *ARS1*, the best characterized yeast origin, has three B elements, B1, B2, and B3. The other origins that have been mapped in their entirety contain some of the same elements, but in different combinations. For instance, *ARS307* contains A, B1 and B2 elements, but no B3 element (Theis and Newlon 1994). *ARS305* contains an A element, a B1 element, a large region that can functionally swap for the B2 element, and a B4 element that can functionally swap with any of the other B elements (Lin and Kowalski 1997) (Huang and Kowalski 1996).

As in *E.coli*, it has been suggested that one of the B elements, the B2 element, acts as a DNA Unwinding Element (Lin and Kowalski 1997). Calculations of helical stability suggest that this region of the origin is thermodynamically unstable. The B2 element at *ARS1* is only 11 bp, and probably has another function as well, because it

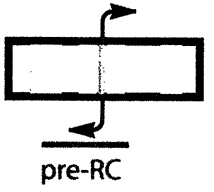
Comparison of Eukaryotic Origins

S. cerevisiae

- average length of origin ~120 bp

-A Element: ARS consensus sequence (ACS), essential; ORC binding site

-B elements: functionally conserved, important sequences

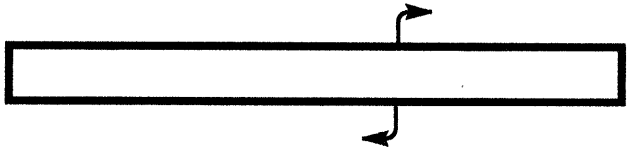


S. Pombe

- average length of origin ~0.5 - 1 kb

- ORC binding site-mutation of this region drastically reduces origin activity

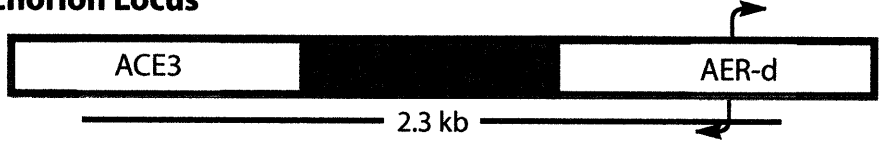
- mutation of this region reduces origin activity



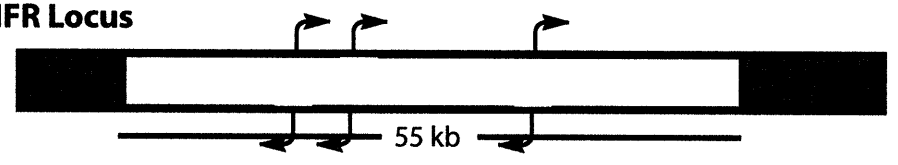
Metazoan

Drosophila - Chorion Locus

ORC binding sites



Hamster - DHFR Locus



Human - Lamin B2

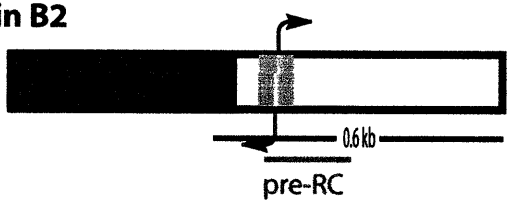


Figure 1. Structure of Replication Origins in Different Organisms. Known ORC binding sites are shown in dark yellow. Known pre-RC forming regions are shown in green. Origins of bi-directional replication are depicted by arrows. The hamster DHFR locus is depicted as an example of a metazoan origin with a large initiation zone, while the human Lamin B2 origin is shown as an example of a metazoan origin with a single initiation site.

cannot be replaced by other easily unwound sequences. At other origins such as *ARS305*, there is no short B2 element, but instead a broad easily unwound region that can swap with the same region of *ARS1*. The *ARS1* B2 element only swaps functionally for this region if its associated AT rich sequences are also included, not in a GC rich context.

Another function of the B elements is to bind transcription factors. The B3 element at *ARS1* is a binding site for Abf1(Diffley and Stillman 1988), and Abf1 binding sites have been found at other origins. However, the transcriptional domain of Abf1 is unnecessary for its origin function (Wiltshire et al. 1997). Instead, binding of Abf1 may help to keep the origin nucleosome-free (Venditti et al. 1994).

S. pombe- Another model eukaryotic origin

The fission yeast *Schizosaccharomyces pombe* is the other organism in which multiple origins have been mapped in some detail. Fission yeast origins are larger than those in *S. cerevisiae*, approximately 0.5 to 1 kb long. *Cerevisiae* and *pombe* origins differ in the mode of ORC binding. *S. pombe* ORC binds to origins through an "AT hook" on spORC4 (Chuang and Kelly 1999; Kong and DePamphilis 2001; Lee et al. 2001), a motif that binds asymmetrical AT tracts. Therefore, *S.pombe* origins do not contain a short essential ACS with a conserved sequence. Instead *S. pombe* origins contain asymmetrical AT tracts that bind spORC *in vitro* and *in vivo* (Kong and DePamphilis 2002; Takahashi et al. 2003). Like *S. cerevisiae* origins, *S. pombe* origins contain more asymmetrical AT tracts than are essential, and the essential tracts utilized *in vivo* must be determined empirically by footprinting and mutational analysis. However,

fission yeast origins appear to have multiple ORC binding sites of varying importance (Kong and DePamphilis 2002; Takahashi et al. 2003).

Four fission yeast origins have been mapped in detail, and they also have multiple short elements that contribute to origin efficiency, but are not ORC binding sites (Clyne and Kelly 1995; Dubey et al. 1996; Kim and Huberman 1998; Kim and Huberman 1999; Okuno et al. 1999). At *ARS3001*, a pre-RC footprint has been observed over an element adjacent to the primary ORC binding site, and the site of initiation of replication mapped to the middle of this region, as in *S. cerevisiae* (Kong and DePamphilis 2002). However, this origin also has a secondary ORC binding site, and another element of unknown function. *Ori2004* also has a site necessary for pre-RC formation adjacent to an ORC binding site, but this element is between two equally important ORC binding sites (Takahashi et al. 2003).

Components of the pre-RC

The formation and activation of the pre-RC controls when and where replication initiates. The regulation of pre-RC assembly throughout the cell cycle varies between organisms and I will discuss these differences in a separate section below. However, the protein components in the pre-RC are conserved in all eukaryotes, and their basic roles in initiating replication appear to be similar in all cases examined (reviewed in (Bell and Dutta 2002)). Below, I will describe their basic activities in budding yeast, referring to other organisms only where there are major differences.

ORC

The Origin Recognition Complex is required for initiation in all eukaryotes, despite large differences in replicator size and structure. ORC is a six subunit complex that binds origins in an ATP-dependent manner (Bell and Stillman 1992). Although all 6 subunits are essential, Orc6 in dispensable for DNA binding in *S. cerevisiae* . ORC remains bound to yeast origins throughout the cell cycle (Diffley et al. 1994; Aparicio et al. 1997; Liang and Stillman 1997; Tanaka et al. 1997). In higher organisms this is not always the case (see below).

Although ORC exhibits sequence conservation with many ATP-dependent molecular machines, no *in vivo* role has yet been determined for ORC beyond binding DNA and serving as a "landing pad" for the pre-RC. Orc1, 4, and 5 are all members of the AAA+ family of ATPases (Neuwald et al. 1999), although only Orc1 and Orc5 bind ATP, and only the Orc1 ATP binding site is necessary for DNA binding (Klemm et al. 1997). ATP hydrolysis is decreased upon ORC binding origin DNA, and increased upon ORC binding ssDNA (Klemm et al. 1997; Lee et al. 2000). Therefore, hydrolysis has been suggested to play a role in disassembly of the pre-RC (Klemm and Bell 2001). A mutation in the *ORC5* ATP binding site causes increased gross chromosomal rearrangements and has therefore been proposed to be involved in maintenance of genome integrity (Huang and Koshland 2003).

Cdc6

Cdc6 is an essential protein necessary for MCM chromatin association (Cocker et al. 1996; Liang and Stillman 1997). Cdc6 is also a member of the AAA+ family of ATPases, and is highly homologous to Orc1. MCM loading requires the Cdc6 ATP

binding motif (Wang et al. 1999; Weinreich et al. 1999; Schepers and Diffley 2001). Mutational analysis has suggested that ATP hydrolysis is also necessary for MCM loading (Donovan et al. 1997; Seki and Diffley 2000). This observation has led to the suggestion that Cdc6 uses the energy associated with ATP hydrolysis to assemble the ring-shaped MCM2-7 complex around the origin DNA. Although CDC6 can only be detected at origins in early G1, its continued presence is required to maintain MCM association with origins until S phase (Donovan et al. 1997; Tanaka et al. 1997) (Cocker et al. 1996; Aparicio et al. 1997).

Cdt1

Cdt1 is a second essential protein required coordinately with Cdc6 for MCM2-7 association with chromatin. Cdt1 was discovered first in *S. pombe*, and thereafter in *Xenopus*, *Drosophila*, and humans (Maiorano et al. 2000; Nishitani et al. 2000; Whittaker et al. 2000; Nishitani et al. 2001). Recently a budding yeast homolog was identified, confirming that all of the known proteins involved in pre-RC formation are conserved throughout eukaryotes (Devault et al. 2002; Tanaka and Diffley 2002b). Cdt1 association with the origin has not yet been identified in budding yeast, and may be transient, but it is associated with chromatin in fission yeast and higher eukaryotes. Therefore, it is unclear whether Cdt1 contributes to the architecture of the pre-RC, or is just needed briefly to form the pre-RC.

MCMs

The six MCM2-7 proteins are related, conserved throughout evolution, and essential for both the initiation and elongation stages of DNA replication (Labib et al. 2000). Consistent with their involvement in both initiation and elongation, after associating with origins during G1 to form the pre-RC, the MCMs travel with the replication fork after initiation, and slowly dissociate from chromatin (Aparicio et al. 1997). All six are also members of the AAA+ ATPase family, and the ATP binding motifs are essential (Schwacha and Bell 2001).

The essential role of the MCMs at the replication fork suggests that they act as the eukaryotic replicative helicase, even though strong helicase activity has yet to be demonstrated for the intact MCM2-7 complex (reviewed in (Labib and Diffley 2001)). Weak helicase activity has been shown to be associated with a subcomplex of MCMs4/6/and 7derived from human and fission yeast cells (Ishimi 1997; Chong et al. 2000; Lee and Hurwitz 2000; Lee and Hurwitz 2001). EM studies of the MCMs from fission yeast suggest that they form a doughnut-like complex with a central cavity that could accommodate DNA (Adachi et al. 1997). In contrast to the weak helicase activity demonstrated in eukaryotic cells, a related archaeal MCM protein displays processive helicase activity and acts as a double-hexamer (Kelman et al. 1999; Chong et al. 2000).

Architecture of the pre-RC

Data at *ARS1* suggests that the B2 element acts in concert with the bipartite ORC binding site of the A and B1 element to load the pre-RC. The pre-RC protects a region including the A to B2 elements of *ARS1* from nuclease digestion *in vivo* (Santocanale and Diffley 1996). Deletion of the B2 element at *ARS1* reduces MCM loading at the origin *in*

vivo as assayed by chromatin Immunoprecipitation (Zou and Stillman 2000; Lipford and Bell 2001). The origin of bi-directional replication, where the first base pairs of DNA are synthesized, has been mapped to a point between the B1 and B2 elements (Bielinsky and Gerbi 1999).

ORC is the only pre-RC component with defined DNA binding activity. However, it is possible that other pre-RC proteins interact with specific DNA sequences that have yet to be defined. The crystal structure of an archael Cdc6 protein has been solved and also contains a winged helix domain similar to DNA binding folds (Liu et al. 2000). However, only non-specific DNA binding properties have been reported for Cdc6, and they are mediated through the non-essential regulatory N terminus (Feng et al. 2000). Mouse Cdt1 has also been reported to have nonspecific DNA binding activity (Yanagi et al. 2002).

Protein-protein interactions may recruit some members of the pre-RC to the origin. Cdc6, for instance, interacts strongly with Orc1 (Wang et al. 1999; Mizushima et al. 2000). Cdt1 in *S. pombe* interacts with Cdc6 (Nishitani et al. 2000). The MCMs can remain associated with the DNA even if all of the rest of the members of the pre-RC are salt-extracted, suggesting that stable association with the origin does not require protein-protein interactions with known factors (Donovan et al. 1997). This may, however, represent topological linkage between the DNA and the MCMs. It is possible that protein-protein interactions could be necessary to initially recruit the MCMs to the origin.

Metazoan Origins

Very few replication origins have been identified in higher organisms, but those that have been identified tend to be much larger than those in yeast (reviewed in (Bogan et al. 2000; Gilbert 2001)) (see Figure 1). This, combined with the larger size of metazoan centromeres, has hampered efforts to identify them simply by their ability to confer autonomous replication on plasmids, as yeast origins were first identified. Different methods to identify origins have yielded contradictory results as to whether higher origins initiate in specific places, as in *S. cerevisiae*, or in broader initiation zones. Various sequence elements have been implicated as possibly contributing to higher origin function, including DUEs, matches to the yeast ACS, bent DNA, sequences that position nucleosomes, transcription factor binding sites, and matrix attachment regions (reviewed in (DePamphilis 1999))(Liu et al. 2003). Therefore, many of the sequence elements identified in yeast origins may act at metazoan origins as well.

Specific ORC binding sites have been mapped at a few well-characterized metazoan origins. Those that are easiest to study are in regions of DNA that are amplified during development. One well-studied origin in *Drosophila* is the chorion gene amplification locus. *Drosophila* ORC binds both *in vitro* and *in vivo* to multiple sites within this locus including the approximately 400 base pair Amplification Control Element (*ACE3*), and to sites within the flanking non-essential amplification enhancing element (*AER-d*)(Austin et al. 1999). Interestingly, when multiple copies of *ACE3* are moved to an ectopic location, ORC binds to *ACE3* and to nearby sites. Therefore, as in *S. pombe*, *Drosophila* origins may contain multiple ORC binding sites, or bind multimers of ORC. ORC from the fly *Sciara coprophila* has also been mapped binding to a specific 80 bp sequence at an amplification element, and a single replication initiation point has

been mapped immediately adjacent to the ORC binding site (Bielinsky et al. 2001). Despite these reports of specific ORC binding *in vitro* and *in vivo*, no specific binding site beyond AT-richness has been reported for dmORC (Chesnokov et al. 2001). A recent report also states that HsORC has no DNA binding specificity in the Xenopus *in vitro* replication system (Vashee et al. 2003). However, others have reported sequence specific DNA footprinting *in vitro* by human ORC (Stefanovic et al. 2003). As has been suggested in the yeasts, there may be proteins that help to localize ORC in higher eukaryotes (Beall et al. 2002) (Royzman et al. 1999; Bosco et al. 2001).

Changing protein complexes have been mapped over one human origin, near the human lamin B2 gene, which are reminiscent of the pre- and post-RC complexes in budding yeast (Dimitrova et al. 1996; Abdurashidova et al. 1998). The 110 bp G1 complex contains at least ORC1/2, Cdc6, and the MCMs and therefore seems comparable with the pre-RC, while the 70 bp S phase complex contains ORC2, consistent with a post-RC containing ORC, and with the established release of ORC1 from chromatin after firing (see below) (Abdurashidova et al. 2003). Finally, in mitosis, there is no protection over the origin, consistent with the release of ORC from chromatin in metazoan mitotic cells (see below). The start site of DNA synthesis has also been mapped to a single nucleotide at this origin, just as in origins in the yeasts and *sciara*, suggesting that there are origins that do not fire in initiation zones (Abdurashidova et al. 2000).

Because of the large size of metazoan origins, mapping of individual sequences necessary for function has been difficult. Various sequence elements have been implicated as possibly contributing to higher origin function, including DUES, matches

to the yeast ACS, bent DNA, sequences that position nucleosomes, transcription factor binding sites, and matrix attachment regions(reviewed in (DePamphilis 1999))(Liu et al. 2003).

Regulation of Initiation

Initiation of DNA replication is activated by the coordinate action of two protein kinases, which allows control over initiation at both a local and a global level. At a local level, an S phase specific kinase, the Dbf4 dependent kinase (DDK) Cdc7, provides a mechanism to control the firing of origins in a defined temporal pattern. At a global level, activity of the Cyclin Dependent Kinase (CDK) Cdc28 drives all cell cycle events. This includes ensuring that every S phase is separated by an intervening M phase. In this section I will describe in more detail how each of these kinases activates DNA replication, and in the next section I will describe how the CDK prevents re-initiation within a single cell cycle.

Local Kinase Activity-DDK

The Cdc7 protein kinase and its activating subunit Dbf4 are required throughout S phase to fire individual replication origins (Bousset and Diffley 1998; Donaldson et al. 1998a). Cdc7 binds to chromatin throughout the cell cycle, whereas Dbf4 is a cell cycle regulated protein that is synthesized at the end of G1 phase, and then is degraded at the metaphase to anaphase transition by the Anaphase Promoting Complex(APC) (Weinreich and Stillman 1999; Ferreira et al. 2000; Nougarede et al. 2000). In yeast, Dbf4 is

probably recruited to the origin at the time of initiation through interactions with ORC (Dowell et al. 1994; Pasero et al. 1999; Ferreira et al. 2000), although Cdc7 chromatin association in *Xenopus* requires the MCM proteins, and not ORC (Jares and Blow 2000; Walter 2000).

Although the *in vivo* targets of DDK activity and the specific phosphorylation sites have not been identified, Mcm2 is most likely an essential substrate for initiation. Mcm2 is the best *in vitro* substrate of Cdc7/Dbf4 (Brown and Kelly 1998). Genetic studies also support the idea that *MCM2* is an essential target of DDK activity, as a *DBF4* mutation can suppress an *MCM2* mutation defective in replication initiation (Lei et al. 1997). Further evidence for the MCM2-7 complex as a DDK target is provided by an allele of *MCM5* that bypasses the requirement for DDK activity altogether (Hardy et al. 1997).

Global Kinase Activity-CDK

Cyclin dependent kinase activity is important for driving all major cell cycle transitions, including initiating DNA replication. CDK activity is controlled by the association of the CDK (or CDKs) with various cyclin partners throughout the cell cycle. In budding yeast the single CDK, Cdc28, is activated by association with any of three different G1 cyclins, or Clns, and six different B-type Cyclins, or Clbs. Most regulation of DNA replication involves the Clbs (Schwob et al. 1994).

Although there is significant redundancy amongst the B-type cyclins, the S phase Clbs, Clb5 and Clb6, usually initiate replication, whereas Clbs 1-4 are specialized for progression through mitosis (reviewed in (Miller and Cross 2001)). Clb5 and 6 levels

rise at the G1 to S transition, Clbs 3 and 4 rise at the end of S phase, and Clbs 1 and 2 rise at the onset of mitosis. Although Clb5 and Clb6 are co-regulated and are very similar in sequence, Clb5 is the more abundant S phase cyclin. Clb5 is able to support initiation from all origins in the absence of Clb6, whereas Clb6 is only able to support initiation from origins that fire early in S phase on its own (Donaldson et al. 1998b). However, if Clb5 and Clb6 are both deleted, the replication program is carried out with normal timing of both early and late origins later in the cell cycle when Clb3 and 4 levels rise. Therefore, although the three pairs of cyclins are specialized to an extent, other cyclins may be able to substitute if their levels are high enough (reviewed in (Roberts 1999)).

Although many CDK targets have been identified, only one so far has proven to be essential for initiation, Sld2 (Masumoto et al. 2002). When all of the CDK phosphorylation sites on Sld2 are mutated, replication initiation is blocked. Phosphorylated Sld2 forms a complex with Dpb11, a protein associated with DNA polymerase epsilon (Kamimura et al. 1998). Overexpression of Dpb11 suppresses the replication defect of the Sld2 phosphorylation site mutant, suggesting that Sld2 phosphorylation regulates formation of this complex to promote initiation (Masumoto et al. 2002).

Regulation of Re-initiation

Concomitant with activation of pre-RCs upon S phase entry, CDK activity also prevents pre-RC reformation. This dual function of CDKs links pre-RC formation to the cell cycle regulated oscillation of B type CDK activity, thereby ensuring that cells must divide before replicating their DNA again. CDK activity prevents premature reformation

of the pre-RC in all organisms, although the mechanisms of regulation, and the ease in which these mechanisms can be subverted differ. CDKs phosphorylate all or most of the members of the pre-RC to regulate their activity. CDKs also interact stably with a subset of pre-RC components in most organisms. The significance of these interactions is less clear, but they may add a local level of regulation to the control over initiation by CDKs. Below I will describe how CDK activity regulates the pre-RC in different organisms (summarized in Table 1).

S. cerevisiae

The strongest evidence that CDK activity controls the replication cycle is that interfering with CDK activity can reset the entire cycle. If CDK activity is transiently inhibited by expression of the CDK inhibitor Sic1 that is normally expressed only in G1, yeast cells will reform pre-RCs when Sic1 is present. When Sic1 is removed again, those pre-RCs are activated and the cells undergo an extra discrete round of DNA replication (Dahmann et al. 1995). These cells have simply skipped M phase, as evidenced by their rebudding.

CDKs prevent re-initiation by at least three mechanisms: phosphorylation of ORC, Cdc6, and the MCMs (Nguyen et al. 2001). The mechanism by which ORC phosphorylation, on Orc2 and Orc6, prevents re-replication is unknown. Phosphorylation of Cdc6 targets it for degradation by the SCF protein complex (Drury et al. 1997; Calzada et al. 2000; Drury et al. 2000; Jang et al. 2001; Perkins et al. 2001). CDK dependent phosphorylation of the MCMs causes their nuclear export, although only after they are

ORGANISM:	ORC:	Cdc6:	Cdt1:	MCM2-7:	CDK-independent:
S. cerevisiae	Orc2/6-?	Yes-Degradation	?- Exported with MCMs	Yes-Nuclear Localization	
S. pombe	Orc2-?	Yes-Degradation	Yes-Degradation	Yes-? constitutively nuclear	
Metazoans:					
H. sapiens	Orc1/2-Orc1 release or proteolysis	Yes-Nuclear Localization (some ?)	Yes-Degradation	Yes-? inhibits *in vitro* helicase, constitutively nuclear	Geminin inhibits Cdt1
Hamster	Orc1/2-Orc1 release or proteolysis	Yes-Nuclear Localization (some ?)	Yes-Degradation	Yes-? constitutively nuclear	Geminin inhibits Cdt1
D. melanogaster			Yes-Degradation		Geminin inhibits Cdt1
C. elegans			Yes-Degradation		Geminin inhibits Cdt1
X. laevis	Orc1/2-?		Yes-Degradation	Yes-? Chromatin release, constitutively nuclear, but controlled by export machinery	Geminin inhibits Cdt1

Table 1. CDK Control of pre-RC components in different organisms. For each pre-RC component, whether they are subject to CDK-dependent phosphorylation to prevent re-replication, and the molecular consequences of this phosphorylation are presented. See text for references.

released from chromatin (Labib et al. 1999; Nguyen et al. 2000). Cdt1 nuclear import is coordinated with MCM nuclear import (Tanaka and Diffley 2002b).

Induction of re-replication in *S. cerevisiae* requires disruption of all three mechanisms mentioned above (Nguyen et al. 2001). When ORC, Cdc6, and the MCM complex are all deregulated from cyclin control, cells re-replicate when arrested in M phase with high CDK levels. This is in contrast to the complete cell cycle resetting imposed by transient Sic1 expression. However, only some origins reinitiate, and the cells only re-replicate approximately half of the genome.

Although most CDK regulation over replication is mediated by Clb-Cdc28 activity, Cln-Cdc28 activity at the end of G1 has recently been implicated in regulating pre-RC formation at this vulnerable time as cells enter S phase (Tanaka and Diffley 2002a). Cln-Cdc28 kinase activity phosphorylates Cdc6 to target it for particularly rapid degradation as the cells enter S phase, and Cln-mediated phosphorylation can also target the MCMs for nuclear export (Tanaka and Diffley 2002a). In contrast to Cdc6 and the MCMs, ORC is not phosphorylated by the Clns, but only by the Clbs (Nguyen et al. 2001; Weinreich et al. 2001).

Both ORC and Cdc6 may interact directly with the cyclins. ORC ColPs with Clb/Cdc28 complexes when co-expressed in insect cells (Weinreich et al. 2001). Cdc6 also interacts with the cylins through a motif in its nonessential regulatory N terminus, and may help regulate CDK activity at the exit from mitosis (Elsasser et al. 1996; Calzada et al. 2001; Weinreich et al. 2001). The significance of these interactions is currently unclear, but the stability suggests a particularly intimate connection between CDKs and pre-RC components that may be important for regulating pre-RC formation.

CDK control over re-replication in yeast is highly redundant, and still more mechanisms may exist that we have not yet discovered. CDKs appear to regulate the function of all of the members of the pre-RC to ensure that no pre-RCs are formed after replication has initiated. It is also possible that there are mechanisms to prevent activation of any pre-RC that forms.

S. pombe

Re-replication in *S. pombe* can also be induced by inhibition of CDK activity (Broek et al. 1991; Hayles et al. 1994; Correa-Bordes and Nurse 1995). However, inhibition of the mitotic cyclin induces multiple rounds of endoreduplication of the genome. This is similar to the rounds of endoreduplication that are a part of the normal life cycle of some cells during development, which is also accompanied by down-regulation of the mitotic cyclin (reviewed in (Edgar and Orr-Weaver 2001)).

There is evidence for CDK control in *S. pombe* over all of the factors regulated in *S. cerevisiae*: ORC, Cdc6, Cdt1, and the MCMs. The role of ORC phosphorylation in preventing re-replication is also unclear in *S. pombe.* Orc2 is phosphorylated by the G2/M cyclin Cdc13/Cdc2 *in vitro* and *in vivo* (Lygerou and Nurse 1999; Vas et al. 2001). Phosphorylation of the Cdc6 homolog Cdc18 leads to ubiquitin-mediated proteolysis via the SCF pathway (Jallepalli et al. 1997; Lopez-Girona et al. 1998), as in *S. cerevisiae.* Cdt1 abundance parallels that of Cdc18 in *S. pombe* and is also controlled by CDK phosphorylation in a manner similar to Cdc18 (Nishitani et al. 2000)((Kelly et al. 1993) The effect of MCM phosphorylation in S. pombe is unclear. MCMs are phosphorylated by CDK activity, but the specific sites have not been identified (Coue et al. 1996). The

MCMs cannot be regulated by nuclear export as in *S. cerevisiae* because they are constitutively nuclear (Okishio et al. 1996; Sherman and Forsburg 1998; Pasion and Forsburg 1999).

Although all of the same factors are regulated in *S. pombe* as in *S. cerevisiae*, M phase re-replication is much easier to induce in *S. pombe*. Overexpression of Cdc18, or co-overexpression of Cdc18 and Cdt1 are sufficient to induce a large amount of DNA re-replication in M phase, as much as 64n (Nishitani and Nurse 1995; Muzi Falconi et al. 1996; Jallepalli et al. 1997; Gopalakrishnan et al. 2001; Yanow et al. 2001). Mutation of Cdt1 or Cdc6 CDK phosphorylation sites also results in increased re-replication in sensitized backgrounds, confirming a direct connection between CDK phosphorylation and re-replication (Gopalakrishnan et al. 2001). However, Cdc18 must be highly overexpressed, not simply deregulated from CDK control to induce re-replication on its own (Jallepalli et al. 1997; Gopalakrishnan et al. 2001). It has been suggested that Cdc18 overexpression may function partly by inhibiting cyclin activity by interfering with the proteolysis machinery (Wolf et al. 1999). Mutation of the ORC CDK phosphorylation sites *in vivo* also results in increased re-replication in a sensitized strain background including stabilized and/or over-expressed Cdc18 (Vas et al. 2001).

The endoreduplication cycles in S. pombe are regulated by a stable interaction between Cdc13/Cdc2 and spOrc2 (Leatherwood et al. 1996; Wuarin et al. 2002). Cdc13 binds origins during M phase to prevent endoreduplication (Wuarin et al. 2002). Cdc18 also interacts strongly with Cdc2, but the function of this interaction is unclear (Brown et al. 1997; Lopez-Girona et al. 1998).

Metazoans

Less in known about CDK prevention of re-replication in higher organisms, but the picture is emerging that the same pre-RC factors are regulated by CDK phosphorylation, if sometimes by novel mechanisms. As in the yeasts, inhibition of the mitotic CDK activity can be sufficient to induce re-replication (Itzhaki et al. 1997; Bates et al. 1998).

Orc1 and Orc2 are phosphorylated in higher organisms, and this phosphorylation probably leads to release of Orc1 from the complex. However, technical and species differences have led to varying reports (reviewed in (DePamphilis 2003)). Both human and hamster ORC have been reported to undergo phosphorylation dependent ubiquitination, which mediates either its release from the DNA or its proteolysis (Kreitz et al. 2001; Mendez et al. 2002) (Natale et al. 2000; Li and DePamphilis 2002). In humans, *Drosophila*, and *Xenopus* ORC also dissociates completely from DNA from M phase through G1 phase (Coleman et al. 1996; Romanowski et al. 1996; Hua and Newport 1998; Rowles et al. 1999; Sun et al. 2002; Abdurashidova et al. 2003). However, it is unclear whether this regulation of ORC chromatin binding is necessary for prevention of re-replication or for chromatin condensation during mitosis.

Cdc6 is stable in higher organisms, unlike in the yeasts, but its function is controlled mainly by nuclear localization. CDK phosphorylation leads to nuclear export of human Cdc6 (Saha et al. 1998; Jiang et al. 1999; Petersen et al. 1999; Pelizon et al. 2000; Delmolino et al. 2001). However, some reports find that at least some Cdc6 is associated with chromatin throughout the cell cycle in hamsters and humans (Coverley et al. 2000; Okuno et al. 2001).

Cdt1 is regulated by proteolysis, as in fission yeast (Wohlschlegel et al. 2000; Nishitani et al. 2001) (Whittaker et al. 2000; Zhong et al. 2003). This degradation is dependent on the Cul-4 ubiquitin ligase in *C. elegans*, as inhibition of this gene by RNAi causes massive Cdt1-dependent re-replication in developing cells (Zhong et al. 2003). Although the mechanisms targeting Cdt1 for degradation are unknown in metazoans, the timing of degradation suggests that it is likely through CDK mediated phosphorylation, again as in *S. pombe*.

CDK phosphorylation clearly regulates function of the MCM2-7 complex in metazoans, although the precise mechanism is unclear. MCM phosphorylation may directly inhibit MCM activity, as MCM4 phosphorylation by Cdk2/Cyclin A inhibits *in vitro* helicase activity of MCM4/6/7 complexes purified from HeLa cells (Ishimi et al. 2000; Ishimi and Komamura-Kohno 2001). However, this phosphorylation also inhibits the ssDNA binding activity, and could have an effect on dsDNA binding activity, so the mechanism of inhibition is unclear. In *Xenopus*, MCM4 is highly phosphorylated during M phase by Cdc2/Cyclin B (Hendrickson et al. 1996; Pereverzeva et al. 2000). This phosphorylation correlates with release from the chromatin and has been suggested to prevent chromatin association (Hendrickson et al. 1996) (Coue et al. 1996; Findeisen et al. 1999; Pereverzeva et al. 2000).

The MCMs are constitutively nuclear in metazoans (Mendez and Stillman 2000; Okuno et al. 2001; Yamaguchi and Newport 2003), but the nuclear export machinery may still regulate their activity (Yamaguchi and Newport 2003). A recent study in frog egg extracts suggests that the nuclear export machinery may inhibit MCM loading onto chromatin in a CDK dependent manner, simply by sequestering the MCMs, not exporting

them. These export proteins are the same ones, Ran-GTP and Crm1, implicated in CDK dependent export of HsCdc6, and possibly HsORC, from the nucleus (Jiang et al. 1999; Delmolino et al. 2001; Laman et al. 2001).

Unlike in *S. pombe*, re-replication cannot be induced by simple over-expression of Cdc6 or Cdt1. However, in human cancer cells, in which the p53 dependent checkpoint has been inactivated, re-replication can be induced by over-expression of Cdc6 or Cdt1, suggesting that checkpoints are yet another mechanism for preventing or repairing DNA damage due to re-replication (Vaziri et al. 2003).

Metazoans also have a CDK-independent mechanism for controlling re-replication, the Cdt1 inhibitor protein geminin (Wohlschlegel et al. 2000; Tada et al. 2001). Geminin is present from S phase through M phase, and is degraded by the Anaphase Promoting Complex (APC) at the metaphase to anaphase transition (McGarry and Kirschner 1998). Simple repression of geminin can be sufficient to induce re-replication in *Drosophila* cells (Quinn et al. 2001). However, in other systems, deletion of geminin is not sufficient, such as in *Xenopus* extracts (McGarry 2002).

ORC and Cdc6 also interact with the cyclins for unknown reasons in higher organims. Xenopus and human ORC both interact stably with cyclins (Romanowski et al. 2000; Mendez et al. 2002). Human Cdc6 also interacts physically with Cyclin A/CDK2 via a Cy (or RXL) motif in the N terminus, and this interaction may simply ensure that Cdc6 is a particularly good substrate for the CDK (Petersen et al. 1999; Delmolino et al. 2001) (Saha et al. 1998).

Thesis Summary

In this thesis, I have characterized the protein complexes that regulate initiation of DNA replication. In chapter two, I describe experiments characterizing the cis acting sequence requirements of an origin of replication. I have found that the *ARS1* B2 element requires A rich DNA on one strand to direct efficient MCM loading. Moreover, functional B2 sequences resemble the binding site for ORC, although they do not bind ORC well *in vitro*. These experiments suggest that this element is not simply a DNA unwinding element, and that a member of the pre-RC might bind specific sequences at the origin. In chapter three, I describe experiments suggesting that an RXL domain on Orc6 interacts stably with a hydrophobic patch on the S phase cyclin Clb5, recruiting Clb5 to the origin after initiation. This interaction prevents re-replication of the genome in M phase in a sensitized strain background. Together, these studies help shed light on the architecture of both the pre-RC and the post-RC at origins of replication throughout the cell cycle.

References

Abdurashidova, G., M.B. Danailov, A. Ochem, G. Triolo, V. Djeliova, S. Radulescu, A. Vindigni, S. Riva, and A. Falaschi. 2003. Localization of proteins bound to a replication origin of human DNA along the cell cycle. *Embo J* **22**: 4294-4303.

Abdurashidova, G., M. Deganuto, R. Klima, S. Riva, G. Biamonti, M. Giacca, and A. Falaschi. 2000. Start sites of bidirectional DNA synthesis at the human lamin B2 origin. *Science* **287**: 2023-6.

Abdurashidova, G., S. Riva, G. Biamonti, M. Giacca, and A. Falaschi. 1998. Cell cycle modulation of protein-DNA interactions at a human replication origin. *Embo J* **17**: 2961-9.

Adachi, Y., J. Usukura, and M. Yanagida. 1997. A globular complex formation by Nda1 and the other five members of the MCM protein family in fission yeast. *Genes Cells* **2**: 467-79.

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

Austin, R.J., T.L. Orr-Weaver, and S.P. Bell. 1999. Drosophila ORC specifically binds to ACE3, an origin of DNA replication control element. *Genes Dev* **13**: 2639-49.

Bates, S., K.M. Ryan, A.C. Phillips, and K.H. Vousden. 1998. Cell cycle arrest and DNA endoreduplication following p21Waf1/Cip1 expression. *Oncogene* **17**: 1691-703.

Beall, E.L., J.R. Manak, S. Zhou, M. Bell, J.S. Lipsick, and M.R. Botchan. 2002. Role for a Drosophila Myb-containing protein complex in site-specific DNA replication. *Nature* **420**: 833-7.

Bell, S.P. and A. Dutta. 2002. DNA replication in eukaryotic cells. *Annu Rev Biochem* **71**: 333-74.

Bell, S.P., R. Kobayashi, and B. Stillman. 1993. Yeast origin recognition complex functions in transcription silencing and DNA replication. *Science* **262**: 1844-9.

Bell, S.P. and B. Stillman. 1992. ATP-dependent recognition of eukaryotic origins of DNA replication by a multiprotein complex. *Nature* **357**: 128-34.

Bielinsky, A.K., H. Blitzblau, E.L. Beall, M. Ezrokhi, H.S. Smith, M.R. Botchan, and S.A. Gerbi. 2001. Origin recognition complex binding to a metazoan replication origin. *Curr Biol* **11**: 1427-31.

Bielinsky, A.K. and S.A. Gerbi. 1999. Chromosomal ARS1 has a single leading strand start site. *Mol Cell* **3**: 477-86.

Bogan, J.A., D.A. Natale, and M.L. Depamphilis. 2000. Initiation of eukaryotic DNA replication: conservative or liberal? *J Cell Physiol* **184**: 139-50.

Bosco, G., W. Du, and T.L. Orr-Weaver. 2001. DNA replication control through interaction of E2F-RB and the origin recognition complex. *Nat Cell Biol* **3**: 289-95.

Bousset, K. and J.F. Diffley. 1998. The Cdc7 protein kinase is required for origin firing during S phase. *Genes Dev* **12**: 480-90.

Bramhill, D. and A. Kornberg. 1988a. Duplex opening by dnaA protein at novel sequences in initiation of replication at the origin of the E. coli chromosome. *Cell* **52**: 743-55.

-. 1988b. A model for initiation at origins of DNA replication. *Cell* **54**: 915-8.

Broek, D., R. Bartlett, K. Crawford, and P. Nurse. 1991. Involvement of p34cdc2 in establishing the dependency of S phase on mitosis. *Nature* **349**: 388-93.

Brown, G.W., P.V. Jallepalli, B.J. Huneycutt, and T.J. Kelly. 1997. Interaction of the S phase regulator cdc18 with cyclin-dependent kinase in fission yeast. *Proc Natl Acad Sci U S A* **94**: 6142-7.

Brown, G.W. and T.J. Kelly. 1998. Purification of Hsk1, a minichromosome maintenance protein kinase from fission yeast. *J Biol Chem* **273**: 22083-90.

Calzada, A., M. Sacristan, E. Sanchez, and A. Bueno. 2001. Cdc6 cooperates with Sic1 and Hct1 to inactivate mitotic cyclin-dependent kinases. *Nature* **412**: 355-8.

Calzada, A., M. Sanchez, E. Sanchez, and A. Bueno. 2000. The stability of the Cdc6 protein is regulated by cyclin-dependent kinase/cyclin B complexes in Saccharomyces cerevisiae. *J Biol Chem* **275**: 9734-41.

Chesnokov, I., D. Remus, and M. Botchan. 2001. Functional analysis of mutant and wild-type Drosophila origin recognition complex. *Proc Natl Acad Sci U S A* **98**: 11997-2002.

Chong, J.P., M.K. Hayashi, M.N. Simon, R.M. Xu, and B. Stillman. 2000. A double-hexamer archaeal minichromosome maintenance protein is an ATP- dependent DNA helicase. *Proc Natl Acad Sci U S A* **97**: 1530-5.

Chuang, R.Y. and T.J. Kelly. 1999. The fission yeast homologue of Orc4p binds to replication origin DNA via multiple AT-hooks. *Proc Natl Acad Sci U S A* **96**: 2656-61.

Clyne, R.K. and T.J. Kelly. 1995. Genetic analysis of an ARS element from the fission yeast Schizosaccharomyces pombe. *Embo J* **14**: 6348-6357.

Cocker, J.H., S. Piatti, C. Santocanale, K. Nasmyth, and J.F. Diffley. 1996. An essential role for the Cdc6 protein in forming the pre-replicative complexes of budding yeast. *Nature* **379**: 180-2.

Coleman, T.R., P.B. Carpenter, and W.G. Dunphy. 1996. The Xenopus Cdc6 protein is essential for the initiation of a single round of DNA replication in cell-free extracts. *Cell* **87**: 53-63.

Correa-Bordes, J. and P. Nurse. 1995. p25rum1 orders S phase and mitosis by acting as an inhibitor of the p34cdc2 mitotic kinase. *Cell* **83**: 1001-9.

Coue, M., S.E. Kearsey, and M. Mechali. 1996. Chromotin binding, nuclear localization and phosphorylation of Xenopus cdc21 are cell-cycle dependent and associated with the control of initiation of DNA replication. *Embo J* **15**: 1085-97.

Coverley, D., C. Pelizon, S. Trewick, and R.A. Laskey. 2000. Chromatin-bound Cdc6 persists in S and G2 phases in human cells, while soluble Cdc6 is destroyed in a cyclin A-cdk2 dependent process. *J Cell Sci* **113**: 1929-38.

Dahmann, C., J.F. Diffley, and K.A. Nasmyth. 1995. S-phase-promoting cyclin-dependent kinases prevent re-replication by inhibiting the transition of replication origins to a pre-replicative state. *Curr Biol* **5**: 1257-69.

Delmolino, L.M., P. Saha, and A. Dutta. 2001. Multiple mechanisms regulate subcellular localization of human CDC6. *J Biol Chem* **276**: 26947-54.

DePamphilis, M.L. 1999. Replication origins in metazoan chromosomes: fact or fiction? *Bioessays* **21**: 5-16.

-. 2003. The 'ORC cycle': a novel pathway for regulating eukaryotic DNA replication. *Gene* **310**: 1-15.

Deshpande, A.M. and C.S. Newlon. 1992. The ARS consensus sequence is required for chromosomal origin function in Saccharomyces cerevisiae. *Mol Cell Biol* **12**: 4305-13.

Devault, A., E.A. Vallen, T. Yuan, S. Green, A. Bensimon, and E. Schwob. 2002. Identification of Tah11/Sid2 as the ortholog of the replication licensing factor Cdt1 in Saccharomyces cerevisiae. *Curr Biol* **12**: 689-94.

Diffley, J.F., J.H. Cocker, S.J. Dowell, and A. Rowley. 1994. Two steps in the assembly of complexes at yeast replication origins in vivo. *Cell* **78**: 303-16.

Diffley, J.F. and B. Stillman. 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**: 2120-4.

Dimitrova, D.S., M. Giacca, F. Demarchi, G. Biamonti, S. Riva, and A. Falaschi. 1996. In vivo protein-DNA interactions at human DNA replication origin. *Proc Natl Acad Sci U S A* **93**: 1498-503.

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

Donaldson, A.D., M.K. Raghuraman, K.L. Friedman, F.R. Cross, B.J. Brewer, and W.L. Fangman. 1998b. CLB5-dependent activation of late replication origins in S. cerevisiae. *Mol Cell* **2**: 173-82.

Donovan, S., J. Harwood, L.S. Drury, and J.F. Diffley. 1997. Cdc6p-dependent loading of Mcm proteins onto pre-replicative chromatin in budding yeast. *Proc Natl Acad Sci U S A* **94**: 5611-6.

Dowell, S.J., P. Romanowski, and J.F. Diffley. 1994. Interaction of Dbf4, the Cdc7 protein kinase regulatory subunit, with yeast replication origins in vivo. *Science* **265**: 1243-6.

Drury, L.S., G. Perkins, and J.F. Diffley. 1997. The Cdc4/34/53 pathway targets Cdc6p for proteolysis in budding yeast. *Embo J* **16**: 5966-76.

-. 2000. The cyclin-dependent kinase Cdc28p regulates distinct modes of Cdc6p proteolysis during the budding yeast cell cycle. *Curr Biol* **10**: 231-40.

Dubey, D.D., S.M. Kim, I.T. Todorov, and J.A. Huberman. 1996. Large, complex modular structure of a fission yeast DNA replication origin. *Curr Biol* **6**: 467-73.

Edgar, B.A. and T.L. Orr-Weaver. 2001. Endoreplication cell cycles: more for less. *Cell* **105**: 297-306.

Elsasser, S., F. Lou, B. Wang, J.L. Campbell, and A. Jong. 1996. Interaction between yeast Cdc6 protein and B-type cyclin/Cdc28 kinases. *Mol Biol Cell* **7**: 1723-35.

Feng, L., B. Wang, B. Driscoll, and A. Jong. 2000. Identification and characterization of Saccharomyces cerevisiae Cdc6 DNA-binding properties. *Mol Biol Cell* **11**: 1673-85.

Ferreira, M.F., C. Santocanale, L.S. Drury, and J.F. Diffley. 2000. Dbf4p, an essential S phase-promoting factor, is targeted for degradation by the anaphase-promoting complex. *Mol Cell Biol* **20**: 242-8.

Findeisen, M., M. El-Denary, T. Kapitza, R. Graf, and U. Strausfeld. 1999. Cyclin A-dependent kinase activity affects chromatin binding of ORC, Cdc6, and MCM in egg extracts of Xenopus laevis. *Eur J Biochem* **264**: 415-26.

Gilbert, D.M. 2001. Making sense of eukaryotic DNA replication origins. *Science* **294**: 96-100.

Gopalakrishnan, V., P. Simancek, C. Houchens, H.A. Snaith, M.G. Frattini, S. Sazer, and T.J. Kelly. 2001. Redundant control of rereplication in fission yeast. *Proc Natl Acad Sci U S A* **98**: 13114-9.

Hardy, C.F., O. Dryga, S. Seematter, P.M. Pahl, and R.A. Sclafani. 1997. mcm5/cdc46-bob1 bypasses the requirement for the S phase activator Cdc7p. *Proc Natl Acad Sci U S A* **94**: 3151-5.

Hayles, J., D. Fisher, A. Woollard, and P. Nurse. 1994. Temporal order of S phase and mitosis in fission yeast is determined by the state of the p34cdc2-mitotic B cyclin complex. *Cell* **78**: 813-22.

Hendrickson, M., M. Madine, S. Dalton, and J. Gautier. 1996. Phosphorylation of MCM4 by cdc2 protein kinase inhibits the activity of the minichromosome maintenance complex. *Proc Natl Acad Sci U S A* **93**: 12223-8.

Hua, X.H. and J. Newport. 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**: 271-81.

Huang, D. and D. Koshland. 2003. Chromosome integrity in Saccharomyces cerevisiae: the interplay of DNA replication initiation factors, elongation factors, and origins. *Genes Dev* **17**: 1741-54.

Huang, R.Y. and D. Kowalski. 1996. Multiple DNA elements in ARS305 determine replication origin activity in a yeast chromosome. *Nucleic Acids Res* **24**: 816-23.

Ishimi, Y. 1997. A DNA helicase activity is associated with an MCM4, -6, and -7 protein complex. *J Biol Chem* **272**: 24508-13.

Ishimi, Y. and Y. Komamura-Kohno. 2001. Phosphorylation of Mcm4 at specific sites by cyclin-dependent kinase leads to loss of Mcm4,6,7 helicase activity. *J Biol Chem* **276**: 34428-33.

Ishimi, Y., Y. Komamura-Kohno, Z. You, A. Omori, and M. Kitagawa. 2000. Inhibition of Mcm4,6,7 helicase activity by phosphorylation with cyclin A/Cdk2. *J Biol Chem* **275**: 16235-41.

Itzhaki, J.E., C.S. Gilbert, and A.C. Porter. 1997. Construction by gene targeting in human cells of a "conditional' CDC2 mutant that rereplicates its DNA. *Nat Genet* **15**: 258-65.

Jacob, F., S. Brenner, and F. Cuzin. 1963. On the regulation of DNA replication in bacteria. *Cold Spring Harbor Symp. Quant. Biol.,* **28**: 329-348.

Jallepalli, P.V., G.W. Brown, M. Muzi-Falconi, D. Tien, and T.J. Kelly. 1997. Regulation of the replication initiator protein p65cdc18 by CDK phosphorylation. *Genes Dev* **11**: 2767-79.

Jang, S.W., S. Elsasser, J.L. Campbell, and J. Kim. 2001. Identification of Cdc6 protein domains involved in interaction with Mcm2 protein and Cdc4 protein in budding yeast cells. *Biochem J* **354**: 655-61.

Jares, P. and J.J. Blow. 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**: 1528-40.

Jiang, W., N.J. Wells, and T. Hunter. 1999. Multistep regulation of DNA replication by Cdk phosphorylation of HsCdc6. *Proc Natl Acad Sci U S A* **96**: 6193-8.

Kamimura, Y., H. Masumoto, A. Sugino, and H. Araki. 1998. Sld2, which interacts with Dpb11 in Saccharomyces cerevisiae, is required for chromosomal DNA replication. *Mol Cell Biol* **18**: 6102-9.

Kelly, T.J., G.S. Martin, S.L. Forsburg, R.J. Stephen, A. Russo, and P. Nurse. 1993. The fission yeast cdc18+ gene product couples S phase to START and mitosis. *Cell* **74**: 371-382.

Kelman, Z., J.K. Lee, and J. Hurwitz. 1999. The single minichromosome maintenance protein of Methanobacterium thermoautotrophicum DeltaH contains DNA helicase activity. *Proc Natl Acad Sci U S A* **96**: 14783-8.

Kim, S.M. and J.A. Huberman. 1998. Multiple orientation-dependent, synergistically interacting, similar domains in the ribosomal DNA replication origin of the fission yeast, Schizosaccharomyces pombe. *Mol Cell Biol* **18**: 7294-303.

-. 1999. Influence of a replication enhancer on the hierarchy of origin efficiencies within a cluster of DNA replication origins. *J Mol Biol* **288**: 867-82.

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

Klemm, R.D. and S.P. Bell. 2001. ATP bound to the origin recognition complex is important for preRC formation. *Proc Natl Acad Sci U S A* **98**: 8361-7.

Kong, D. and M.L. DePamphilis. 2001. Site-specific DNA binding of the Schizosaccharomyces pombe origin recognition complex is determined by the Orc4 subunit. *Mol Cell Biol* **21**: 8095-103.

-. 2002. Site-specific ORC binding, pre-replication complex assembly and DNA synthesis at Schizosaccharomyces pombe replication origins. *Embo J* **21**: 5567-76.

Kornberg, A. and T.A. Baker. 1992. *DNA Replication: second edition.* W. H. Freeman and Company, New York.

Kowalski, D. and M.J. Eddy. 1989. The DNA unwinding element: a novel, cis-acting component that facilitates opening of the Escherichia coli replication origin. *Embo J* **8**: 4335-44.

Kreitz, S., M. Ritzi, M. Baack, and R. Knippers. 2001. The human origin recognition complex protein 1 dissociates from chromatin during S phase in HeLa cells. *J Biol Chem* **276**: 6337-42.

Labib, K. and J.F. Diffley. 2001. Is the MCM2-7 complex the eukaryotic DNA replication fork helicase? *Curr Opin Genet Dev* **11**: 64-70.

Labib, K., J.F. Diffley, and S.E. Kearsey. 1999. G1-phase and B-type cyclins exclude the DNA-replication factor Mcm4 from the nucleus. *Nat Cell Biol* **1**: 415-22.

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

Laman, H., G. Peters, and N. Jones. 2001. Cyclin-mediated export of human Orc1. *Exp Cell Res* **271**: 230-7.

Leatherwood, J., G.A. Lopez, and P. Russell. 1996. Interaction of Cdc2 and Cdc18 with a fission yeast ORC2-like protein. *Nature* **379**: 360-363.

Lee, D.G. and S.P. Bell. 1997. Architecture of the yeast origin recognition complex bound to origins of DNA replication. *Mol Cell Biol* **17**: 7159-68.

Lee, D.G., A.M. Makhov, R.D. Klemm, J.D. Griffith, and S.P. Bell. 2000. Regulation of origin recognition complex conformation and ATPase activity: differential effects of single-stranded and double-stranded DNA binding. *Embo J* **19**: 4774-82.

Lee, J.K. and J. Hurwitz. 2000. Isolation and characterization of various complexes of the minichromosome maintenance proteins of Schizosaccharomyces pombe. *J Biol Chem* **275**: 18871-8.

-. 2001. Processive DNA helicase activity of the minichromosome maintenance proteins 4, 6, and 7 complex requires forked DNA structures. *Proc Natl Acad Sci U S A* **98**: 54-9.

Lee, J.K., K.Y. Moon, Y. Jiang, and J. Hurwitz. 2001. The Schizosaccharomyces pombe origin recognition complex interacts with multiple AT-rich regions of the

replication origin DNA by means of the AT-hook domains of the spOrc4 protein. *Proc Natl Acad Sci U S A* **98**: 13589-94.

Lei, M., Y. Kawasaki, M.R. Young, M. Kihara, A. Sugino, and B.K. Tye. 1997. Mcm2 is a target of regulation by Cdc7-Dbf4 during the initiation of DNA synthesis. *Genes Dev* **11**: 3365-74.

Li, C.J. and M.L. DePamphilis. 2002. Mammalian Orc1 protein is selectively released from chromatin and ubiquitinated during the S-to-M transition in the cell division cycle. *Mol Cell Biol* **22**: 105-16.

Liang, C. and B. Stillman. 1997. Persistent initiation of DNA replication and chromatin-bound MCM proteins during the cell cycle in cdc6 mutants. *Genes Dev* **11**: 3375-86.

Lin, S. and D. Kowalski. 1997. Functional equivalency and diversity of cis-acting elements among yeast replication origins. *Mol Cell Biol* **17**: 5473-84.

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

Liu, G., M. Malott, and M. Leffak. 2003. Multiple functional elements comprise a Mammalian chromosomal replicator. *Mol Cell Biol* **23**: 1832-42.

Liu, J., C.L. Smith, D. DeRyckere, K. DeAngelis, G.S. Martin, and J.M. Berger. 2000. Structure and function of Cdc6/Cdc18: implications for origin recognition and checkpoint control. *Mol Cell* **6**: 637-48.

Lopez-Girona, A., O. Mondesert, J. Leatherwood, and P. Russell. 1998. Negative regulation of Cdc18 DNA replication protein by Cdc2. *Mol Biol Cell* **9**: 63-73.

Lygerou, Z. and P. Nurse. 1999. The fission yeast origin recognition complex is constitutively associated with chromatin and is differentially modified through the cell cycle. *J Cell Sci* **112 (Pt 21)**: 3703-12.

Maiorano, D., J. Moreau, and M. Mechali. 2000. XCDT1 is required for the assembly of pre-replicative complexes in Xenopus laevis. *Nature* **404**: 622-5.

Marahrens, Y. and B. Stillman. 1992. A yeast chromosomal origin of DNA replication defined by multiple functional elements. *Science* **255**: 817-23.

Masumoto, H., S. Muramatsu, Y. Kamimura, and H. Araki. 2002. S-Cdk-dependent phosphorylation of Sld2 essential for chromosomal DNA replication in budding yeast. *Nature* **415**: 651-5.

McGarry, T.J. 2002. Geminin deficiency causes a Chk1-dependent G2 arrest in Xenopus. *Mol Biol Cell* **13**: 3662-71.

McGarry, T.J. and M.W. Kirschner. 1998. Geminin, an inhibitor of DNA replication, is degraded during mitosis. *Cell* **93**: 1043-53.

Mendez, J. and B. Stillman. 2000. Chromatin association of human origin recognition complex, cdc6, and minichromosome maintenance proteins during the cell cycle: assembly of prereplication complexes in late mitosis. *Mol Cell Biol* **20**: 8602-12.

Mendez, J., X.H. Zou-Yang, S.Y. Kim, M. Hidaka, W.P. Tansey, and B. Stillman. 2002. Human origin recognition complex large subunit is degraded by ubiquitin-mediated proteolysis after initiation of DNA replication. *Mol Cell* **9**: 481-91.

Messer, W., F. Blaesing, D. Jakimowicz, M. Krause, J. Majka, J. Nardmann, S. Schaper, H. Seitz, C. Speck, C. Weigel, G. Wegrzyn, M. Welzeck, and J. Zakrzewska-Czerwinska. 2001. Bacterial replication initiator DnaA. Rules for DnaA binding and roles of DnaA in origin unwinding and helicase loading. *Biochimie* **83**: 5-12.

Miller, C.A. and D. Kowalski. 1993. cis-acting components in the replication origin from ribosomal DNA of Saccharomyces cerevisiae. *Mol Cell Biol* **13**: 5360-9.

Miller, M.E. and F.R. Cross. 2001. Cyclin specificity: how many wheels do you need on a unicycle? *J Cell Sci* **114**: 1811-20.

Mizushima, T., N. Takahashi, and B. Stillman. 2000. Cdc6p modulates the structure and DNA binding activity of the origin recognition complex in vitro. *Genes Dev* **14**: 1631-41.

Muzi Falconi, M., G.W. Brown, and T.J. Kelly. 1996. cdc18+ regulates initiation of DNA replication in Schizosaccharomyces pombe. *Proc Natl Acad Sci U S A* **93**: 1566-70.

Natale, D.A., C.J. Li, W.H. Sun, and M.L. DePamphilis. 2000. Selective instability of Orc1 protein accounts for the absence of functional origin recognition complexes during the M-G(1) transition in mammals. *Embo J* **19**: 2728-38.

Neuwald, A.F., L. Aravind, J.L. Spouge, and E.V. Koonin. 1999. AAA+: A class of chaperone-like ATPases associated with the assembly, operation, and disassembly of protein complexes. *Genome Res* **9**: 27-43.

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

Nguyen, V.Q., C. Co, and J.J. Li. 2001. Cyclin-dependent kinases prevent DNA re-replication through multiple mechanisms. *Nature* **411**: 1068-73.

Nishitani, H., Z. Lygerou, T. Nishimoto, and P. Nurse. 2000. The Cdt1 protein is required to license DNA for replication in fission yeast. *Nature* **404**: 625-8.

Nishitani, H. and P. Nurse. 1995. p65cdc18 plays a major role controlling the initiation of DNA replication in fission yeast. *Cell* **83**: 397-405.

Nishitani, H., S. Taraviras, Z. Lygerou, and T. Nishimoto. 2001. The human licensing factor for DNA replication Cdt1 accumulates in G1 and is destabilized after initiation of S-phase. *J Biol Chem* **276**: 44905-11.

Nougarede, R., F. Della Seta, P. Zarzov, and E. Schwob. 2000. Hierarchy of S-phase-promoting factors: yeast Dbf4-Cdc7 kinase requires prior S-phase cyclin-dependent kinase activation. *Mol Cell Biol* **20**: 3795-806.

Okishio, N., Y. Adachi, and M. Yanagida. 1996. Fission yeast Nda1 and Nda4, MCM homologs required for DNA replication, are constitutive nuclear proteins. *J Cell Sci* **109 (Pt 2)**: 319-26.

Okuno, Y., A.J. McNairn, N. den Elzen, J. Pines, and D.M. Gilbert. 2001. Stability, chromatin association and functional activity of mammalian pre-replication complex proteins during the cell cycle. *Embo J* **20**: 4263-77.

Okuno, Y., H. Satoh, M. Sekiguchi, and H. Masukata. 1999. Clustered adenine/thymine stretches are essential for function of a fission yeast replication origin. *Mol Cell Biol* **19**: 6699-709.

Pasero, P., B.P. Duncker, E. Schwob, and S.M. Gasser. 1999. A role for the Cdc7 kinase regulatory subunit Dbf4p in the formation of initiation-competent origins of replication. *Genes Dev* **13**: 2159-76.

Pasion, S.G. and S.L. Forsburg. 1999. Nuclear localization of Schizosaccharomyces pombe Mcm2/Cdc19p requires MCM complex assembly. *Mol Biol Cell* **10**: 4043-57.

Pelizon, C., M.A. Madine, P. Romanowski, and R.A. Laskey. 2000. Unphosphorylatable mutants of Cdc6 disrupt its nuclear export but still support DNA replication once per cell cycle. *Genes Dev* **14**: 2526-33.

Pereverzeva, I., E. Whitmire, B. Khan, and M. Coue. 2000. Distinct phosphoisoforms of the Xenopus Mcm4 protein regulate the function of the Mcm complex. *Mol Cell Biol* **20**: 3667-76.

Perkins, G., L.S. Drury, and J.F. Diffley. 2001. Separate SCF(CDC4) recognition elements target Cdc6 for proteolysis in S phase and mitosis. *Embo J* **20**: 4836-45.

Petersen, B.O., J. Lukas, C.S. Sorensen, J. Bartek, and K. Helin. 1999. Phosphorylation of mammalian CDC6 by cyclin A/CDK2 regulates its subcellular localization. *Embo J* **18**: 396-410.

Quinn, L.M., A. Herr, T.J. McGarry, and H. Richardson. 2001. The Drosophila Geminin homolog: roles for Geminin in limiting DNA replication, in anaphase and in neurogenesis. *Genes Dev* **15**: 2741-54.

Raghuraman, M.K., E.A. Winzeler, D. Collingwood, S. Hunt, L. Wodicka, A. Conway, D.J. Lockhart, R.W. Davis, B.J. Brewer, and W.L. Fangman. 2001. Replication dynamics of the yeast genome. *Science* **294**: 115-21.

Rao, H., Y. Marahrens, and B. Stillman. 1994. Functional conservation of multiple elements in yeast chromosomal replicators. *Mol Cell Biol* **14**: 7643-51.

Roberts, J.M. 1999. Evolving ideas about cyclins. *Cell* **98**: 129-32.

Romanowski, P., M.A. Madine, A. Rowles, J.J. Blow, and R.A. Laskey. 1996. The Xenopus origin recognition complex is essential for DNA replication and MCM binding to chromatin. *Curr Biol* **6**: 1416-25.

Romanowski, P., J. Marr, M.A. Madine, A. Rowles, J.J. Blow, J. Gautier, and R.A. Laskey. 2000. Interaction of Xenopus Cdc2 x cyclin A1 with the origin recognition complex. *J Biol Chem* **275**: 4239-43.

Rowles, A., S. Tada, and J.J. Blow. 1999. Changes in association of the Xenopus origin recognition complex with chromatin on licensing of replication origins. *J Cell Sci* **112 (Pt 12)**: 2011-8.

Royzman, I., R.J. Austin, G. Bosco, S.P. Bell, and T.L. Orr-Weaver. 1999. ORC localization in Drosophila follicle cells and the effects of mutations in dE2F and dDP. *Genes Dev* **13**: 827-40.

Saha, P., J. Chen, K.C. Thome, S.J. Lawlis, Z.H. Hou, M. Hendricks, J.D. Parvin, and A. Dutta. 1998. Human CDC6/Cdc18 associates with Orc1 and cyclin-cdk and is selectively eliminated from the nucleus at the onset of S phase. *Mol Cell Biol* **18**: 2758-67.

Santocanale, C. and J.F. Diffley. 1996. ORC- and Cdc6-dependent complexes at active and inactive chromosomal replication origins in Saccharomyces cerevisiae. *Embo J* **15**: 6671-9.

Schepers, A. and J.F. Diffley. 2001. Mutational analysis of conserved sequence motifs in the budding yeast Cdc6 protein. *J Mol Biol* **308**: 597-608.

Schwacha, A. and S.P. Bell. 2001. Interactions between two catalytically distinct MCM subgroups are essential for coordinated ATP hydrolysis and DNA replication. *Mol Cell* **8**: 1093-104.

Schwob, E., T. Bohm, M.D. Mendenhall, and K. Nasmyth. 1994. The B-type cyclin kinase inhibitor p40SIC1 controls the G1 to S transition in S. cerevisiae. *Cell* **79**: 233-44.

Seki, T. and J.F. Diffley. 2000. Stepwise assembly of initiation proteins at budding yeast replication origins in vitro. *Proc Natl Acad Sci U S A* **97**: 14115-20.

Sherman, D.A. and S.L. Forsburg. 1998. Schizosaccharomyces pombe Mcm3p, an essential nuclear protein, associates tightly with Nda4p (Mcm5p). *Nucleic Acids Res* **26**: 3955-60.

Stefanovic, D., S. Stanojcic, A. Vindigni, A. Ochem, and A. Falaschi. 2003. In vitro protein-DNA interactions at the human lamin B2 replication origin. *J Biol Chem.*

Sun, W.H., T.R. Coleman, and M.L. DePamphilis. 2002. Cell cycle-dependent regulation of the association between origin recognition proteins and somatic cell chromatin. *Embo J* **21**: 1437-46.

Tada, S., A. Li, D. Maiorano, M. Mechali, and J.J. Blow. 2001. Repression of origin assembly in metaphase depends on inhibition of RLF- B/Cdt1 by geminin. *Nat Cell Biol* **3**: 107-13.

Takahashi, T., E. Ohara, H. Nishitani, and H. Masukata. 2003. Multiple ORC-binding sites are required for efficient MCM loading and origin firing in fission yeast. *Embo J* **22**: 964-74.

Tanaka, S. and J.F. Diffley. 2002a. Deregulated G1-cyclin expression induces genomic instability by preventing efficient pre-RC formation. *Genes Dev* **16**: 2639-49.

-. 2002b. Interdependent nuclear accumulation of budding yeast Cdt1 and Mcm2-7 during G1 phase. *Nat Cell Biol* **4**: 198-207.

Tanaka, T., D. Knapp, and K. Nasmyth. 1997. Loading of an Mcm protein onto DNA replication origins is regulated by Cdc6p and CDKs. *Cell* **90**: 649-60.

Theis, J.F. and C.S. Newlon. 1994. Domain B of ARS307 contains two functional elements and contributes to chromosomal replication origin function. *Mol Cell Biol* **14**: 7652-9.

-. 1997. The ARS309 chromosomal replicator of Saccharomyces cerevisiae depends on an exceptional ARS consensus sequence. *Proc Natl Acad Sci U S A* **94**: 10786-91.

Vas, A., W. Mok, and J. Leatherwood. 2001. Control of DNA rereplication via Cdc2 phosphorylation sites in the origin recognition complex. *Mol Cell Biol* **21**: 5767-77.

Vashee, S., C. Cvetic, W. Lu, P. Simancek, T.J. Kelly, and J.C. Walter. 2003. Sequence-independent DNA binding and replication initiation by the human origin recognition complex. *Genes Dev* **17**: 1894-908.

Vaziri, C., S. Saxena, Y. Jeon, C. Lee, K. Murata, Y. Machida, N. Wagle, D.S. Hwang, and A. Dutta. 2003. A p53-dependent checkpoint pathway prevents rereplication. *Mol Cell* **11**: 997-1008.

Venditti, P., G. Costanzo, R. Negri, and G. Camilloni. 1994. ABFI contributes to the chromatin organization of Saccharomyces cerevisiae ARS1 B-domain. *Biochim Biophys Acta* **1219**: 677-89.

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**: 39773-8.

Wang, B., L. Feng, Y. Hu, S.H. Huang, C.P. Reynolds, L. Wu, and A.Y. Jong. 1999. The essential role of Saccharomyces cerevisiae CDC6 nucleotide-binding site in cell growth, DNA synthesis, and Orc1 association. *J Biol Chem* **274**: 8291-8.

Weinreich, M., C. Liang, H.H. Chen, and B. Stillman. 2001. Binding of cyclin-dependent kinases to ORC and Cdc6p regulates the chromosome replication cycle. *Proc Natl Acad Sci U S A* **98**: 11211-7.

Weinreich, M., C. Liang, and B. Stillman. 1999. The Cdc6p nucleotide-binding motif is required for loading mcm proteins onto chromatin. *Proc Natl Acad Sci U S A* **96**: 441-6.

Weinreich, M. and B. Stillman. 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**: 5334-46.

Whittaker, A.J., I. Royzman, and T.L. Orr-Weaver. 2000. Drosophila double parked: a conserved, essential replication protein that colocalizes with the origin recognition complex and links DNA replication with mitosis and the down-regulation of S phase transcripts. *Genes Dev* **14**: 1765-76.

Wiltshire, S., S. Raychaudhuri, and S. Eisenberg. 1997. An Abf1p C-terminal region lacking transcriptional activation potential stimulates a yeast origin of replication. *Nucleic Acids Res* **25**: 4250-6.

Wohlschlegel, J.A., B.T. Dwyer, S.K. Dhar, C. Cvetic, J.C. Walter, and A. Dutta. 2000. Inhibition of eukaryotic DNA replication by geminin binding to Cdt1. *Science* **290**: 2309-12.

Wolf, D.A., F. McKeon, and P.K. Jackson. 1999. Budding yeast Cdc6p induces re-replication in fission yeast by inhibition of SCF(Pop)-mediated proteolysis. *Mol Gen Genet* **262**: 473-80.

Wuarin, J., V. Buck, P. Nurse, and J.B. Millar. 2002. Stable association of mitotic cyclin B/Cdc2 to replication origins prevents endoreduplication. *Cell* **111**: 419-31.

Wyrick, J.J., J.G. Aparicio, T. Chen, J.D. Barnett, E.G. Jennings, R.A. Young, S.P. Bell, and O.M. Aparicio. 2001. Genome-wide distribution of ORC and MCM proteins in S. cerevisiae: high-resolution mapping of replication origins. *Science* **294**: 2357-60.

Yamaguchi, R. and J. Newport. 2003. A role for Ran-GTP and Crm1 in blocking re-replication. *Cell* **113**: 115-25.

Yanagi, K., T. Mizuno, Z. You, and F. Hanaoka. 2002. Mouse geminin inhibits not only Cdt1-MCM6 interactions but also a novel intrinsic Cdt1 DNA binding activity. *J Biol Chem* **277**: 40871-80.

Yanow, S.K., Z. Lygerou, and P. Nurse. 2001. Expression of Cdc18/Cdc6 and Cdt1 during G2 phase induces initiation of DNA replication. *Embo J* **20**: 4648-56.

Zhong, W., H. Feng, F.E. Santiago, and E.T. Kipreos. 2003. CUL-4 ubiquitin ligase maintains genome stability by restraining DNA-replication licensing. *Nature* **423**: 885-9.

Zou, L. and B. Stillman. 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**: 3086-96.

Chapter II

The B2 element of the *S. cerevisiae ARS1* origin of replication requires specific sequences to facilitate pre-RC formation

An earlier version of this chapter was published in 2001 under the same title (PNAS 99(1): 101-6). The authors were Gwendolyn M. Wilmes and Stephen P. Bell.

I would like to thank Richard Klemm for ORC and Orc1-5p, Anthony Schwacha for MCM antibody, Rick Austin for CDC6 plasmids, Milan de Vries for assistance with statistical analyses, and Tania Baker for critical reading of the manuscript.

Summary

The minimal requirements for a eukaryotic origin of replication are an initiator binding site and a region of helically unstable DNA (DNA Unwinding Element, DUE). Budding yeast origins consist of modular elements, and one of these elements, B2, has been proposed to act as a DUE. To test this hypothesis, we screened for sequences that function at the B2 element of *ARS1*. We found that the B2 element required A-rich sequences, but that the function of these identified sequences did not correlate with helical instability. Instead, the sequences that substituted fully for B2 function showed similarity to the ARS Consensus Sequence (ACS). The ACS is the binding site for the initiator Origin Recognition Complex (ORC), but the selected sequences are not strong ORC binding sites *in vitro*. Non-functional B2 sequences show a corresponding loss in Mcm2-7p origin association. The function of these mutant sequences is rescued by Cdc6p over-expression. We propose that the B2 element requires specific sequences to bind a component of the pre-RC.

Introduction

Origins of replication provide a site for DNA unwinding and the loading of the DNA replication machinery. The DNA sequences required for an origin of replication are referred to as a replicator and contain a binding site for an initiator protein combined with a structural DNA Unwinding Element (DUE) (DePamphilis 1999). The eukaryotic initiator is the Origin Recognition Complex (ORC), which has been shown to bind origins in several species (Bell and Stillman 1992; Austin et al. 1999; Ogawa et al. 1999). DUEs have been identified at origins in many organisms (Kowalski and Eddy 1989; Natale et al. 1993; Lin and Kowalski 1994) and are defined as helically unstable DNA sequences that can be replaced by any easily unwound DNA. Although an initiator binding site and a DUE appear to be present at all origins, other sequence elements are likely to be necessary for origin function.

Saccharomyces cerevisiae replicator sequences were identified based upon their ability to confer autonomous replication on episomes and have been extensively characterized. Mutagenesis of these sequences revealed that they are composed of multiple 10-15 base pair (bp) elements within a 100-150 bp region. These elements include the essential A element, containing the 11 bp ARS Consensus Sequences (ACS), ORC's binding site. ORC is bound to the origin throughout the cell cycle and is required to recruit the remaining DNA replication machinery to the origin. The A element is insufficient for origin function, and a variable number of B elements also contribute to function (Marahrens and Stillman 1992; Rao et al. 1994; Theis and Newlon 1994; Huang and Kowalski 1996). B elements were identified by analysis of multiple origins and named based on their ability to functionally substitute for each other. The B1 element

cooperates with the ACS to form ORC's bipartite binding site. The function of the B2 element is unknown, but may include either binding a specific protein or acting as a site for initial unwinding of the origin DNA.

Whether the B2 element functions structurally or in a sequence-specific manner, significant evidence supports a role for this sequence in pre-Replicative Complex (pre-RC) formation. The pre-RC protects the A, B1, and B2 region of *ARS1 in vivo* during G1 (Santocanale and Diffley 1996) and contains ORC, Cdc6p, and the MCM proteins (Diffley et al. 1995; Aparicio et al. 1997; Donovan et al. 1997; Tanaka et al. 1997). A linker scan mutation in the B2 element of *ARS1* reduces MCM loading *in vivo* but has no effect on ORC binding (Zou and Stillman 2000; Lipford and Bell 2001). The MCM proteins are loaded onto the origin by Cdc6p (Aparicio et al. 1997; Donovan et al. 1997; Tanaka et al. 1997) in an ATP-dependent manner (Seki and Diffley 2000). In *S. pombe, X. laevis*, and *D. melanogaster*, Cdt1p also associates with Cdc6p and is required for MCM loading (Maiorano et al. 2000; Nishitani et al. 2000; Whittaker et al. 2000)

The B2 element has been proposed to act structurally as a DUE. Consistent with this model, the region of *ARS305* that can functionally replace the B2 elements at *ARS1* and *ARS307* is over 50 bp long, and has no short sequence elements that can be identified by linker scan mutations. The *ARS305* B2 region can be replaced with a random easily unwound sequence (Huang and Kowalski 1993; Huang and Kowalski 1996). The much shorter 11 bp *ARS1* B2 element also has some characteristics of a DUE, as single point mutations have no effect (Rao et al. 1994) and *in vitro* RPA-dependent unwinding of the *ARS1* origin is dependent upon the B2 element (Matsumoto and Ishimi 1994). Similarly, the shorter 20 bp *ARS307* B2 element has been proposed to function as a DUE based on a

correlation between the helical stability and replication defects of several B2 mutations (Lin and Kowalski 1997).

Other evidence suggests that the B2 element has a function other than a DUE. At *ARS307*, a specific sequence requirement is suggested by a three bp mutation that has little effect on helical stability but a large effect on origin function (Rao et al. 1994; Lin and Kowalski 1997). In addition, the *ARS1* B2 element cannot be inverted or replaced with a sequence of equal helical stability (Lin and Kowalski 1997). Together, these data suggest that the B2 element has additional sequence requirements and binds one or more replication factors, such as components of the pre-RC.

Here, we describe a genetic screen to identify the sequence requirements of the *ARS1* B2 element. Sequences that functioned as B2 elements were found to each include a partial inverted match to the ACS, and not simply helically unstable DNA. Despite their similarity to the ACS, these sequences were not specifically bound by ORC in vitro. Origin function of the mutant B2 sequences correlated with Mcm2-7p loading and function of defective mutants was significantly rescued by over-expressing Cdc6p. We propose a model whereby all yeast origins require large DUEs with a sequence related to the ACS embedded within them to function as a binding site for a pre-RC component.

Results

A screen for sequences that affect origin function.

To determine the sequence requirements at the B2 element of *S. cerevisiae* origins, we took advantage of the well-defined B2 element of *ARS1*. Previous linker scan mutagenesis of *ARS1* had identified B2 as a short 11 bp sequence (Marahrens and Stillman 1992) that can functionally substitute for the longer B2 elements at *ARS307* and *ARS305* (Rao et al. 1994; Lin and Kowalski 1997). To identify additional sequences that can function at this site, we constructed an *ARS1* plasmid library with randomized sequences at the 11 bp B2 element (Fig. 1a). This library was amplified in *E. coli*, and found to have a complexity of ~ 40,000 clones. Because this represents a small fraction of all possible 11 bp sequences, we sequenced 25 clones to determine whether the library was enriched for any particular base. A slight enrichment of Ts and depletion of Gs on the bottom strand as depicted in Fig. 1a was observed (we will refer to the sequence of this strand throughout, Fig. 1b). However, the diversity of the library was sufficient to represent both functional and non-functional origins (see below and Fig. 1c, d).

Plasmid loss rates were used as a measure of function of the B2 mutant origins. Plasmids containing B2 element sequences that functioned poorly were identified using a colorimetric assay for loss of the *ADE2* gene (see Materials and Methods). Thirty-one plasmids were recovered with loss rates higher than the pARS1/802-810 B2 linker scan mutant (7.8±1.0 %/ generation, Fig. 1c and Table 3). B2 elements from these plasmids are termed $B2^-$ sequences. B2 sequences that functioned at wild-type levels were found in plasmids that were lost at a low rate and therefore retained for ten days (see Materials and Methods). Thirty-one plasmids were recovered with plasmid loss

Figure 1

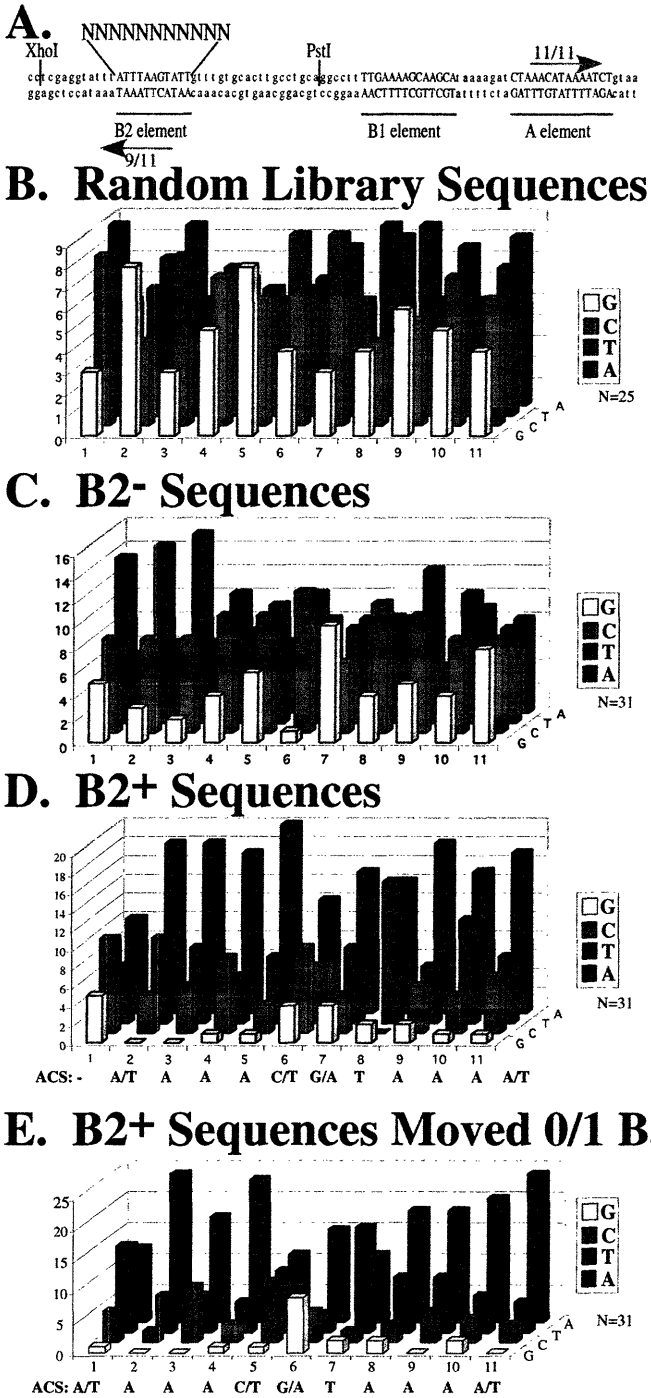

A.
NNNNNNNNNNN
XhoI
PstI
11/11
B2 element
B1 element
A element
9/11
B. Random Library Sequences
N=25
C. B2- Sequences
N=31
D. B2+ Sequences
N=31
ACS: - A/T A A A C/T G/A T A A A A/T
E. B2+ Sequences Moved 0/1 Bases
N=31
ACS: A/T A A A C/T G/A T A A A A/T

Figure 1. *ARS1* B2 mutant sequences.

(A) The B2 region of *ARS1*. A XhoI to PstI fragment of *ARS1* with randomized bases at the 11 bp B2 element was cloned into the XhoI to PstI region of pARS1/785-92. Note that the strand referred to throughout this paper is the bottom strand in this figure, the A-rich strand of the B2 element, read from right to left 5' to 3'. The A and B2 element ACS matches are shown as red arrows.

(B-E) The frequency of each nucleotide on the y axis is plotted against each of the 11 randomized positions on the x axis.

(B) The library represents all four bases at all eleven positions. Twenty-five clones from the *E. coli* library were sequenced.

(C) The $B2^-$ sequences are representative of the library as a whole.

(D) The $B2^+$ sequences are A-rich and weakly match up to the ACS starting at the second nucleotide. The ACS is aligned underneath the graph for comparison.

(E) The $B2^+$ sequences form a consensus that strongly matches the ACS if two thirds of the sequences are shifted one base to the right. The $B2^+$ sequences from part D were examined to find the best match to the ACS within one base of the randomized 11 bases. The ACS is provided below for comparison.

rates within one standard deviation of wild-type *ARS1* (3.0±1.1%/ generation, Fig. 1d and Table 3). The stable maintenance of these plasmids was confirmed by measuring their plasmid loss rates at least three times for each plasmid, and elements isolated in this selection are termed $B2^+$ sequences.

$B2^+$ sequences are A:T rich and contain a sequence related to the ACS.

To characterize the $B2^+$ and $B2^-$ sequences, each group was compared to randomly sequenced clones from the library. A two-tailed student's t–test was applied to the data in each case to calculate the probability that the differences between the selected and random sequences were due to chance alone (p-value). A p-value of less than 0.05 was considered significant.

The $B2^+$ sequences had a significant bias for A:T rich DNA. They contained an average of 5.6 ± 1.4 As on the bottom strand, which was much higher than the composition of the library as a whole ($p= 6.6 \times 10^{-8}$, Table 1). In contrast, there was no selection for Ts on the bottom strand (Table 1). Although these sequences had calculated helical stabilities lower than those of random library sequences, most of this helical instability could be accounted for by the bias for A-rich DNA (Table 1). Furthermore, there was no correlation between the rate of plasmid loss per generation and calculated helical stability (Fig. 2).

As originally defined, the wild-type *ARS1* B2 element is a 9/11 match to the ACS in an inverse orientation relative to the A element ACS. This raised the possibility that a sequence similar to the ACS was important for B2 function. To test this hypothesis, we

	Best Match to ACS/11	Calculated Helical Stability	# of A's/11	# of T's/11
Avg. of $B2^+$ Sequences:	7.4 ± 0.9	5.3 ± 1.6 Kcal/mol	5.6 ± 1.4	2.8 ± 1.3
Avg. of $B2^-$ Sequences:	4.5 ± 1.2	7.9 ± 3.6 Kcal/mol	2.5 ± 1.4	3.9 ± 1.5
Avg. of Random Library Sequences:	4.6 ± 1.1	8.2 ± 5.3 Kcal/mol	2.7 ± 1.2	3.2 ± 1.5
Avg. of Randomly Generated A-rich Sequences:	6.4 ± 1.7	6.2 ± 1.7 Kcal/mol	5.4 ± 1.6	2.3 ± 1.2
P value $B2^+$ v. Random:	*3.3E-05*	*6.6E-08*	*2.9E-11*	0.27
P value $B2^-$ v. Random:	0.91	0.53	0.42	0.080
P value $B2^+$ v. Random A-rich:	*8.8E-05*	*0.0060*	0.52	*0.041*

Table 1. Characteristics of $B2^+$ and $B2^-$ sequences. Best match to ACS is either at the defined B2 element or shifted one bp to the left (based on Fig. 1a). Calculations were as described in Materials and Methods. Significant p-values are in italics.

Figure 2

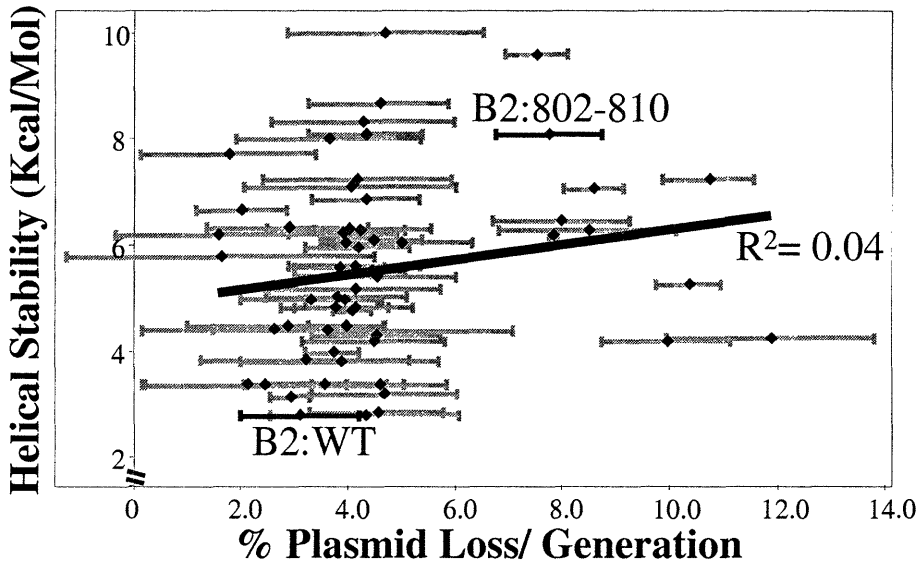

Helical Stability (Kcal/Mol)
10
8
6
4
2
B2:802-810
R2= 0.04
B2:WT
0
2.0
4.0
6.0
8.0
10.0
12.0
14.0
% Plasmid Loss/ Generation

Figure 2. Helical stability does not correlate with origin function of *ARS1* B2 mutants.

The helical stability of the mutant B2 element sequences was calculated as described in Materials and Methods. The helical stability was plotted against origin function for all B2 element mutants whose plasmid stability had been measured more than once, and a linear regression trend line and correlation value (R^2) were assigned by Excel (Microsoft). The error bars represent the confidence intervals of at least two plasmid loss assays.

examined the incidences of inverse matches to the ACS in the $B2^+$ sequences. There was no bias for any particular base at the first position, and the majority (~ 2/3) of the sequences formed a better match to the ACS if shifted one base away from the A element to include the flanking A (Fig. 1d). Because wild-type *ARS1* function is not altered by a one bp shift in B2 element location (Marahrens and Stillman 1992), we examined the best matches to the ACS within one base of the defined B2 element. The most frequent base at each position formed a match to the ACS at almost every position (Fig. 1e). Using this approach, the $B2^+$ sequences included an average match to the ACS of 7.4 ± 0.9 bases, a number that is much greater than the number of matches found in the similarly shifted randomly sequenced clones (4.6 ± 1.1, Table 1).

The above analyses suggested that the selection for functional B2 element sequences identified both A-rich DNA and, more specifically, matches to the A-rich ACS. One explanation for this dual selection was that the matches to the ACS were simply due to a preference for A-rich DNA. To test this hypothesis, we computer-generated a population of random 11 bp sequences with the same percentage of As as the $B2^+$ sequences (Table 1). We found that there was a statistically greater match to the ACS in the $B2^+$ sequences than in the randomly generated A-rich sequences ($p= 8.8 \times 10^{-5}$, Table 1). Therefore, the $B2^+$ sequences contain not simply A:T-rich DNA, but instead are selected to include a sequence similar to the ACS.

In contrast to the selection for matches to the ACS in the $B2^+$ sequences, the $B2^-$ sequences are indistinguishable from randomly sequenced library clones by all measures

examined (Table 1). These sequences have a similar composition, calculated helical stability (Natale et al. 1992) , and incidence of matches to the ACS as the sequences from the starting library (Table 1).

ORC does not protect $B2^+$ sequences from DNase I cleavage *in vitro*.

The selection for a close match to the ACS in the $B2^+$ sequences suggested that a second inverse ORC binding site at the B2 element was important for origin function. The wild-type B2 element is a 9/11 match for the ACS, and DNase I protection assays have demonstrated that ORC can bind to this site *in vitro* if the A element is mutated (Bell and Stillman 1992). ORC will also bind to both the A and B2 elements at the same time if the smallest subunit (Orc6p), which is not required for DNA binding (Lee and Bell 1997), is missing from the complex (R. Klemm and S.P.B., unpublished results, and Fig. 3 lanes 4-6). Therefore, we examined whether Orc1-5p would protect representative $B2^+$ sequences (Table 2) from DNase I digestion. In each case, Orc1-5p strongly protected the A element. In contrast, Orc1-5p failed to protect the $B2^+$ sequences or produce the characteristic hypersensitive site seen at the wild-type B2 element, although some small differences in the cleavage patterns at the $B2^+$ sequences can be noted when ORC1-5p is present (Fig. 3). Likewise, wild-type ORC did not footprint over mutant B2 origin sequences when the adjacent A element was disrupted with a linker scan mutation (865-872, data not shown). This finding confirmed that the lack of Orc1-5p association with the $B2^+$ sequences was not due to the absence of Orc6p.

Sequence Name	% Plasmid Loss/ Generation	Sequence	Best Match to ACS/11:	Helical Stability (Kcal/Mol):	# of As/11:	# of Ts/11:
WILDTYPE	3.1±1.1	AATACTTAAAT	9	2.8	6	4
B2-802-10	7.8±1.0	CTCGAGGAAAT	6	8.1	4	2
B2-M1	2.4±2.1	AAACAAATATA	8	3.4	8	2
B2-M2	2.9±0.4	AAAATGATATA	8	3.16	7	3
B2-M3	3.0±1.9	AATTACGAAAT	8	4.5	6	3
B2-M4	4.6±1.3	ATAAACCGCCA	7	8.7	5	1
B2-M5	7.1±2.2	CTTGGCTCGCC	3	12.3	0	3
B2-M6	7.5±0.6	TTTCGTACCGC	4	9.6	1	4
B2-M7	10.4±0.6	TCTTTTAGCAT	4	5.3	2	6
B2-M8	10.7±1.0	CCAAGTTTCTT	4	7.2	2	5
B2-M9	15.0±6.6	CTTCCTGGCTT	4	9.4	0	5
B2-M10	23.1±6.4	TGATCCTCACA	6	8.2	3	3

Table 2. Representative B2 mutant sequences.

Plasmid loss assays were performed at least three times. Best match to the ACS and helical stability were calculated as in Table 1.

Table 3. B2 mutant sequences. See Table 2. S.D.= Standard Deviation.

Sequence	% Plasmid Loss/ Generation	S.D. of plasmid loss	Best Match to ACS/ 11	Helical Stability (Kcal/Mol)	Number of As/ 11	Number of Ts/11
CCAATCTTTAA	1.6	1.9	7	6.2	4	4
AAATGCTAATC	1.6	2.9	6	5.8	5	3
AATGCGAAAAC	1.8	1.6	7	7.7	6	1
CTAAACCTCTA	2.0	0.9	8	6.7	4	3
AAACAAATATA	2.1	1.9	8	3.4	8	2
AAACAAATATA	2.5	2.3	8	3.4	8	2
TAACATAACAA	2.6	1.1	8	4.4	7	2
ATTATCGTTAA	2.9	1.8	8	4.5	4	5
GTTTACAAAAC	2.9	1.5	7	6.3	5	3
AAAATGATATA	2.9	0.4	8	3.2	7	3
AACTACTAAAT	3.2	2.0	7	3.8	6	3
CATACATAACT	3.3	1.3	8	5.0	5	3
AAACAAATATA	3.6	1.5	8	3.4	8	2
ATTAAAATGCT	3.6	3.5	6	4.4	5	3
CCAAACAAAAC	3.7	1.7	8	8.0	7	0
TACCAATTATA	3.7	0.5	6	4.0	5	4
TCAATGTTTAA	3.8	1.0	8	4.8	4	5
CAAAAAGATTA	3.8	1.3	7	5.0	7	2

GTTAAAAGACT	3.9	0.8	6	5.6	5	3
CTAAATAGATA	3.9	1.9	9	3.8	6	3
CACAAACTTAA	3.9	1.0	7	6.2	6	2
TATTCCCTAAT	3.9	0.7	6	5.0	3	5
AATTACGAAAT	4.0	0.7	7	4.5	6	3
CTAAACAAAAG	4.0	0.5	9	6.1	7	1
GCAAATGTTAA	4.0	1.5	9	6.3	5	3
GACATAAACAC	4.1	2.0	6	7.1	6	1
ATAAAAATGTC	4.1	0.4	7	4.8	6	3
TACATTCAAAA	4.1	1.1	8	4.8	6	3
CAACAGTAAAT	4.1	1.2	8	5.6	6	2
TAACTAAACGT	4.2	1.6	7	5.2	5	3
TTAAATTCGCG	4.2	1.8	6	7.3	3	4
CAACATGAATA	4.2	1.0	8	6.0	6	2
GTTTATTTCCA	4.2	0.9	6	6.3	2	6
CAACAAGACGT	4.3	1.7	5	8.3	5	1
TATAAAATTAC	4.3	1.8	7	2.8	6	4
GTTAATGTACC	4.3	1.0	8	6.9	3	4
CGGCTAAATAC	4.4	1.1	4	8.1	4	2
TGATAAAACGT	4.5	0.6	5	5.5	5	3
CGAATCTTAAA	4.5	0.9	8	6.1	4	5
ATGAATTTAGT	4.5	1.3	7	4.2	5	3
CTAAATACTGA	4.5	1.5	8	5.4	5	4

GATTAATTAAG	4.6	1.2	6	4.3	5	5
TAAAAGTATTT	4.6	1.2	8	2.9	7	3
ATAAACCGCCA	4.6	1.3	7	8.7	5	5
AATTAAAACAT	4.6	1.3	6	3.4	4	1
TGAATTATTAA	4.7	1.4	8	3.2	3	4
GAAACCGCTGA	4.7	1.8	7	10.0	7	1
TAAAAACAGGA	5.0	1.3	7	6.1	3	4
CTTTACGTATA	5.3	2.0	8	5.3	5	1
AGTACCTAAAA	5.4	3.3	8	5.7	6	2
CTTAAATTGTT	5.6	3.1	5	4.6	6	3
TCTCATGATTG	6.6		4	6.8	2	5
CTTGGCTCGCC	7.1	2.2	3	12.3	0	5
TTTCGTACCGC	7.5	0.6	4	9.6	1	4
TTCACAGACTG	7.6		5	8.1	3	3
TACCCTATTAC	7.9	0.1	6	6.2	3	4
TTACACTCCTA	8.0	1.3	6	6.5	7	4
GTTTATTTCCA	8.5	1.7	6	6.3	2	6
GTTAAACCCAT	8.6	0.6	5	7.1	2	6
TAGTGACTTCG	9.3		3	7.8	4	5
ATCACGATGAC	9.4		6	8.3	4	3
TACATTAATTC	10.0	1.2	5	4.2	2	4
GCACAAGCACG	10.1		5	11.0	4	2
CCAATCTTTAA	10.1	6.2	7	6.2	0	3

TCTTTTAGCAT	10.4	0.6	4	5.3	2	7
CCCTTCACTAT	10.4		4	7.8	4	0
AACGTAGATCG	10.5		5	8.0	3	2
CCAAGTTTCTT	10.7	1	5	7.2	2	6
TTTTACGAGTG	10.9	5.6	5	7.0	2	4
TTGACCTGAGA	10.9		6	8.3	4	2
CTTGCACCTCA	11.2		3	9.8	3	3
CCTCGTGTTTA	11.4		5	8.5	2	3
ATTTTCGATAG	11.7	3.1	5	5.7	1	5
TTATTTACTTG	11.9	1.9	5	4.3	2	5
TCCTGACTTGC	12.5		2	9.5	2	3
GTTGTCAACCA	13.5		5	8.9	3	3
GATTCCGCGGT	13.8	5.4	4	10.9	1	3
TGCTCCCAAAC	15.0	4.3	5	9.8	3	3
CTTCCTGGCTT	15.0	6.6	4	9.4	4	4
AGTCCTTTCAT	16.0	3.3	5	6.8	0	5
AATCTACCTTC	16.4	5.4	4	6.8	2	5
CTTCTCGGGTT	17.5		4	9.5	0	5
TGATCCTCACA	23.1	6.4	6	8.2	3	4

Figure 3

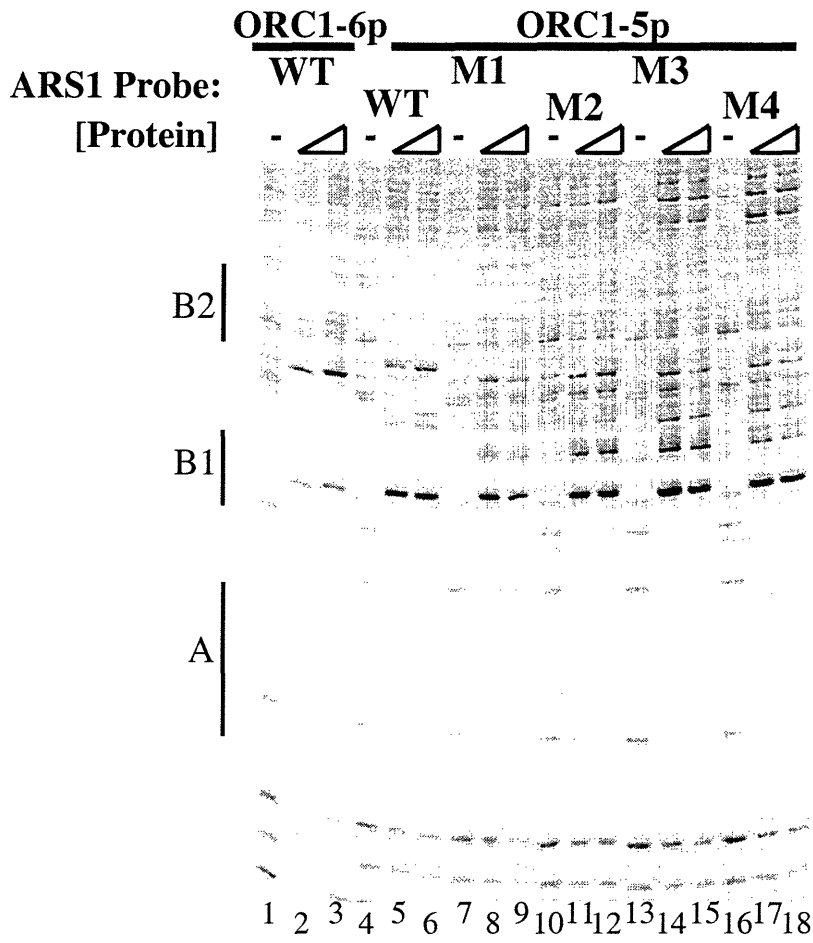

ORC1-6p
ORC1-5p
ARS1 Probe:
WT
WT
M1
M2
M3
M4
[Protein]
- - - - - -
B2
B1
A
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

Figure 3. Orc1-5p cannot bind representative $B2^+$ sequences *in vitro*. DNase I protection assays were performed with wild-type Orc1-6p (lanes 1-3), or Orc1-5p (lanes 4-18). Orc1-6p protects the A and B1 elements of wild-type *ARS1* DNA (lanes 1-3), while Orc1-5p protects the A element and the B2 element of wild-type *ARS1* DNA (lanes 4-6), with characteristic hypersensitive sites in between. In contrast, Orc1-5p does not protect the B2 elements of the $B2^+$ *ARS1* DNAs (lanes7-18). Lanes 1,4,7,10,13,and 16 contain no protein. Lanes 2, 5, 8, 11, 14, and 17 contain 50 ng protein. Lanes 3, 6, 9, 12, 15, and 18 contain 250 ng of protein. M1-M4 refer to the B2 mutants listed in Table 2.

MCM loading at the chromosomal *ARS1* correlates with plasmid origin function.

Because we could not detect an ORC interaction with the B2 element, we investigated the effect of the B2 mutants on the formation of the pre-RC *in vivo*. Previous reports have shown that Mcm2-7p loading is reduced in a B2 linker scan mutant *in vivo* when assayed by chromatin immunoprecipitation (ChIP) (Zou and Stillman 2000; Lipford and Bell 2001). To determine if B2 element function correlated with MCM loading, we integrated a series of representative mutants (Table 2) into the chromosome at *ARS1* and performed ChIP with a monoclonal antibody that recognizes all six MCM proteins (A. Schwacha and S.P.B., unpublished results). $B2^-$ sequences associate with low levels of MCM proteins during G1, whereas $B2^+$ sequences recruit Mcm2-7p to the origin at high levels (Fig. 4). By quantitation, MCM binding at the different *ARS1* B2 mutants is bimodal: either bound at wild-type levels, or at levels near that of the non-functional A element mutation. In contrast, the MCM proteins are recruited to a wild-type origin, *ARS305*, in all strains, and never to a non-origin sequence, *URA3* (Fig. 4). This data indicates that the plasmid stability data presented above is representative of origin function at the natural chromosomal locus and correlates with pre-RC formation.

Cdc6p over-expression rescues B2 mutant origins.

Because $B2^-$ origins have defects in MCM loading, a step requiring Cdc6p, we reasoned that they might be improved by over-expression of Cdc6p. Therefore, we introduced plasmids containing $B2^-$ origins into a strain containing an extra copy of

CDC6 under control of the *Gal1-10* promoter. Wild-type plasmids were lost at a slightly higher rate when the strain was grown in galactose, presumably due to titration of another factor. In contrast, the B2 linker scan mutation, and a series of other $B2^-$ mutations, were rescued to levels indistinguishable from wild-type in galactose media (Fig. 5a,b). Notably, a particularly strong B2 mutation, B2-m10, was significantly rescued, although not to wild-type, suggesting that over-expression of Cdc6p cannot completely substitute for the B2 element (Fig. 5b). Over-expression of Cdc6K114Ep, which does not associate with chromatin, and is non-functional *in vivo* (Elsasser et al. 1996; Perkins and Diffley 1998; Weinreich et al. 1999) had no effect on either the wild-type or the mutant origins (Fig. 5a).

Figure 4

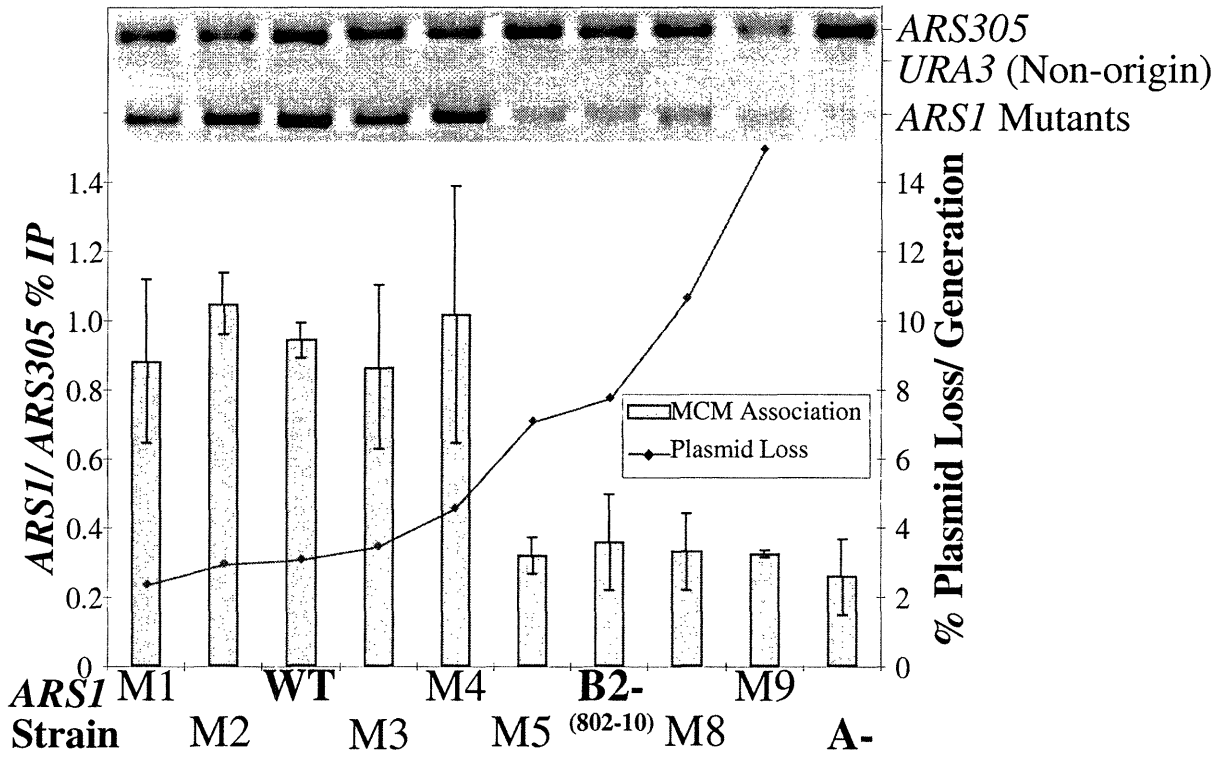

ARS305
URA3 (Non-origin)
ARS1 Mutants
ARS1/ARS305 % IP
% Plasmid Loss/ Generation
MCM Association
Plasmid Loss
ARS1 Strain
M1
M2
WT
M3
M4
M5
B2- (802-10)
M8
M9
A-

Figure 4. MCM origin association correlates with B2 mutant origin function.

Strains containing *ARS1* mutations were arrested in the G1 phase of the cell cycle with alpha factor and assayed for MCM association by ChIP. PCR was performed on both the immunoprecipitated (IPed) DNA and the input DNA with primers to the origins *ARS305* and *ARS1*, and the non-origin sequence *URA3*. Sample IPed PCR is shown above. The graph represents the quantitation of the (*ARS1* IP/ *ARS1* Input) / (*ARS305* IP/ *ARS305* Input) for four individual experiments. Origin function is plotted on the second axis. The A element mutation is ARS1/865-872 and does not have a plasmid loss rate because plasmids containing this origin cannot transform. M1-M9 refer to the B2 mutants listed in Table 2.

Figure 5

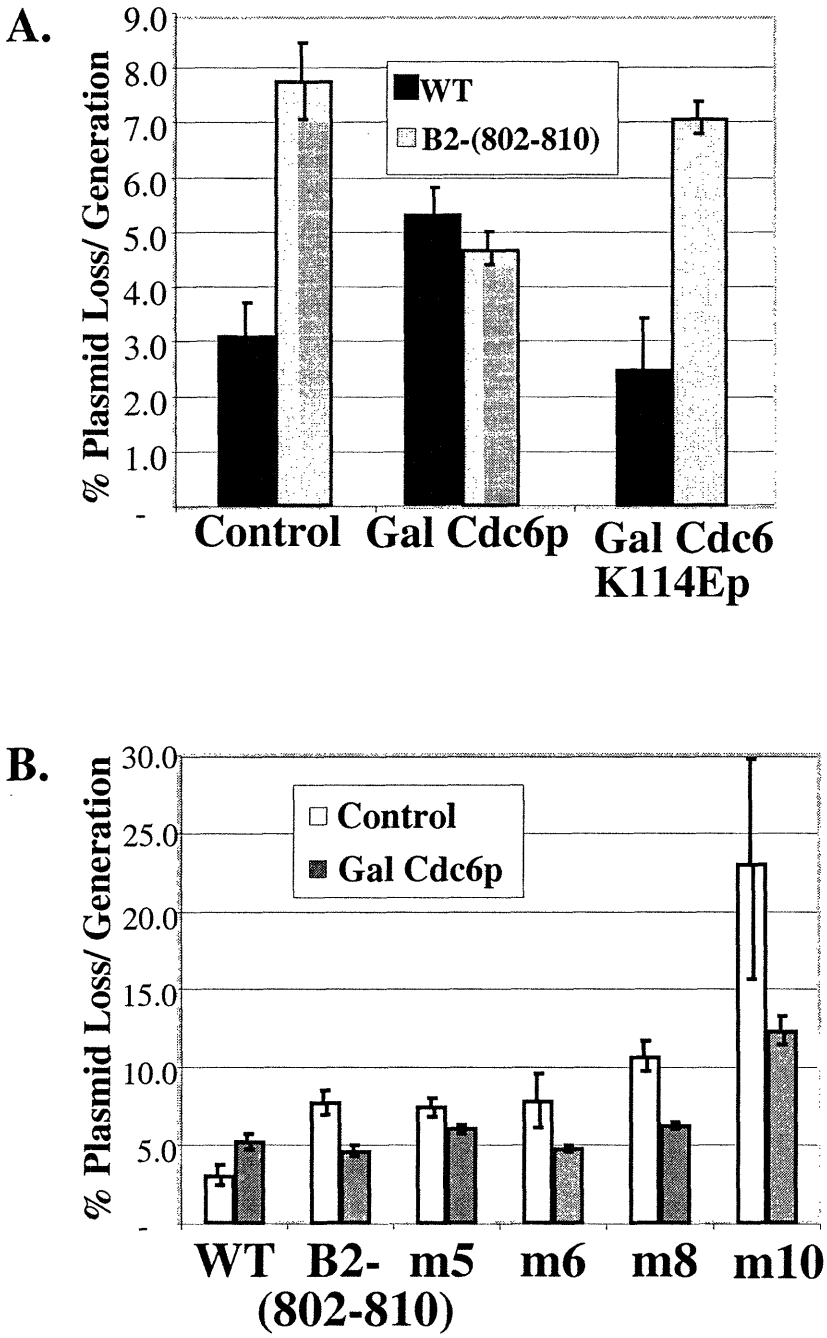

A.
% Plasmid Loss/ Generation
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
-
WT
B2-(802-810)
Control
Gal Cdc6p
Gal Cdc6 K114Ep
B.
% Plasmid Loss/ Generation
30.0
25.0
20.0
15.0
10.0
5.0
-
Control
Gal Cdc6p
WT
B2-(802-810)
m5
m6
m8
m10

Figure 5. Over-expression of wild-type but not mutant Cdc6p rescues B2 mutant origins.

(A) A wild-type plasmid (pARS1/785-92) is lost at a higher rate when wild-type Cdc6p is over-expressed, while the plasmid loss of a B2 mutant plasmid (pARS1/802-810) decreases to the wild-type level. Plasmids were transformed into strains containing wild-type or mutant CDC6 under control of the *Gal1-10* promoter, and the cells grown in media containing 2% galactose.

(B) A series of representative B2 mutant plasmids are improved by galactose-induced over-expression of Cdc6p. Control= no over-expression. Gal= galactose. M5-M10 refer to the B2 mutants listed in Table 2.

Discussion

Using a genetic screen for novel B2 elements, we identified specific sequences required for the function of this element at *ARS1*. These sequences were found to be A-rich and specifically related to the ACS in a manner not explained by their high A-content. Analysis of these sequences suggested that the *ARS1* B2 element is not a DUE, but instead requires a specific sequence. B2 element function correlates with MCM origin loading, and B2 element defects can be ameliorated by over-expressing Cdc6p. These data, combined with the observation that the pre-RC forms a footprint over the B2 element during G1 *in vivo* (Santocanale and Diffley 1996), suggest that B2 is a binding site for a component of the pre-RC.

Sequence and Structure of the B2 Element

Our selection for sequences that can function in place of the B2 element provided evidence against a simple structural role, such as helical instability or DNA bending. The identified $B2^+$ sequences could not have been selected based solely upon helical instability, as they showed a strong bias for A-rich DNA, despite a starting library that was biased for Ts. The nearest neighbor base pair interactions predicted to have the lowest helical stability are 5'-TA-3' and 5'-AT-3', not 5'-AA-3' (Breslauer et al. 1986; SantaLucia et al. 1996; Sugimoto et al. 1996). Moreover, low helical stability is not a predictor of origin function in our sequences, as we find no correlation between origin function and helical instability at *ARS1* (R^2=0.03, Fig. 2). This is in contrast to previous analyses at *ARS307* (Lin and Kowalski 1997). These differences may reflect differences in the two origins, but those analyses also identified outlying points that did not correlate and incorporated a smaller panel of less diverse mutations. Although A-rich DNA is

often bent, the wild-type B2 element is not intrinsically bent (Marahrens and Stillman 1992; Marilley 2000), nor do the sequences we isolated have high calculated curvatures (data not shown, (Munteanu et al. 1998)). Finally, the observation that the B2 element is necessary for pre-RC formation suggests a non-structural role for this sequence as no DNA unwinding has been detected until yeast cells enter S-phase, and the pre-RC is dismantled (Geraghty et al. 2000). Furthermore, the 11 bp *ARS1* B2 element cannot itself be a DUE because it can only substitute for the DUE of *ARS305* in the presence of its AT-rich flanking sequences (Lin and Kowalski 1997).

Although the formal possibility remains that this A-rich DNA is performing an unknown structural function, our data suggest that B2 is a protein binding site related to the ACS. Matches to the ACS were more prevalent in the $B2^+$ sequences than in random computer-generated A-rich sequences (Table 1). The parameters for this selection can be seen in figure 1, where it is apparent that although there is an overall selection for As, certain positions are enriched for Ts, Gs, or Cs. Importantly, these non-A positions match the sequence of the ACS. Also, those positions with the strongest selection for As (positions 2 and 4) are those that are least tolerant of mutation within the A element at *ARS307* (Van Houten and Newlon 1990). Any sequence with a 7/11 match to the ACS functions well as a B2 element, and all of the $B2^+$ sequences contain at least a 6/11 match (Table 3). The B2 element can be shifted slightly in either direction with no effect on origin function (Marahrens and Stillman 1992; Lin and Kowalski 1997), and about 2/3 of our sequences had better matches to the ACS when shifted over one base. This is not surprising because the presence of an A flanking the randomized sequences facilitated the selection of an ACS incorporating only 10 randomized bases.

This flexibility in positioning of the B2 element, the frequent occurrence of near matches to the ACS at origins, and the AT-richness of the ACS have all made uncovering of the functional importance of these sequences difficult at most origins. The presence of many more extra inverse ACS matches than would be expected given the AT-rich nature of yeast origins has been noted (Palzkill and Newlon 1988). It is striking that *ARS1* is the only well-characterized origin with a single additional inverse match to the ACS greater than 7/11 bases in the region over which the pre-RC forms, and this match identifies the B2 element. Therefore, a requirement for extra inverse sequences that match the ACS may not be unique to *ARS1*, but simply the most easily identified.

Several prior studies addressed the role of partial matches to the ACS at other yeast origins. The *H4 ARS* B domain can be replaced by an unrelated easily unwound pBR322 sequence (Umek and Kowalski 1988), and an attempt was made to remove all 8/11 or better matches to the ACS (Holmes and Smith 1989). However, both of these approaches left behind 7/11 and 8/11 matches to the ACS, by the current definition (Van Houten and Newlon 1990), at the approximate distance of the B2 element from the A element. A similar analysis at the *rDNA ARS* only mutated 9/11 matches to the ACS (Walker et al. 1991). Given the uncertainty in where an extra ACS match would have to be placed to function as a B2 element, and that many 7/11 matches were functional in our study, these studies have not fully addressed the importance of a redundant consensus similar to the ACS at other origins.

Function of the B2 Element: pre-RC component binding site?

The simplest interpretation of our sequence data is that the B2 element serves as a second binding site for ORC, because ORC binds the ACS. Multimerization of ORC during G1 would be analogous to the binding of *E.coli* DnaA-ATP to the 13mers embedded within the *E.coli* DUE during initation to facilitate open complex formation (Bramhill and Kornberg 1988; Speck and Messer 2001). The rescue of $B2^-$ origin function by Cdc6p could be mediated indirectly through improved ORC binding, as Cdc6p over-expression also rescues an *Orc5-1* mutation (Liang et al. 1995). If ORC were to bind this site *in vivo* it would have to be assisted by other factors, and bind in a different conformation. Two purified ORCs cannot bind to the A and B2 elements at the same time (Bell and Stillman 1992) unless 2-3 extra bps are inserted between them (R. Austin and S.P.B., unpublished results), or the smallest subunit, Orc6p, is left out of the complex (R. Klemm and S.P.B., unpublished results and Fig. 3). Also, the binding of ORC to the $B2^+$ sequences *in vitro* was not detected within the stringency of the Dnase I protection assay (Fig. 3). We cannot rule out the binding of a second ORC to the B2 element *in vivo* as a possibility, and current *in vivo* binding assays (e.g. ChIP) cannot detect the stoichiometry of ORC in the pre-RC.

Another candidate B2-binding protein is the Orc1p-related protein Cdc6p. The recent crystal structure of the archaeon *Pyrobaculum aerophilium* Cdc6p revealed a winged-helix DNA binding domain that is conserved in *S. cerevisiae* Cdc6p, Orc1p, Orc4p, and in the *S. pombe* ortholog Cdc18p. Furthermore, this conservation in CDC6 proteins is important for function, as mutations in the putative DNA binding domain of

Cdc18p cause cell cycle arrests or null phenotypes (Liu et al. 2000). This raises the possibility that Cdc6p is recruited to the origin not solely through its interactions with ORC (Wang et al. 1999; Mizushima et al. 2000) but also by binding the sequence related to the ACS at the B2 element. This is supported by the requirement for the B2 element in MCM loading, and by the observation that over-expressing Cdc6p ameliorates the defects in a wide range of B2 mutants. On the other hand, although DNA binding activity has been observed for purified Cdc6p, it is non-specific and mediated by the non-essential N-terminus (Feng et al. 2000). Moreover, ORC interactions may be sufficient to recruit Cdc6p to the origin, and the winged helix domain could be involved in protein-protein interactions instead of protein-DNA interactions (Gajiwala and Burley 2000). Although CDC6 appears to be a good candidate protein for binding the B2 element, we have not been able to use ChIP to directly test this model because of the low protein levels and tight cell cycle control of origin association.

Finally, it is possible that the B2 element is a binding site for another protein in the pre-RC, such as the Mcm2-7p complex, Mcm10p, or an *S. cerevisiae CDT1* ortholog. It is reasonable that the MCM proteins, which are the putative eukaryotic replicative helicase, would associate with DNA embedded within the easily unwound region of the origin. Furthermore, the rescue of the $B2^-$ plasmids by Cdc6p over-expression could also have been mediated through increased MCM loading, as has previously been observed on bulk chromatin (Perkins and Diffley 1998). Very little is known about how Mcm10p and Cdt1p associate with the origin.

By specifically randomizing the well-defined B2 element at *ARS1,* we have identified an ACS-related sequence requirement for this origin component. Because of

redundancy at most origins this ACS-related sequence has not previously been identified as functionally important. We also found that the *ARS1* B2 element is not simply a DUE, and suggest that specific A-rich sequences like the B2 element are embedded within broad DUEs at yeast origins. We suggest that ORC, Cdc6p, or the MCMs are the most likely proteins to bind this site. *In vitro* eukaryotic DNA replication systems should allow the direct testing of this model, along with a clearer picture of the stoichiometry and architecture of the pre-RC.

Experimental Procedures

Plasmids, Strains, and Library Construction

Strains (W303 background) and plasmids were prepared using standard laboratory methods (Ausubel 1992). Plasmids pARS1/785-92ADE2 and pARS1/802-810ADE2 were created by cloning the SalI/NcoI fragment of pASZ10 (Stotz and Linder 1990) containing the *ADE2* gene into the SalI and NcoI sites of pARS1/785-92 and pARS1/802-810 (Marahrens and Stillman 1992), replacing part of the *URA3* gene. pARS1/785-92 was used instead of pARS1WT because the XhoI site created by the mutation facilitates cloning, but has no effect on origin function (Marahrens and Stillman 1992). The plasmid pARS1/785-92ADE2SIR3 was created by cloning the XhoI to PstI fragment of *SIR3* into the XhoI to PstI sites of pARS1/785-92ADE2. This plasmid facilitated cloning of the library because the large fragment of the library was easier to cut out completely from the vector than the small 20 base pair fragment it replaced. The *ARS1* B2 mutant library was created by primer extension of the primers B2MUT1 (TATTACCTCGAGGTATTTNNNNNNNNNNGTTTGTGCACTTGCCTGCAGGCC TTTT) and B2MUT2 (AAAAGGCCTGCAGGCAAGTGC), synthesized by Integrated DNA Technologies, and cloned into the XhoI and PstI sites of pARS1/785-92ADE2SIR3. Strains used in ChIP were created as described (Lipford and Bell 2001). Cdc6p over-expression strains were created as described (Klemm and Bell 2001).

$B2^-$ Selection and $B2^+$ Screen

$B2^-$ plasmids were identified by streaking yeast colonies from a transformation with the library onto minimal complete media, growing for two days at 30°C, and then incubating for a week at 4°C to allow the red color indicative of an *ade2* mutation to develop. Yeast that turned red were losing the *ADE2* plasmid at a high rate, because the B2 elements did not allow for efficient replication of the plasmid. Under these conditions, streaks from pARS1/785-92ADE2 (wild-type B2 element) transformants were white, streaks from pARS1/802-810ADE2 (mutant B2 element) transformants were pink, and streaks from pARS1/785-92ADE2SIR3 (large insertion in the origin) transformants were red. Streaks were replica plated to plates lacking adenine, and plasmids were recovered from replicas of streaks that turned red on minimal complete media. $B2^+$ plasmids were selected by growing pools of approximately 1000 yeast library transformants together in liquid media including supplemental adenine. The cultures were maintained in log phase for ten days by dilution, and then plated onto media lacking adenine.

Plasmid Loss Assays

Plasmid loss assays for origin function were performed as described (Marahrens and Stillman 1992), with the exception that selection was for the *ADE2* gene, not *URA3*. Media was supplemented with 0.1 mg/ml adenine. Plasmid loss rates with adenine selection were consistently lower than those with uracil selection , but the relative measurements were the same (data not shown).

Calculations

Helical stability calculations were done with the program WebThermodyn (http://wings.buffalo.edu/gsa/dna/dk/DWEBTHERMODYN/ (Natale et al. 1992)), using the data sets of Santa Lucia et al. (SantaLucia et al. 1996). Calculations included the 11 bases of the B2 element plus one base on either side. Statistical analyses, random sequence generations, and calculations of matches to the ACS were done by the program Excel (Microsoft). The means of different samples were compared with two-tailed student's t-tests, assuming equal variance.

Chromatin Immunoprecipitation (ChIP)

ChIP was performed as described (Klemm and Bell 2001), except PCR was performed for 28 cycles on $1/50^{th}$ of the IP or $1/10{,}000^{th}$ of the input. Quantitation was done with a ChemiImager5500 (Alpha Innotech) and accompanying software.

DNase I Protection Assays

DNase I protection assays were performed as described (Bell et al. 1995) except that all reactions contained 1.4 mM ATP. Baculovirus ORC, and ORC1-5p were purified as described previously (Lee and Bell 1997).

References

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

Austin, R.J., T.L. Orr-Weaver, and S.P. Bell. 1999. Drosophila ORC specifically binds to ACE3, an origin of DNA replication control element. *Genes Dev* **13**: 2639-49.

Ausubel, F.M. 1992. *Current protocols in molecular biology*. Published by Greene Pub. Associates and Wiley-Interscience : J. Wiley, New York.

Bell, S.P., J. Mitchell, J. Leber, R. Kobayashi, and B. Stillman. 1995. The multidomain structure of Orc1p reveals similarity to regulators of DNA replication and transcriptional silencing. *Cell* **83**: 563-8.

Bell, S.P. and B. Stillman. 1992. ATP-dependent recognition of eukaryotic origins of DNA replication by a multiprotein complex. *Nature* **357**: 128-34.

Bramhill, D. and A. Kornberg. 1988. Duplex opening by dnaA protein at novel sequences in initiation of replication at the origin of the E. coli chromosome. *Cell* **52**: 743-55.

Breslauer, K.J., R. Frank, H. Blocker, and L.A. Marky. 1986. Predicting DNA duplex stability from the base sequence. *Proc Natl Acad Sci U S A* **83**: 3746-50.

DePamphilis, M.L. 1999. Replication origins in metazoan chromosomes: fact or fiction? *Bioessays* **21**: 5-16.

Diffley, J.F., J.H. Cocker, S.J. Dowell, J. Harwood, and A. Rowley. 1995. Stepwise assembly of initiation complexes at budding yeast replication origins during the cell cycle. *J Cell Sci Suppl* **19**: 67-72.

Donovan, S., J. Harwood, L.S. Drury, and J.F. Diffley. 1997. Cdc6p-dependent loading of Mcm proteins onto pre-replicative chromatin in budding yeast. *Proc Natl Acad Sci U S A* **94**: 5611-6.

Elsasser, S., F. Lou, B. Wang, J.L. Campbell, and A. Jong. 1996. Interaction between yeast Cdc6 protein and B-type cyclin/Cdc28 kinases. *Mol Biol Cell* **7**: 1723-35.

Feng, L., B. Wang, B. Driscoll, and A. Jong. 2000. Identification and characterization of Saccharomyces cerevisiae Cdc6 DNA-binding properties. *Mol Biol Cell* **11**: 1673-85.

Gajiwala, K.S. and S.K. Burley. 2000. Winged helix proteins. *Curr Opin Struct Biol* **10**: 110-6.

Geraghty, D.S., M. Ding, N.H. Heintz, and D.S. Pederson. 2000. Premature structural changes at replication origins in a yeast minichromosome maintenance (MCM) mutant. *J Biol Chem* **275**: 18011-21.

Holmes, S.G. and M.M. Smith. 1989. Interaction of the H4 autonomously replicating sequence core consensus sequence and its 3'-flanking domain. *Mol Cell Biol* **9**: 5464-72.

Huang, R.Y. and D. Kowalski. 1993. A DNA unwinding element and an ARS consensus comprise a replication origin within a yeast chromosome. *Embo J* **12**: 4521-31.

-. 1996. Multiple DNA elements in ARS305 determine replication origin activity in a yeast chromosome. *Nucleic Acids Res* **24**: 816-23.

Klemm, R.D. and S.P. Bell. 2001. ATP bound to the origin recognition complex is important for preRC formation. *Proc Natl Acad Sci U S A* **98**: 8361-7.

Kowalski, D. and M.J. Eddy. 1989. The DNA unwinding element: a novel, cis-acting component that facilitates opening of the Escherichia coli replication origin. *Embo J* **8**: 4335-44.

Lee, D.G. and S.P. Bell. 1997. Architecture of the yeast origin recognition complex bound to origins of DNA replication. *Mol Cell Biol* **17**: 7159-68.

Liang, C., M. Weinreich, and B. Stillman. 1995. ORC and Cdc6p interact and determine the frequency of initiation of DNA replication in the genome. *Cell* **81**: 667-76.

Lin, S. and D. Kowalski. 1994. DNA helical instability facilitates initiation at the SV40 replication origin. *J Mol Biol* **235**: 496-507.

-. 1997. Functional equivalency and diversity of cis-acting elements among yeast replication origins. *Mol Cell Biol* **17**: 5473-84.

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

Liu, J., C.L. Smith, D. DeRyckere, K. DeAngelis, G.S. Martin, and J.M. Berger. 2000. Structure and function of Cdc6/Cdc18: implications for origin recognition and checkpoint control. *Mol Cell* **6**: 637-48.

Maiorano, D., J. Moreau, and M. Mechali. 2000. XCDT1 is required for the assembly of pre-replicative complexes in Xenopus laevis. *Nature* **404**: 622-5.

Marahrens, Y. and B. Stillman. 1992. A yeast chromosomal origin of DNA replication defined by multiple functional elements. *Science* **255**: 817-23.

Marilley, M. 2000. Structure-function relationships in replication origins of the yeast Saccharomyces cerevisiae: higher-order structural organization of DNA in regions flanking the ARS consensus sequence. *Mol Gen Genet* **263**: 854-66.

Matsumoto, K. and Y. Ishimi. 1994. Single-stranded-DNA-binding protein-dependent DNA unwinding of the yeast ARS1 region. *Mol Cell Biol* **14**: 4624-32.

Mizushima, T., N. Takahashi, and B. Stillman. 2000. Cdc6p modulates the structure and DNA binding activity of the origin recognition complex in vitro. *Genes Dev* **14**: 1631-41.

Munteanu, M.G., K. Vlahovicek, S. Parthasarathy, I. Simon, and S. Pongor. 1998. Rod models of DNA: sequence-dependent anisotropic elastic modelling of local bending phenomena. *Trends Biochem Sci* **23**: 341-7.

Natale, D.A., A.E. Schubert, and D. Kowalski. 1992. DNA helical stability accounts for mutational defects in a yeast replication origin. *Proc Natl Acad Sci U S A* **89**: 2654-8.

Natale, D.A., R.M. Umek, and D. Kowalski. 1993. Ease of DNA unwinding is a conserved property of yeast replication origins. *Nucleic Acids Res* **21**: 555-60.

Nishitani, H., Z. Lygerou, T. Nishimoto, and P. Nurse. 2000. The Cdt1 protein is required to license DNA for replication in fission yeast. *Nature* **404**: 625-8.

Ogawa, Y., T. Takahashi, and H. Masukata. 1999. Association of fission yeast Orp1 and Mcm6 proteins with chromosomal replication origins. *Mol Cell Biol* **19**: 7228-36.

Palzkill, T.G. and C.S. Newlon. 1988. A yeast replication origin consists of multiple copies of a small conserved sequence. *Cell* **53**: 441-50.

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

Rao, H., Y. Marahrens, and B. Stillman. 1994. Functional conservation of multiple elements in yeast chromosomal replicators. *Mol Cell Biol* **14**: 7643-51.

SantaLucia, J., Jr., H.T. Allawi, and P.A. Seneviratne. 1996. Improved nearest-neighbor parameters for predicting DNA duplex stability. *Biochemistry* **35**: 3555-62.

Santocanale, C. and J.F. Diffley. 1996. ORC- and Cdc6-dependent complexes at active and inactive chromosomal replication origins in Saccharomyces cerevisiae. *Embo J* **15**: 6671-9.

Seki, T. and J.F. Diffley. 2000. Stepwise assembly of initiation proteins at budding yeast replication origins in vitro. *Proc Natl Acad Sci U S A* **97**: 14115-20.

Speck, C. and W. Messer. 2001. Mechanism of origin unwinding: sequential binding of DnaA to double- and single-stranded DNA. *Embo J* **20**: 1469-76.

Stotz, A. and P. Linder. 1990. The ADE2 gene from Saccharomyces cerevisiae: sequence and new vectors. *Gene* **95**: 91-8.

Sugimoto, N., S. Nakano, M. Yoneyama, and K. Honda. 1996. Improved thermodynamic parameters and helix initiation factor to predict stability of DNA duplexes. *Nucleic Acids Res* **24**: 4501-5.

Tanaka, T., D. Knapp, and K. Nasmyth. 1997. Loading of an Mcm protein onto DNA replication origins is regulated by Cdc6p and CDKs. *Cell* **90**: 649-60.

Theis, J.F. and C.S. Newlon. 1994. Domain B of ARS307 contains two functional elements and contributes to chromosomal replication origin function. *Mol Cell Biol* **14**: 7652-9.

Umek, R.M. and D. Kowalski. 1988. The ease of DNA unwinding as a determinant of initiation at yeast replication origins. *Cell* **52**: 559-67.

Van Houten, J.V. and C.S. Newlon. 1990. Mutational analysis of the consensus sequence of a replication origin from yeast chromosome III. *Mol Cell Biol* **10**: 3917-25.

Walker, S.S., A.K. Malik, and S. Eisenberg. 1991. Analysis of the interactions of functional domains of a nuclear origin of replication from Saccharomyces cerevisiae. *Nucleic Acids Res* **19**: 6255-62.

Wang, B., L. Feng, Y. Hu, S.H. Huang, C.P. Reynolds, L. Wu, and A.Y. Jong. 1999. The essential role of Saccharomyces cerevisiae CDC6 nucleotide-binding site in cell growth, DNA synthesis, and Orc1 association. *J Biol Chem* **274**: 8291-8.

Weinreich, M., C. Liang, and B. Stillman. 1999. The Cdc6p nucleotide-binding motif is required for loading mcm proteins onto chromatin. *Proc Natl Acad Sci U S A* **96**: 441-6.

Whittaker, A.J., I. Royzman, and T.L. Orr-Weaver. 2000. Drosophila double parked: a conserved, essential replication protein that colocalizes with the origin recognition complex and links DNA replication with mitosis and the down-regulation of S phase transcripts. *Genes Dev* **14**: 1765-76.

Zou, L. and B. Stillman. 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**: 3086-96.

Chapter III

Interaction of the S-phase cyclin Clb5 with an 'RXL' docking sequence in the initiator protein Orc6 is a mechanism to prevent re-replication

A revised version of this chapter will be submitted to Genes and Development with the following authors: Gwendolyn M. Wilmes, Vincent Archambault, Richard J. Austin, Matthew D. Jacobson, Stephen P. Bell, Fred R. Cross. Experiments shown in Figures 4, 5, and 7 were performed by G.M.W. The experiments shown in Figure 2 were performed by G.M.W. and R.J.A. Experiments shown in Figures 3 and 6 were performed by V.A. The experiment shown in Figure 1 was performed by M.D.J. and F.R.C.

I would like to thank Joachim Li for the gift of ORC2-ps plasmids and MCM7NLS plasmids. I would also like to thank Hannah Blitzblau, John Randell, Robyn Tanny, and Milan de Vries, for helpful discussions.

Summary

We show that the S-phase cyclin Clb5 binds stably and directly to the origin recognition complex (ORC). This interaction is mediated by the recognition of an 'RXL' target sequence in the Orc6 subunit by the 'hydrophobic patch' region on Clb5. This interaction is unique to Clb5, as the mitotic cyclin Clb2 does not show RXL-dependent interaction with Orc6. Chromatin immunoprecipitation studies show that the Clb5-Orc6 interaction occurs at origins of replication close to the time of initiation and is maintained throughout the remainder of S phase and into M phase. Eliminating the Clb5-Orc6 interaction sensitizes cells to lethal over-replication. We propose that the Clb5-Orc6 interaction and pre-Replicative Complex formation are mutually exclusive, yielding an origin-localized replication control switch.

Introduction

The eukaryotic cell cycle is controlled by oscillations in cyclin-dependent kinase (CDK) activity, resulting from a complex web of regulated cyclin transcription, proteolysis, and inhibitor accumulation. CDK activity oscillations are required because critical cell-cycle steps are both positively and negatively regulated by CDK activity. A well-characterized example is DNA replication (Bell and Dutta 2002): CDK activity is required for origin firing, but at the same time CDK activity inhibits the assembly of essential initiation components onto origins of replication. As a result, there is an obligatory oscillation of CDK activity to obtain one complete round of replication. A similar ratcheting mechanism coupling the events of mitosis to CDK activity results in alternating cycles of replication and chromosome segregation; this alternation is essential to maintain normal ploidy.

In budding yeast, origins of replication are bound throughout the cell cycle by the six-member Origin Recognition Complex (ORC) (Diffley et al. 1994; Aparicio et al. 1997; Liang and Stillman 1997; Tanaka et al. 1997). The Cdc6 and Cdt1 proteins interact with the ORC-bound origin and direct the loading of the six-member Mcm2-7 complex (Aparicio et al. 1997; Tanaka et al. 1997; Devault et al. 2002; Tanaka and Diffley 2002). The resulting structure is called the 'pre-Replicative Complex' or pre-RC. Elevated levels of CDK activity inhibit pre-RC formation and therefore these structures only form during late mitosis and the G1 phase of the cell cycle when CDK activity is low. As cells enter S phase, elevated levels of CDK activity stimulate initiation of DNA replication from these structures and simultaneously blocks any new pre-RC formation during the

remainder of the cell cycle. After initiation, ORC remains at the origins in the absence of other members of the pre-RC, forming a post-Replicative Complex (post-RC).

The mechanism by which CDK activity regulates DNA replication is not fully understood. Many components of the replication machinery are phosphorylated by CDKs, including ORC, the Mcm2-7 complex and Cdc6, however, only the phosphorylation of the Sld2/Drc1 replication protein has been demonstrated to be essential for replication initiation (Masumoto et al. 2002). More is known about the mechanisms by which CDK activity inhibits pre-RC formation. In *S. cerevisiae*, this inhibition is mediated by phosphorylation of Cdc6, ORC and the Mcm2-7 complex (Nguyen et al. 2001). Phosphorylation of Cdc6 results in its SCF-dependent proteolysis (Drury et al. 1997; Calzada et al. 2000; Drury et al. 2000; Jang et al. 2001; Perkins et al. 2001), and phosphorylation of Mcm2-7 complex leads to its exclusion from the nucleus (Labib et al. 1999; Nguyen et al. 2000). Two ORC subunits are modified by CDK activity and mutation of these phosphorylation sites is required to observe inappropriate re-replication in *S. cerevisiae* (Nguyen et al. 2001). Unlike the modification of Cdc6 and the Mcm2-7 complex, the mechanism(s) by which these modifications inhibit re-replication remains undetermined.

Most CDK regulation of replication in budding yeast involves the six B-type cyclins *CLB1,2,3,4,5,6* (Schwob et al. 1994), all of which bind to the same CDK catalytic subunit, Cdc28. These proteins overlap in their functions but still have significant specificity: Clb1,2,3 and 4 largely drive mitosis, while Clb5 and 6 largely trigger replication (Miller and Cross 2001). Clb5 and 6 are expressed earlier in the cell cycle than Clb1,2,3 and 4, but the functional differences between these cyclin classes are

not simply the result of differences in cell-cycle timing of expression. Therefore, some intrinsic difference in the cyclin protein leads to functional differences. The particular cyclins involved in the inhibition of pre-RC formation have not been clearly defined.

It has been proposed that different cyclins may enhance CDK phosphorylation of different targets, by the action of substrate-targeting regions on the cyclins. In support of this hypothesis, the 'hydrophobic patch' region of cyclins interacts with known targets containing the 'RXL' or 'Cy' motif (Schulman et al. 1998; Cross and Jacobson 2000; Wohlschlegel et al. 2001). For example, the hydrophobic patch region of cyclin A interacts with 'RXL' motifs found in cyclin A-CDK2 phosphorylation targets and inhibitors, including the p27 inhibitor (Schulman et al. 1998; Adams et al. 1999; Takeda et al. 2001; Wohlschlegel et al. 2001). The residues making up the hydrophobic patch are highly conserved in the cyclin superfamily and this motif is required for efficient function of the yeast S phase cyclin Clb5 (Cross and Jacobson 2000). Although the hydrophobic patch motif is clearly important for substrate recognition, it remains unclear whether this motif mediates the differential substrate-recognition that contributes to cyclin functional specificity.

Here we report that the Clb5 hydrophobic patch mediates an interaction with an RXL sequence (and CDK phosphorylation sites) in Orc6. In contrast, the mitotic Clb2 cyclin interacts only weakly with Orc6, in an RXL-independent manner. We show that this interaction occurs at origins of replication in a manner that correlates with the time of replication initiation and is maintained at origins throughout S phase, suggesting that Clb5/Cdc28 is part of the post-RC. Analysis of cells containing a mutation in the Orc6 RXL motif and phosphorylation sites shows that these cells are much more vulnerable to

lethal re-replication (synthetic lethality with other known re-replication mutants and an increase past 2C DNA content by flow cytometry). We propose that the association of Clb5 with ORC occurs only after the pre-RC is dismantled, rapidly protecting origins that have undergone initiation or passive replication from new pre-RC formation.

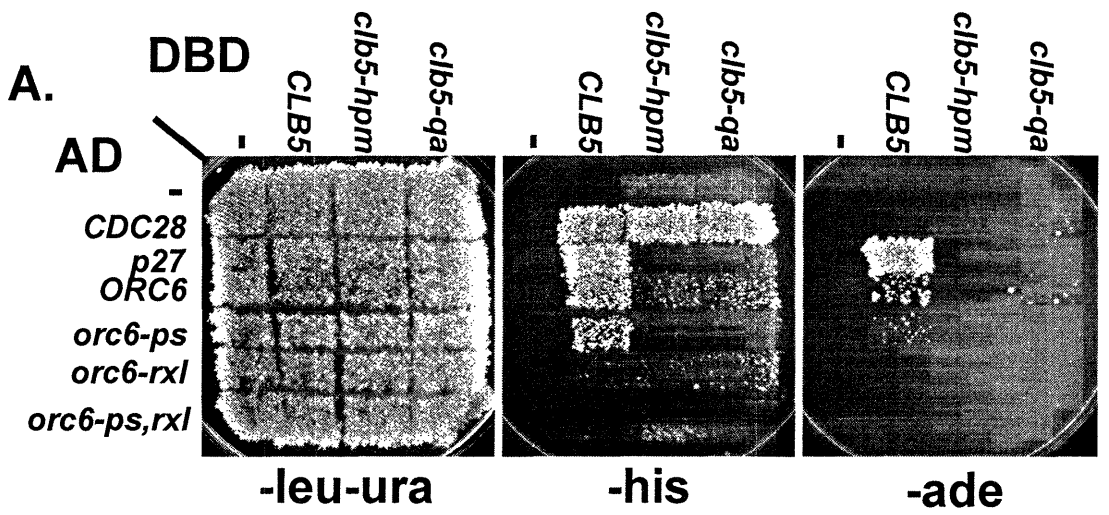
Results

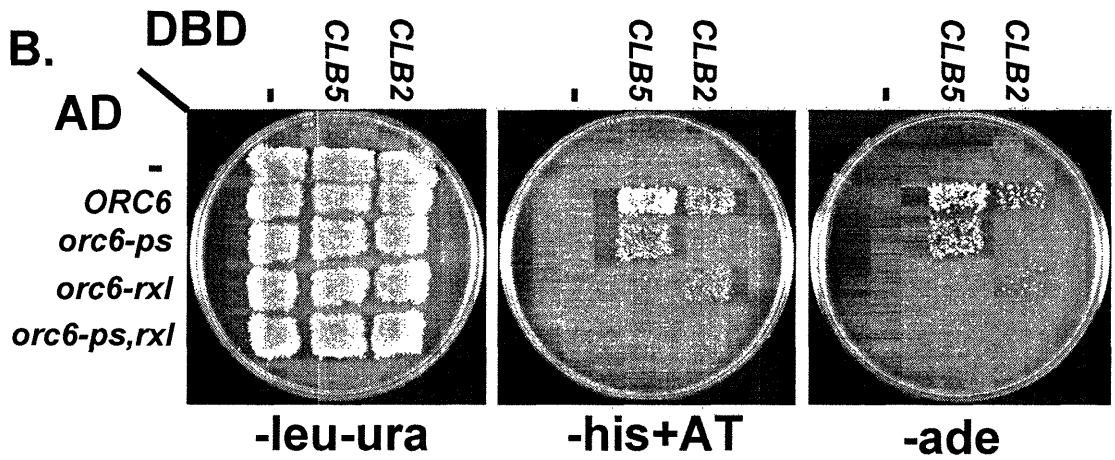
Identification of Orc6 as an RXL-containing binding partner of Clb5.

Endogenous CDK targets recognized by the Clb5 hydrophobic patch are unknown. Based on the functional importance of the Clb5 patch, such endogenous targets seemed likely to be important for Clb5-regulated pathways. To identify such targets, we carried out a 2-hybrid screen using two different bait constructs: a full-length Clb5-Gal4 DNA-binding domain (Clb5-DBD) and a Clb5-DBD with a triply mutated hydrophobic patch (M196A L201A W204A or 'hpm'). The hpm mutation removes most of the hydrophobic residues that interact with the hydrophobic 'RXL' target (Russo et al. 1996; Schulman et al. 1998; Brown et al. 1999). Using a library of yeast DNA segments fused to the Gal4 activation domain (AD) (James et al. 1996), we looked for AD fusions that would activate transcription in a hydrophobic patch-dependent manner. Screening of the library resulted in only the identification of an Orc6-AD clone that activated transcription when present with Clb5-DBD but not with Clb5-DBD-hpm (Figure 1A).

To confirm dependence of the Clb5-DBD interaction with Orc6-AD on the Clb5 hydrophobic patch, we tested the Q241A ('qa') mutation that eliminates a hydrogen-bonding interaction in the binding pocket (Brown et al. 1999). This mutation thus inactivates the function of the binding pocket by a biochemically distinct mechanism. The Orc6-Clb5 interaction was almost as strongly dependent on the Clb5 binding pocket as was the p27-Clb5 interaction (Fig. 1A). As a control, the mutant Clb5-DBD constructs interacted as well as wild-type Clb5-DBD with Cdc28-AD. Therefore, the hydrophobic patch-defective Clb5-DBD mutants had a specific Orc6 interaction defect.

Figure 1





C.

```
                                  A  A
S. cer.  --IPELPPMQTNESPSITRRKLAFEEDEDEDEEEPGND--
S. bay.  --IPELPPAQNPETPSTARRKLAFEEEEEEEEAEDNDL--
S. mik.  --IPVLPPMQTNDAPPVTRRKLAFEEEETHDEELGSKC--
S. par.  --IPELPPMQTNESPPITRRKLAFEEEDDDEEESENSN--
S. cas.  --VKLSPGNLNTDSPIKARRKLAFEDSQSDSEQDEDSH--
                  *        *    *******       *
```

Figure 1. 2-hybrid analysis of Clb5-Orc6 interaction.

(A) Clb5 interaction with Orc6 is dependent on the Clb5 hydrophobic patch and an 'RXL' motif in Orc6. Interaction of Clb5, Clb5-hpm, and Clb5-qa (Q241A) with *CDC28*-AD, p27-AD (Cross and Jacobson, 2000), the *ORC6*-AD clone identified from the GAD-fusion library (James et al., 1996) and its mutagenized derivatives ('*ps*': S106A, S116A, S123A, T146A; '*rxl*': R178A L180A; '*ps,rxl*': combined *ps*, *rxl*) is detected by growth on -his and -ade. Interaction of Clb5-DBD with Cdc28-AD is hydrophobic-patch independent, while interaction with p27-AD is hydrophobic-patch-dependent (Cross and Jacobson, 2000). (For unknown reasons the Clb5-Cdc28 interaction is relatively weak, and is essentially not detected using the -ade reporter.)

(B) Interaction of Orc6 with Clb2 is weaker than interaction with Clb5, and depends on the Orc6 phosphorylation sites but not the Orc6 RXL motif. Methods as in (A), except that 10 mM amino-triazole had to be included in the -his selection due to weak endogenous activating activity of Clb2-DBD (data not shown).

(C) The 'RXL' motif is conserved in budding yeasts. Alignment of *ORC6* homologs from *S. cerevisae*, *S. bayanus*, *S. mikatae,S. paradoxus*, and *S. castellii* (Cliften et al. 2003; Kellis et al. 2003) in the vicinity of R178; complete conservation is indicated by '*'. The location of the R178A, L180A mutation is shown above.)

Orc6 has been proposed to be a Clb5-Cdc28 phosphorylation target (Weinreich et al. 2001). Therefore, we asked whether CDK phosphorylation sites in Orc6 (Nguyen et al. 2001) contributed to the Clb5-Orc6 2-hybrid interaction. Mutation of four CDK consensus phosphorylation sites (S106A, S116A, S123A, T146A) in Orc6 reduced the interaction. The weak residual interaction between Orc6-AD and the Clb5-DBD 'hpm' and 'qa' mutants was completely dependent on the Orc6 phosphorylation sites (Figure 1A).

We identified a candidate 'RXL' consensus sequence from Orc6 at positions 178-180 (RRKLA) that is highly conserved among budding yeast species (Figure 1C). Mutation of this sequence to RAKAA (Orc6-rxl-AD) strongly reduced the 2-hybrid interaction, similarly to the effect of the Clb5 hpm or qa mutations. Residual binding of Clb5-DBD to the Orc6-rxl-AD mutant was completely eliminated by mutation of the Orc6 phosphorylation sites (Orc6-ps,rxl-AD; Figure 1A).

Orc6-AD interacted strongly with Clb5-DBD, but more weakly with Clb2-DBD (Figure 1B). Clb2-DBD interaction with Orc6-AD was dependent on the Orc6 phosphorylation sites but did not require the Orc6 RXL (Figure 1B). Clb2-DBD interaction with Orc6-AD was also independent of the Clb2 hydrophobic patch (data not shown). As a control, Clb2-DBD interacted better with Cdc28-AD than did Clb5-DBD (data not shown), so the partial defect of Clb2-DBD compared to Clb5-DBD in Orc6-AD interaction is specific.

Overall, the 2-hybrid data are consistent with the Clb5-Orc6 interaction depending primarily on the Orc6 RXL interacting with the Clb5 hydrophobic patch, and secondarily on the Orc6 phosphorylation sites interacting with Clb5 in a hydrophobic-

patch-independent manner. The hydrophobic patch-RXL interaction was specific to Clb5, while the Orc6 phosphorylation-site-dependent interaction was common to Clb5 and Clb2. The fact that the Orc6 RXL interacted specifically with the Clb5 hydrophobic patch and not the Clb2 hydrophobic patch confirms the proposal (Cross and Jacobson, 2000)(Brown et al. 1999; Takeda et al. 2001; Wohlschlegel et al. 2001) that this region of cyclins confers cyclin-specific binding.

Clb5/Cdc28 Interacts with ORC *in vitro*

To determine if the interactions identified in the two hybrid screen were direct, we tested whether the proteins interacted *in vitro*. We purified Clb5/Cdc28 and Clb5hpm/Cdc28 from baculovirus infected insect cells using a GST tag on Cdc28. We then tested whether recombinant Clb5/Cdc28 could interact with an ORC-origin DNA complex by means of an electrophoretic mobility shift assay (EMSA, Figure 2A). We found that wild-type Clb5-Cdc28 shifted an ORC-origin DNA complex whereas an equal amount of Clb5hpm/Cdc28 did not. Next, we tested whether this interaction between ORC and Clb5/Cdc28 was dependent upon the Orc6 subunit by testing whether wild-type Clb5/Cdc28 could shift an ORC/origin complex lacking Orc6 (Orc1-5). Orc1-5 still binds DNA as well as wild-type ORC (Lee and Bell 1997; Wilmes and Bell 2002). Addition of Clb5/Cdc28 to Orc1-5 DNA binding assays showed no evidence of a Clb5/Cdc28- dependent supershift.

It remained possible that the altered mobility of the ORC-origin complex was a result of an Orc6-dependent modification of ORC by Clb5-Cdc28 rather than a direct

Figure 2

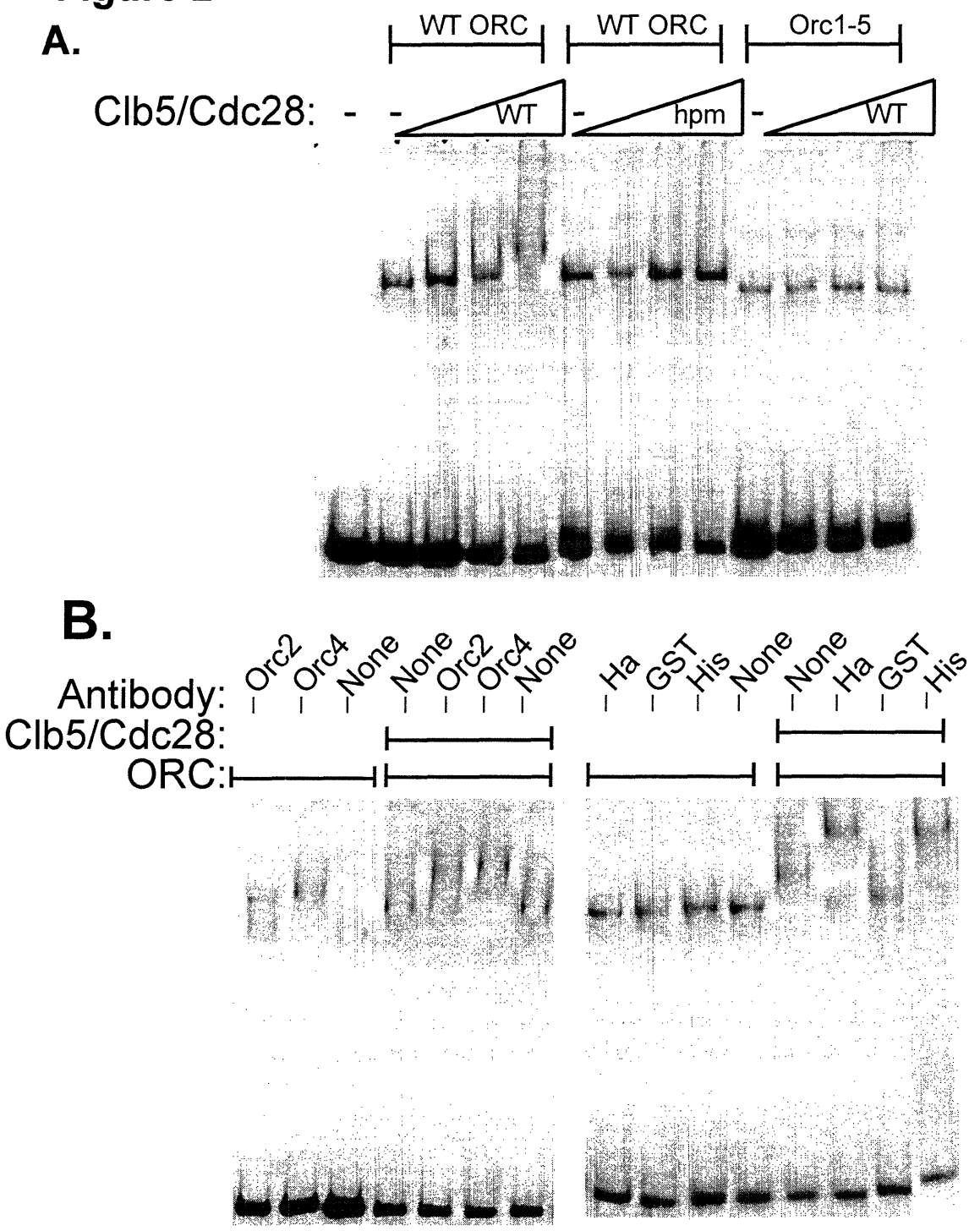

A.
WT ORC
WT ORC
Orc1-5
Clb5/Cdc28:
WT
hpm
WT
B.
Antibody:
Clb5/Cdc28:
ORC:
Orc2
Orc4
None
None
Orc2
Orc4
None
Ha
GST
His
None
None
Ha
GST
His

Figure 2

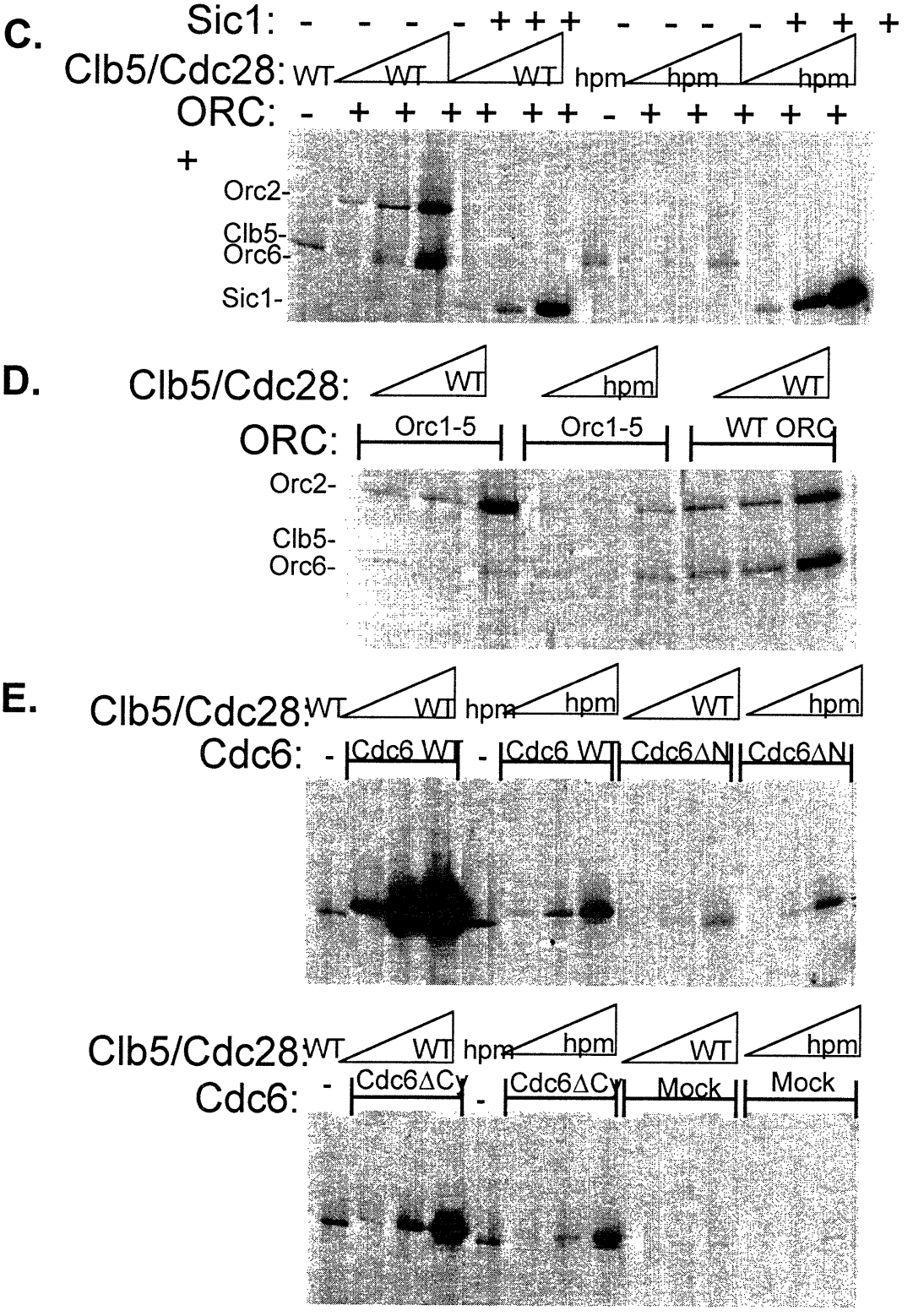

C.
Sic1: - - - - + + + - - - - + + +
Clb5/Cdc28: WT WT WT hpm hpm hpm
ORC: - + + + + + + - + + + + + +
+
Orc2-
Clb5-
Orc6-
Sic1-
D.
Clb5/Cdc28: WT hpm WT
ORC: Orc1-5 Orc1-5 WT ORC
Orc2-
Clb5-
Orc6-
E.
Clb5/Cdc28: WT WT hpm hpm WT hpm
Cdc6: - Cdc6 WT - Cdc6 WT Cdc6ΔN Cdc6ΔN
Clb5/Cdc28: WT WT hpm hpm WT hpm
Cdc6: - Cdc6ΔCy - Cdc6ΔCy Mock Mock

Figure 2. *In vitro* interaction between Clb5/Cdc28 and Orc6.

(A) Clb5/Cdc28 binds an ORC/Origin DNA complex in a hydrophobic patch and Orc6 dependent manner. 20 ng of ORC shifted a radiolabeled RI/HIII fragment of *ARS1*. Addition of wt but not hpm Clb5/Cdc28 supershifts the ORC/origin complex, but not an ORC/origin complex lacking Orc6. Amounts of Clb5/Cdc28 added were 80, 330, and 1320 ng, although the Clb5 was substoichiometric. The higher band present in the ORC/origin DNA lanes represents a multimer of ORC or Orc1-5.

(B) Clb5, Cdc28, and ORC are all present in the Clb5-dependent supershift. 600 ng of Clb5/Cdc28 was added to 20 ng of ORC and the radiolabeled *ARS1* probe as in A. Clb5 is HA and His tagged. Cdc28 is HA and GST tagged. Antibodies added were ORC2: 1 µl 1:150 SB61 , 1 µl 1:150 ORC4 SB6 , HA: 1 µl Ha.11 (Babco, Richmond, CA), GST: 0.2 µl (Amersham Pharmacia, Piscataway, NJ), His: 0.2 µl (Amersham Pharmacia, Piscataway, NJ).

(C) Clb5/Cdc28 phosphorylates ORC better than Clb5hpm/Cdc28 does, but both are inhibited by and phosphorylate Sic1 to equal levels. Amounts of Clb5 or Clb5hpm/Cdc28 are 10, 50, and 250 ng, with 250 ng in lanes 1 and 8. Sic1 and ORC are both present at 250 ng.

(D) Clb5/Cdc28 phosphorylates Orc2 in an hpm dependent manner in a complex lacking Orc6. Amounts of Clb5/Cdc28 are as in (C). Note that Clb5 is approximately the same size as Orc6. The lower band in the middle lanes is Clb5, which is auto-phosyphorylated at a higher rate when there are fewer exogenous substrates present.

(E) Cdc6 phosphorylation by Clb5/Cdc28 is dependent upon the Clb5 hp and the Cdc6 phosphorylation site and RXL (Cy) motif. Cdc6ΔN= Cdc6Δ2-49. Cdc6ΔCy=Cdc6Δ8-17. Mock= buffer alone.

interaction between the two proteins. We tested whether antibodies that recognized Clb5 or Cdc28 could alter the shifted complex. We found that antibodies directed against Orc2, Orc4, HA (Clb5 and Cdc28), and His (Clb5) all further shifted this Clb5/Cdc28-dependent supershift, indicating that all of these proteins were present in the original complex (Figure 2B). We found that an antibody against the GST tag on Cdc28 abolished the Clb5/Cdc28 dependent ORC supershift, also suggesting that Cdc28 was present in the original interaction.

One reason for the lack of interaction between Clb5hpm/Cdc28 and ORC in this assay could simply be that the protein was misfolded or unstable, so we tested whether Clb5hpm/Cdc28 retained kinase activity. We found that Clb5hpm/Cdc28 is equally adept at phosphorylating non-specific substrates such as histone H1 (data not shown). Similarly, both wild-type and mutant Clb5/Cdc28 were able to modify Sic1 (Figure 2C) or autophosphorylate the Clb5 subunit (Figure 2C and data not shown) with approximately equal effectiveness. The lack of an effect of the hpm mutation on Sic1 phosphorylation by Clb5 is consistent with earlier observations that Sic1 does not interact with Clb5 via the hydrophobic patch in a 2 hybrid assay (Cross and Jacobson 2000). We also tested whether the inability to interact stably with ORC altered the ability of Clb5hpm/Cdc28 to phosphorylate ORC *in vitro*. In contrast to the above substrates, Clb5hpm/Cdc28 does not phosphorylate either Orc2 or Orc6 of ORC as well as Clb5/Cdc28 does (Figure 2C). Sic1 specifically inhibited all of the kinase activity of wild-type or mutant Clb5/Cdc28 against ORC, suggesting that we are looking at *bona fide* Cdc28 kinase activity. These results are consistent with previous results *in vivo* suggesting that Clb5hpm retains the ability to activate Cdc28 kinase activity, but is

defective in targeting all specific substrates (Cross and Jacobson 2000). Clb5hpm is also defective in phosphorylating specific substrates other than Orc6, such as Orc2, even when Orc6 is not present, or Cdc6 (Figure 2C,D). This is consistent with the similarity in phenotype between Clb5hpm and a Clb5Δ *in vivo* (Cross and Jacobson 2000), and the observation that Clb5hpm has a replication defect, whereas Orc6rxl does not (see Figure 5 below).

Orc6 and Clb5 co-purify from yeast cells

We verified that Orc6 and Clb5 can associate when expressed in yeast at endogenous levels. For this purpose, we constructed strains that expressed Orc6 fused at its C-terminus to protein A and Clb5 fused at its N-terminus to 9 repeats of the Myc epitope. Each fusion protein was expressed from its cognate promoter at endogenous levels. Protein A affinity purifications were performed using extracts derived from these strains and eluates were probed for Myc and Protein A by Western blotting. Orc6-PrA interacted with Clb5 fused to 9Myc (Figure 3A), in agreement with the 2-hybrid (Figure 1A,B) and EMSA data (Figure 2A). This association was not dependent on the two proteins being present in the same cell. The same association could be observed when extracts that independently expressed Orc6-PrA and Myc-Clb5 were mixed (data not shown).

We also tested the effects of mutations in Orc6 and the Clb5 hydrophobic patch on the ability of Orc6 to co-precipitate Clb5, and compared these interactions to interaction with Clb2. Inclusion of either the Clb5-hpm or the Orc6-rxl mutation led to a dramatic reduction in co-precipitated Clb5 protein (Figure 3A). In each case, however, a

Figure 3

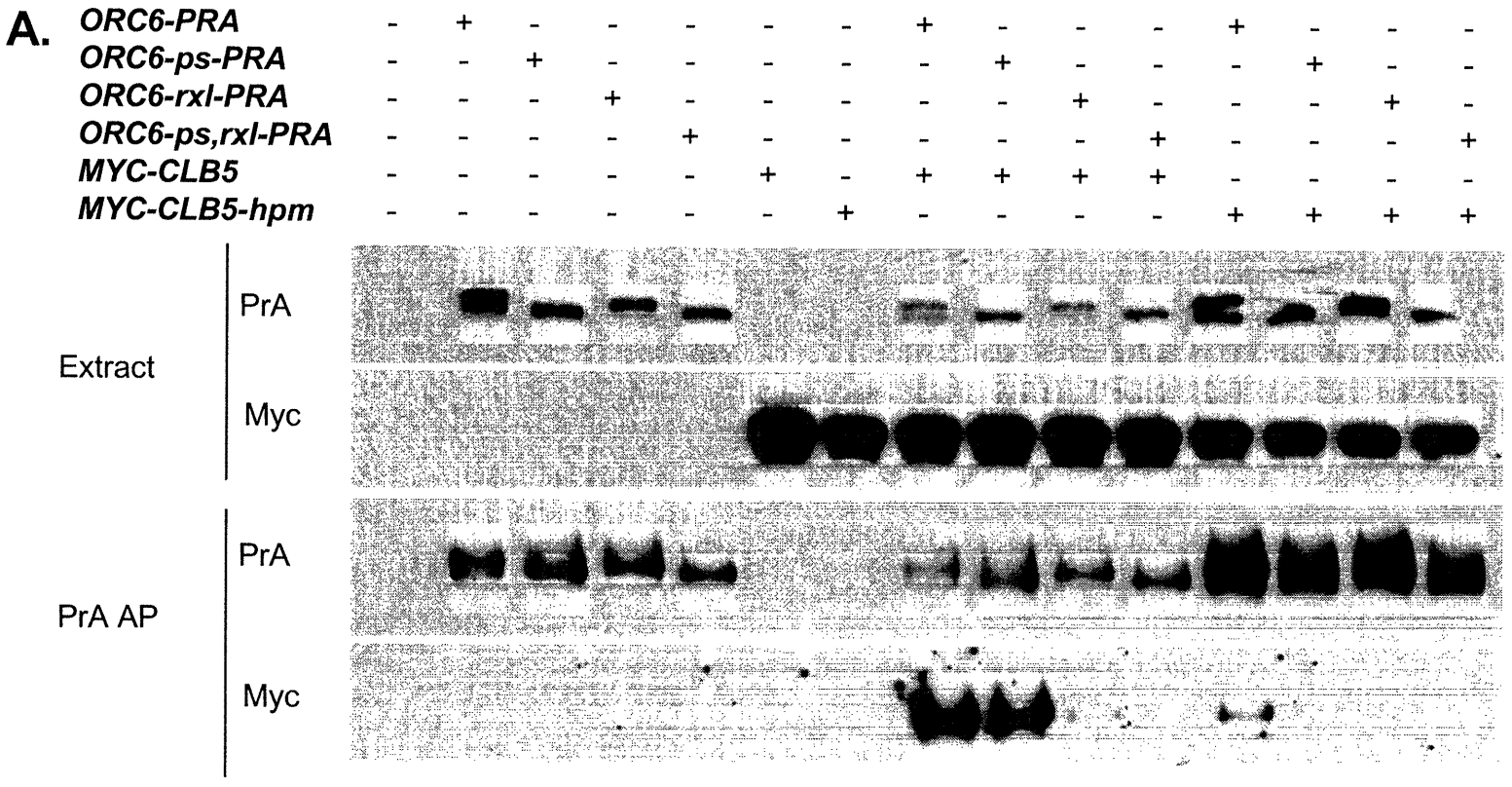

A.
ORC6-PRA - + - - - - - + - - - + - - -
ORC6-ps-PRA - - + - - - - - + - - - + - -
ORC6-rxl-PRA - - - + - - - - - + - - - + -
ORC6-ps,rxl-PRA - - - - + - - - - - + - - - +
MYC-CLB5 - - - - - + - + + + + - - - -
MYC-CLB5-hpm - - - - - - + - - - - + + + +
Extract
PrA
Myc
PrA AP
PrA
Myc

Figure 3

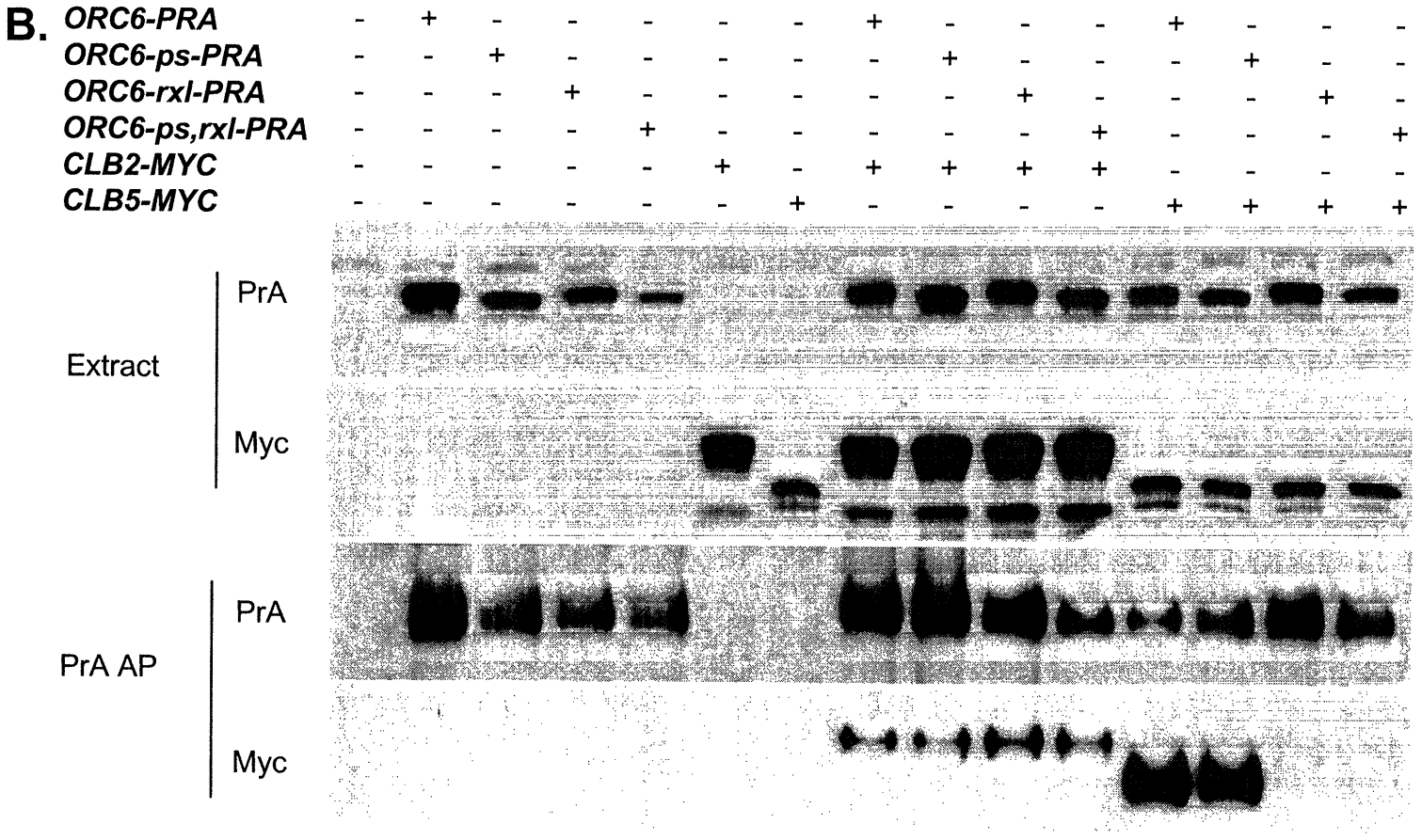

B.
ORC6-PRA
ORC6-ps-PRA
ORC6-rxl-PRA
ORC6-ps,rxl-PRA
CLB2-MYC
CLB5-MYC
Extract
PrA
Myc
PrA AP
PrA
Myc

Figure 3. Orc6 and Clb5 are co-purified from yeast cells with specific sequence requirements. Cells expressing the indicated fusions from their endogenous promoters, alone or in combinations were subjected to protein-A (PrA) affinity purifications (Aps) and to Western blotting analysis (as in Materials and Methods).

(A) The Orc6-Clb5 association depends strongly Clb5 hydrophobic patch motif and on the RXL motif and weakly on CDK consensus site residues in Orc6 (S106, S116, S123, T146).

(B) Orc6 and Clb2 are co-purified from yeast cells. Clb2 association with Orc6 is weaker than the Clb5-Orc6 association. In this assay, Clb2 associates with Orc6 with no detectable dependence on the RXL motif or on the CDK consensus sites in Orc6 (S106, S116, S123, T146).

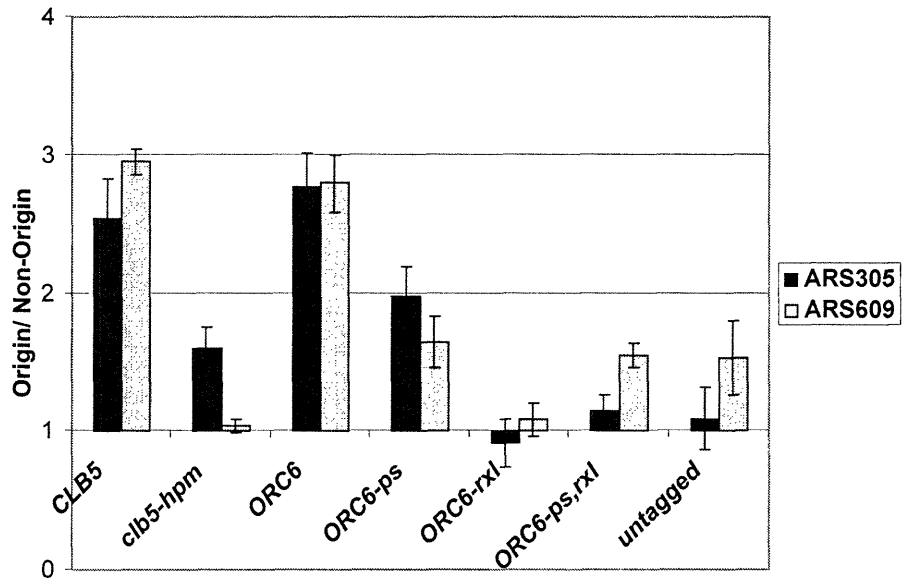
small amount of residual association was observed. Studies of Orc6 with mutated CDK consensus phosphorylation sites (Orc6-ps-PRA, note the faster mobility of these proteins) eliminated this residual interaction. Thus, Orc6 mutated at both the RXL and phosphorylation sites showed no interaction with wild type Clb5. Similarly, the residual interaction between Clb5-hpm and Orc6 was eliminated in the strains carrying the Orc6-ps mutation. In contrast, although Orc6 co-precipitated a small amount of Clb2, this interaction was not affected by mutations in Orc6 (Figure 3B). Overall, these studies closely parallel our two hybrid and EMSA results and support the hypothesis that these interactions occur in the cell.

Clb5 Associates with Origins of Replication *in vivo*

Since Clb5 and Orc6 can interact *in vivo*, we used chromatin immunoprecipitation (ChIP) to ask if this interaction occurred when ORC was bound to origins of replication. Myc-tagged Clb5 was immunoprecipitated from formaldehyde cross-linked cells, and the associated DNA sequences analyzed by quantitative real-time PCR (Figure 4). Clb5 was associated with both early and late-firing origins of DNA replication in asynchronously growing cells (Figure 4A). This association was dependent on the hydrophobic patch on Clb5, the epitope tag on Clb5, and the RXL motif on Orc6 (Figure 4A). As in the studies above, mutation of the phosphorylation sites on Orc6 had a weaker effect on Clb5 origin association. This Clb5-origin association also depended on a wild-type ORC binding site at the origin. Mutations that eliminated the ORC binding site at *ARS1* eliminated association of Clb5 with *ARS1* but not other origins in the cell (Figure 4B).

Figure 4

A.


Origin/ Non-Origin
4
3
2
1
0
CLB5
clb5-hpm
ORC6
ORC6-ps
ORC6-rxl
ORC6-ps,rxl
untagged
ARS305
ARS609

B.

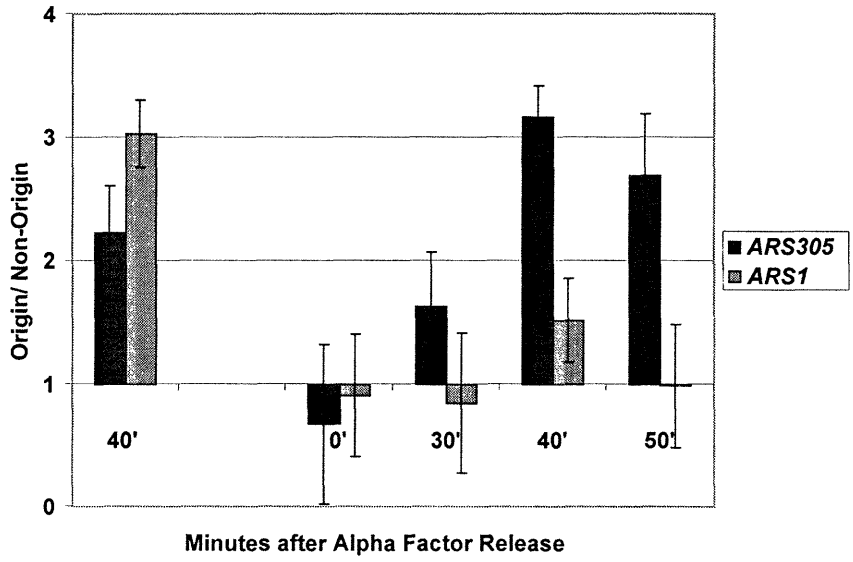
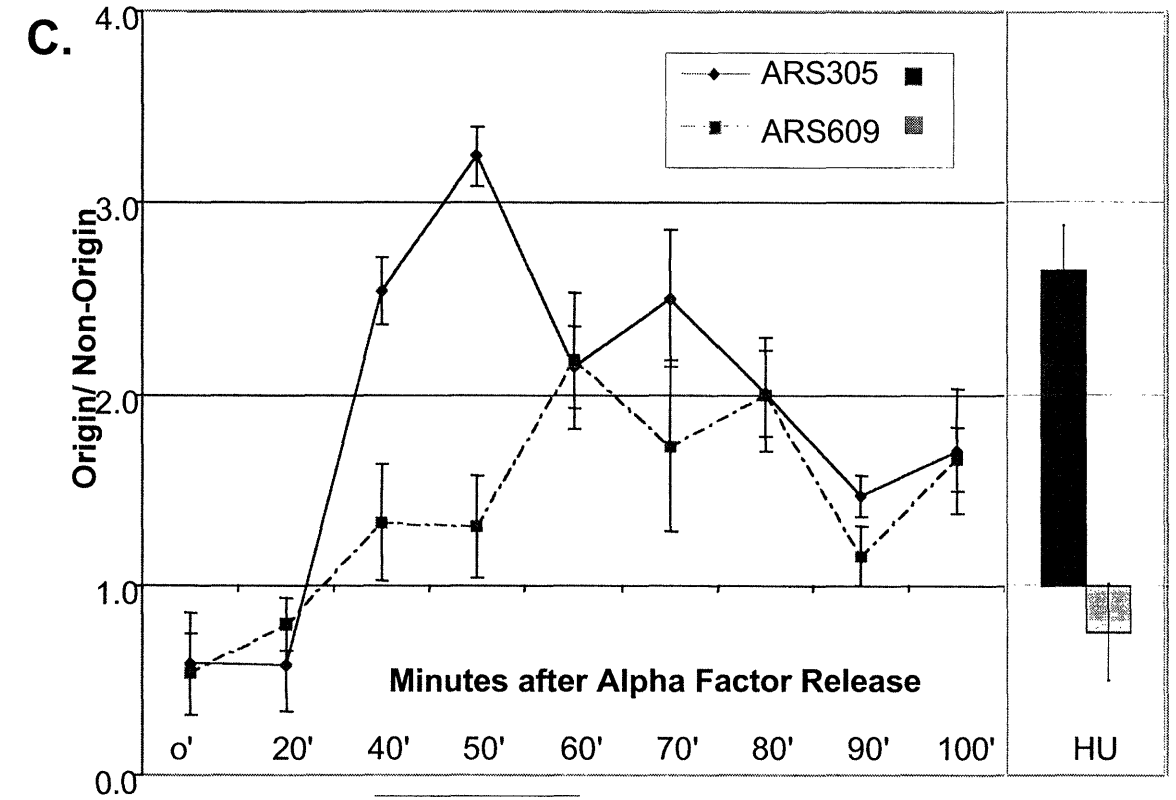
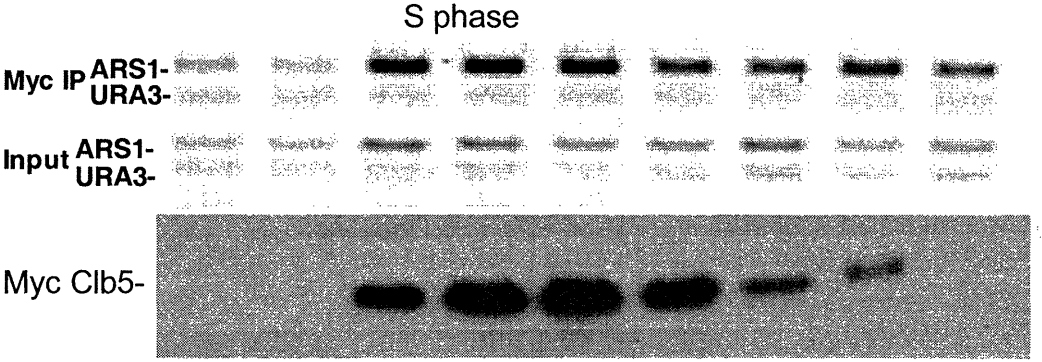

Origin/ Non-Origin
4
3
2
1
0
40'
0'
30'
40'
50'
ARS305
ARS1
Minutes after Alpha Factor Release

Figure 4


C.
4.0
3.0
2.0
1.0
0.0
Origin/ Non-Origin
ARS305
ARS609
Minutes after Alpha Factor Release
o'
20'
40'
50'
60'
70'
80'
90'
100'
HU
S phase


Myc IP ARS1-
URA3-
Input ARS1-
URA3-
Myc Clb5-

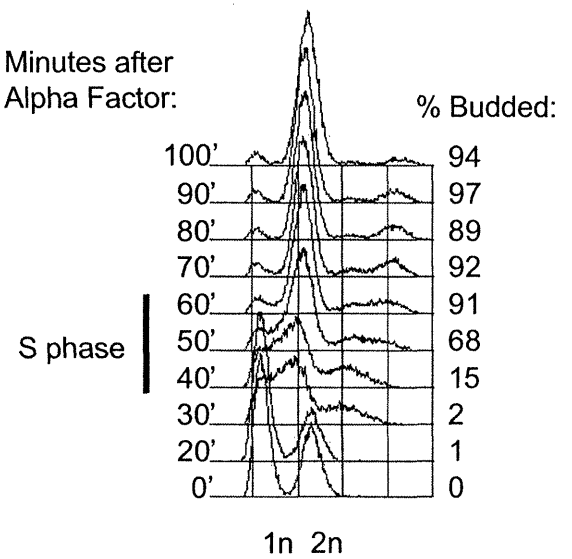

Minutes after Alpha Factor:
% Budded:
100' 94
90' 97
80' 89
70' 92
60' 91
50' 68
40' 15
30' 2
20' 1
0' 0
S phase
1n 2n

Figure 4. *In vivo* interaction between Clb5 and origins of replication.

(A) Clb5 associates with origins of replication *in vivo* in a Clb5 HP and Orc6 –RXL-dependent manner. All strains except the untagged have an N-terminal 9Myc tag on the only copy of Clb5. Asynchronous cultures were analyzed by chromatin immunoprecipitation with a monoclonal anti-Myc antibody. Shown are the results of quantitative PCR of a representative experiment. The error bars represent the error of the PCR. The non-origin DNA sequence is a sequence halfway between *ARS305* and *ARS306*.

(B) Clb5 does not associate with a non-functional origin that does not bind ORC. Myc-Clb5 cells with or without a linker scanner mutation in the A element of *ARS1* (Marahrens and Stillman) were arrested in G1 with alpha factor and released into S phase at room temperature. Clb5 did not associate with the mutated origin, but did with a wild-type origin (*ARS305*) in the same strain. DNA was analyzed as in A, except that the origin/non-origin DNA is the ratio of %IPs, not simply IPs, to take into account the changing amounts of different sequences in the inputs in a timecourse. However, the data looked similar either way.

(C) Clb5 binds origins of replication at the time that they fire, and then persists at the origin. Clb5 associates first with the early firing origins *ARS1* and *ARS305* and later with the later firing origin *ARS609*, and then persists at all of the origins. MycClb5 cells were arrested in G1 with alpha factor and released into the cell cycle at room temperature. Samples were analyzed as in 4b, except that the non-origin sequence shown is URA3. Below the real time quantitative PCR is a representative gel showing conventional PCR of another early origin, *ARS1*, and of *URA3*. In the right half of the panel, the same cells

were arrested in 200 mM HU, after early origins had fired, but before late origins had, and Clb5 associates only with early origins. Below the PCR data is an anti-myc western blot showing Clb5 abundance at each timepoint.

Cell cycle regulation of Clb5 association with Origins

The cell cycle timing of the association of Clb5 with origin DNA was of particular interest. Clb5 is only present during a portion of the cell cycle (from the G1/S transition to metaphase) making it likely that the association with origins is cell cycle regulated. However, the exact pattern of association was likely to be informative concerning the possible functions of Clb5 at the origin. Clb5 is known to have a role in initiating DNA replication, so one possibility was that Clb5 associated with origins briefly when they initiated. Cyclin-dependent kinase activity also acts to prevent re-initiation after an origin has been replicated, raising the possibility that Clb5 associated with origins only after they initiated. Finally, it was possible that Clb5 associated with origins whenever the protein is present in the cell.

To investigate when Clb5 was associated with origins of replication, we arrested the cells in G1 with alpha factor, and then released them into S phase. We found that Clb5 associated with origins that fired early in the cell cycle, such as *ARS305*, around the time that the origin fired (40' after release from alpha factor) (Figure 4C). When we looked at Clb5 association with an origin that fired later in the cell cycle (*ARS609*), we found that the peak of Clb5 association did not occur until 60' after release from alpha factor, when those origins are expected to initiate. Interestingly, for each origin, once associated, Clb5 persisted at the origin until at least the time that Clb5 was degraded in M phase.

The above results suggest that Clb5 associates with origins at the time that they initiate replication and then persists at the origin for some time after initiation. To assess this possibility using a different approach, we arrested cells in hydroxyurea prior to

analysis. This drug causes cells to arrest after early origins have initiated replication but before late origins have done so. At the hydroxyurea arrest point, Clb5 was associated with early origins that had initiated replication, such as *ARS305* (Figure 4C), supporting the idea that the Clb5 persists at the origin after initiation. Conversely, Clb5 was not associated with the late firing origin *ARS609*, suggesting that Clb5 does not associate with origins that have yet to initiate replication.

Mutations that prevent Clb5 origin association do not interfere with replication initiation.

The association of Clb5 with origins at or near their time of initiation could impact either of two replication events; initiation of replication and/or prevention of re-replication. We first tested whether the Clb5/Orc6 interaction contributed to initiation of replication. A severe defect in initiation would be expected to interfere with timely passage through S phase. To test this possibility we used FACS analysis to measure changes in DNA content. Orc6-rxl or Orc6-ps-rxl mutations that prevented association of Clb5 with the origin had no defects in bulk DNA synthesis (data not shown). Measurement of plasmid stability represents a much more sensitive assay for defects in the initiation of replication. We tested strains containing the same two *ORC6* mutations for their ability to maintain an origin containing plasmid (Figure 5). The *ORC6* mutants had at most a very slight defect in retaining plasmids containing early firing (*ARS1*) origins of replication and no defect in retaining plasmids containing late firing (*ARS301*) origins of replication. In contrast, strains with *CLB5* mutations had defects in maintaining the late firing plasmids, but not the early firing plasmids. The effects of the

Figure 5

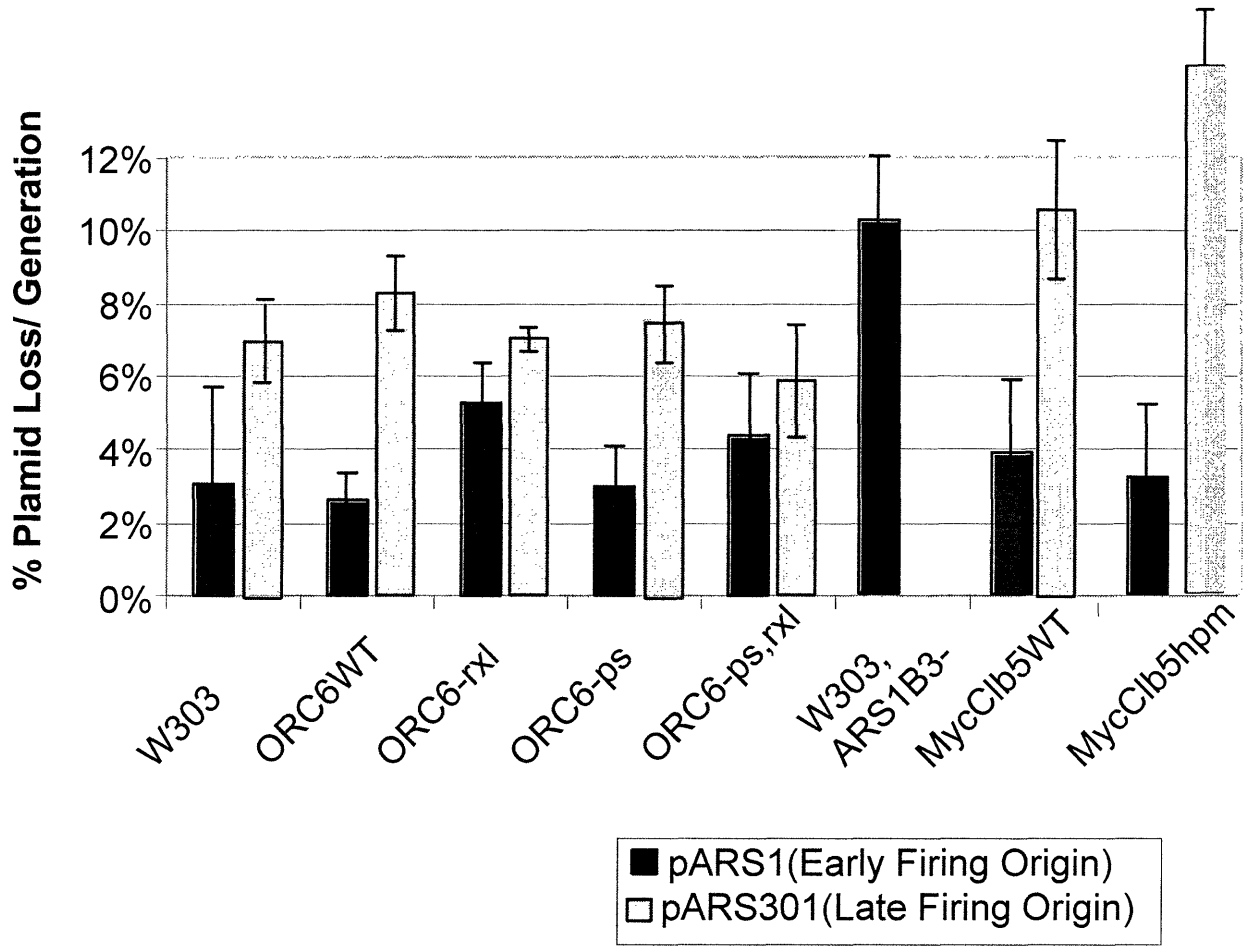


Figure 5. Orc6 RXL mutants do not have a significant initiation defect.
Error bars represent the standard deviations from at least three plasmid loss experiments.
pARS1=pARS1WT, ARS1B3-=pARS1757-764 (Marahrens ad Stillman, 1992).

Clb5 mutant are consistent with previous studies indicating that Clb5 is required for that activation of late but not early origins of replication, and that the phenotype of the *CLB5* hydrophobic patch mutant mirrors that of a *clb5Δ* (Donaldson et al. 1998; Cross and Jacobson 2000). These findings suggest that the interaction of Clb5 and Orc6 does not play an important role in the initiation of replication and that the requirement of Clb5 for initiation from late firing origins is independent of this interaction.

ORC6 and *CLB5* mutations interact with mutations stabilizing Cdc6.

Clb-CDK activity is required both for initiation of DNA replication and for inhibition of a second round of replication before mitosis. Inhibition of re-replication depends on Clb-CDK phosphorylation of Orc2 and Orc6, of the MCM complex leading to its exit from the nucleus, and of the Cdc6 replication factor leading to its degradation (Drury et al. 1997; Labib et al. 1999; Drury et al. 2000; Nguyen et al. 2000; Nguyen et al. 2001). These controls block formation of a new pre-RC.

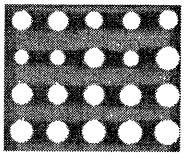
To probe for involvement of the Orc6 RXL-Clb5 hp interaction in control of re-replication, we eliminated elements of this control in the absence of the Orc6 RXL. The Cdc6 N-terminus is required for CDK control of Cdc6 abundance and Cdc6 lacking the N terminus is stable throughout the cell cycle (Donovan et al. 1997; Drury et al. 1997; Perkins et al. 2001). Combining *ORC6-rxl* and chromosomal deletion of the Cdc6 N-terminal 49 amino acids resulted in a striking slow-growth phenotype in tetrad analysis (Figure 6A). This phenotype was enhanced by mutating the Orc6 CDK phosphorylation sites (*ORC6-ps,rxl*) (Figure 6A). *ORC6-ps CDC6Δ2-49* mutants with an intact Orc6

Figure 6

A.

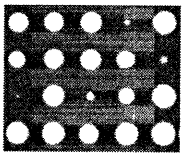
ORC6 mutant (m) (as indicated) or *wt* (+) ; *CDC6ΔN* (m) or *CDC6-wt* (+)

ORC6-wt



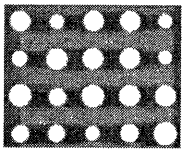
+;+	+;+	+;+	+;m	+;m
+;+	+;m	+;+	+;m	+;+
+;m	+;m	+;m	+;+	+;m
+;m	+;+	+;m	+;+	+;+

ORC6-rxl



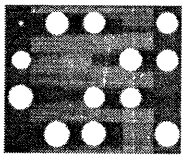
+;+	+;m	+;+	m;m	m;+
m;+	m;+	+;m	m;+	m;m
m;m	+;m	m;m	+;m	+;m
+;m	m;+	m;+	+;+	+;+

ORC6-ps



+;+	m;m	+;m	m;+	m;m
m;+	+;+	+;+	+;+	m;+
+;m	+;+	m;+	+;m	+;m
m;m	m;m	m;m	m;m	+;+

ORC6-ps,rxl



m;m	+;+	m;+	m;m	+;m
+;+	m;m	m;m	+;+	m;+
+;+	m;m	+;+	+;+	m;m
m;m	+;+	+;m	m;m	+;+

B.

All *clb5::HIS3 ura3::URA3::GAL-CLB5*

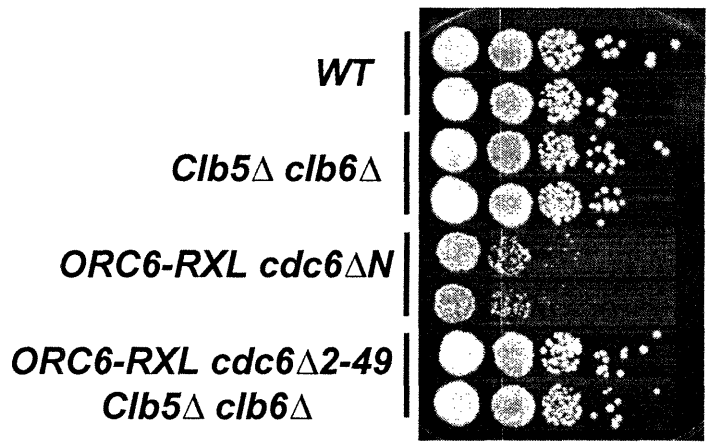
YEPGal YEPD

CDC6-wt

CDC6ΔN

Figure 6

C.


WT
Clb5Δ clb6Δ
ORC6-RXL cdc6ΔN
ORC6-RXL cdc6Δ2-49
Clb5Δ clb6Δ

D.

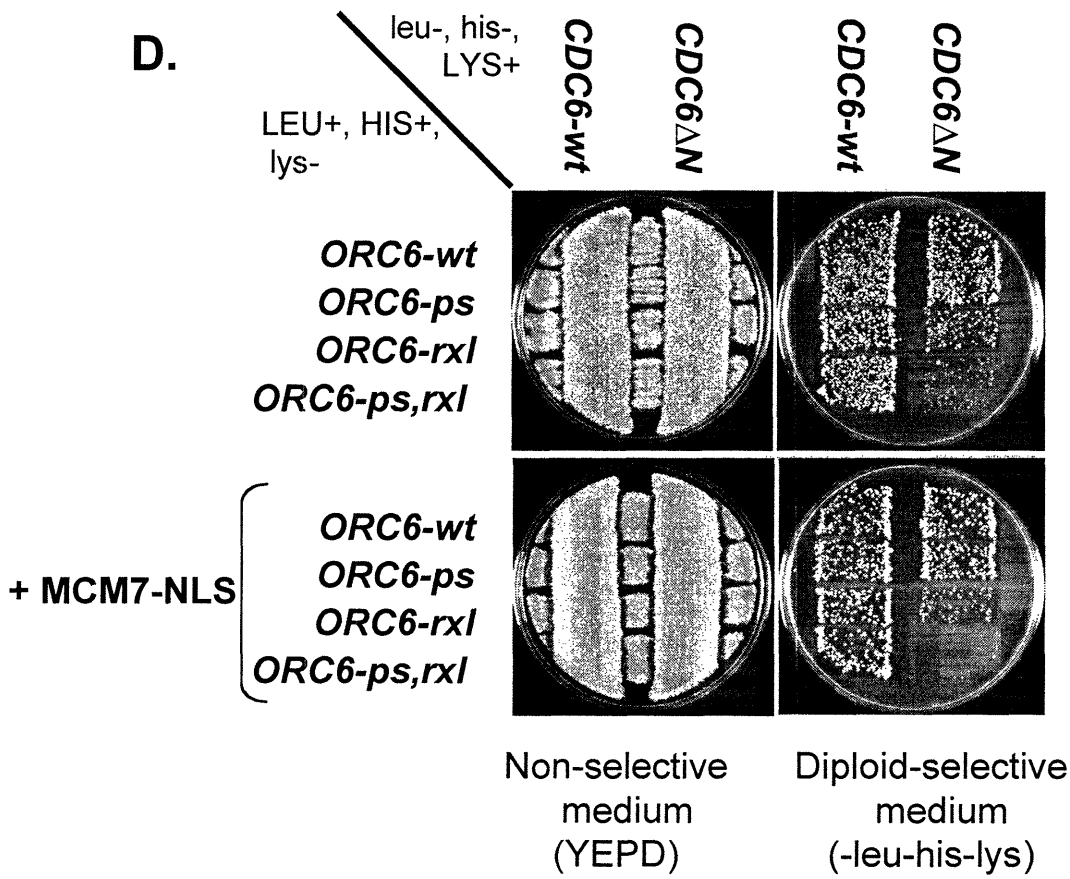

leu-, his-, LYS+
LEU+, HIS+, lys-
CDC6-wt
CDC6ΔN
CDC6-wt
CDC6ΔN
ORC6-wt
ORC6-ps
ORC6-rxl
ORC6-ps,rxl
+ MCM7-NLS
ORC6-wt
ORC6-ps
ORC6-rxl
ORC6-ps,rxl
Non-selective medium (YEPD)
Diploid-selective medium (-leu-his-lys)

Figure 6. Network of genetic interactions between *ORC6, CDC6, CLB5, CLB6* and *MCM7*.

(A) Interaction between *ORC6* alleles and *CDC6_2-49*. *ORC6-m::LEU2::HIS3/ORC6 CDC6_2-49/CDC6-wt* diploid strains were sporulated and tetrads were dissected on YEPD plates, incubated at 30°C for 4 days and photographed. Subsequent replica-plating and PCR tests allowed the detection of *ORC6* alleles and of *CDC6_2-49,* respectively. Inviable spores were genotyped by assuming 2:2 segregation. The presence of the marked *ORC6* mutant allele assayed (m) or *ORC6-wt* (+) (left symbol) and the presence of the *CDC6_2-49* allele (m) or *CDC6-wt* (+) (right symbol) in each colony is indicated. In the control cross (upper left) where the marked *ORC6::LEU2::HIS3* allele was *ORC6-wt*, a '+' was used for both *ORC6* alleles, because the marked and unmarked wild-type alleles behaved identically.

(B) Synthetic lethality of *CDC6_2-49* and *clb5Δ*. *clb5Δ GAL-CLB5* cells that were either *CDC6-wt* or *CDC6_2-49* were streaked on YEPGal or YEPD medium and incubated at 30°C for 2 days.

(C) *ORC6-rxl CDC6_2-49* mutants are rescued by deletion of *CLB5* and *CLB6*. Cells with the indicated genotype were cultured in 2 ml of liquid YEPD until they reached stationary phase. Cell density was normalized by OD_{600} and serial 10-fold serial dilutions were made using cell equivalents. These dilutions were plated on YEPD and incubated at 30°C for 3 days. (Note: to avoid accumulation of second-site suppressors, the *ORC6-rxl CDC6_2-49* stocks used were pre-screened for retention of the characteristic slow-growth phenotype documented in A before this test).

(D) Dominance of *ORC6-rxl, ORC6-ps,rxl, CDC6_2-49*and *MCM7-NLS*. Strains containing the indicated alleles were mated on YEPD plates (left) and replica-plated on SCD-his-leu-lys for diploid selection (right), and incubated at 30°C for 2 days. Diploids appear at the intersections of the streaks.

RXL motif showed only a slight growth defect. Other N-terminally mutated *CDC6* alleles with weaker stabilizing effects had similar but more modest interactions with the *ORC6* alleles (data not shown). This suggests that *CDC6Δ2-49* yields a semi-lethal phenotype when combined with *ORC6-rxl* or *ORC6-ps,rxl* due to stability of N-terminally deleted Cdc6.

The semi-lethal phenotype was dominant for both *CDC6_2-49* and *ORC6-rxl* or *ORC6-ps,rxl* based on transformation experiments with low-copy plasmids encoding wild-type and mutant *CDC6* and *ORC6* (data not shown). Additional wild-type *CDC6* actually enhanced the phenotype (data not shown). Dominance was also suggested by frequent loss of heterozygosity from *CDC6_2-49/CDC6* to *CDC6/CDC6* in *ORC6-rxl/ORC6* diploids (data not shown).

The results above suggested that the Clb5 interaction with Orc6 might be required for cell viability in the presence of stabilized Cdc6Δ2-49. Indeed, *clb5Δ CDC6Δ2-49* double mutants were inviable in tetrad analysis (data not shown). We constructed *GAL-CLB5 clb5Δ CDC6Δ2-49* strains, which grew well on galactose but failed to proliferate when plated on glucose to repress *GAL-CLB5* (Figure 6B). *CDC6Δ2-49* was dominant to wild-type *CDC6* for this phenotype (data not shown). We also observed a less severe slow growth phenotype for *CLB5-hpm CDC6Δ2-49 cells* (data not shown). These results are consistent with the idea that the Clb5-Orc6 interaction is required for viability in the presence of stabilized Cdc6.

Clb5 and Clb6 are similar proteins that are co-regulated, and they generally have redundant function (Schwob and Nasmyth 1993). Surprisingly, though, we found that *CDC6Δ2-49 clb6Δ* mutants, both *clb5Δ* and *CLB5*, were viable, occurred at normal

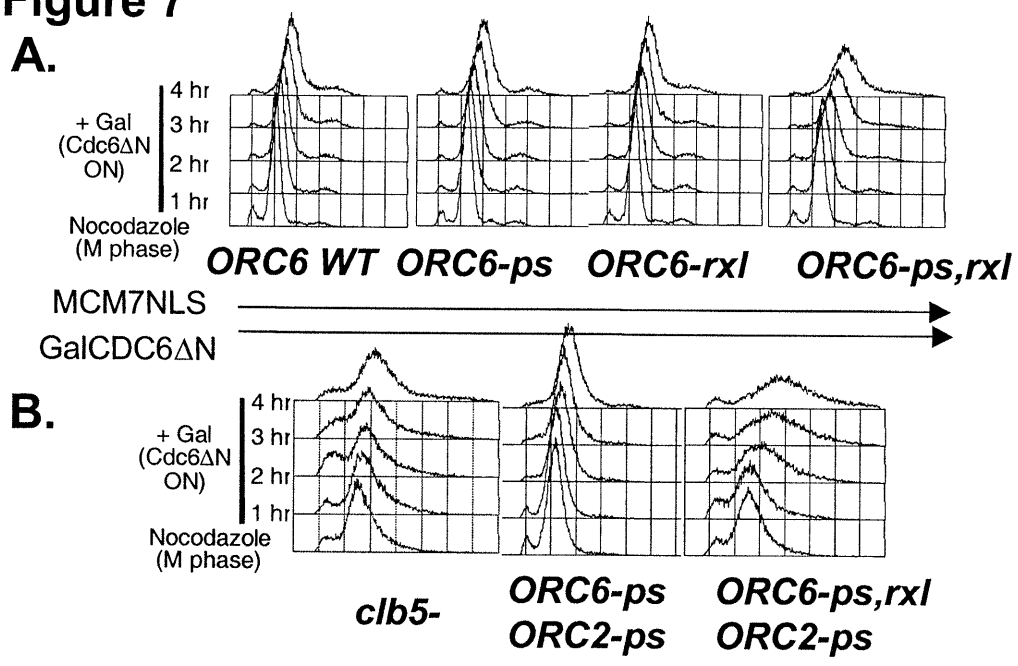
Mendelian frequencies, and had no obvious growth defect (data not shown). Thus, the inviability of *clb5Δ CDC6Δ2-49* cells (see above) requires Clb6.

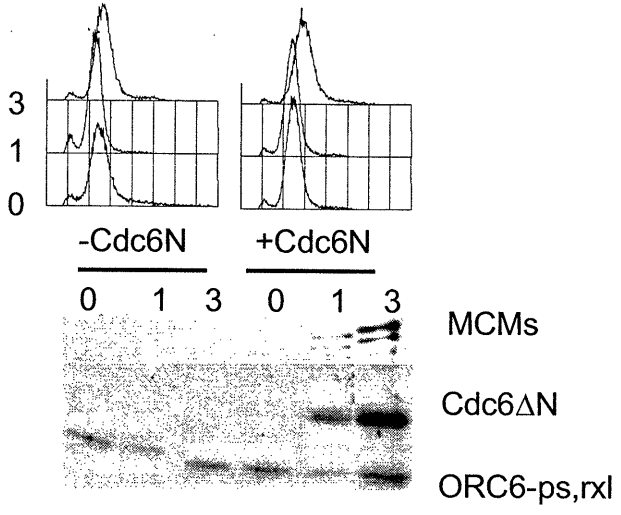
This result suggests that deleting both *CLB5* and *CLB6* should also rescue the slow growth phenotype of *CDC6Δ2-49 ORC6-rxl* or *ORC6-ps,rxl* cells. This prediction was confirmed (Figure 6C and data not shown). As expected, we could not recover viable *clb5Δ CLB6 CDC6Δ2-49 ORC6-rxl* or *ORC6-ps,rxl* cells in tetrad analysis (data not shown). *CLB5 clb6Δ CDC6Δ2-49 ORC6-rxl* or *ORC6-ps,rxl* cells had a semi-lethal phenotype similar to *CLB5 CLB6 CDC6Δ2-49 ORC6-rxl* or *ORC6-ps,rxl* cells (data not shown)

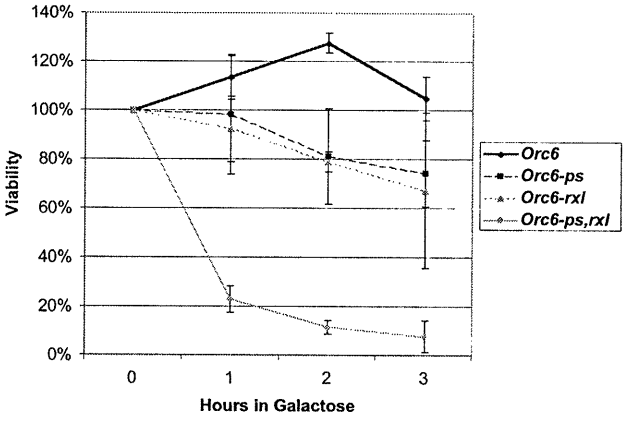
Interference with the Orc6-Clb5 interaction stimulates inappropriate re-replication of the genome.

Given that the *ORC6* mutants are synthetically lethal with mutations known to be involved in re-replication, we asked if the same ORC6 mutations would substitute for alterations in ORC previously shown to stimulate re-replication. To this end, we placed *CDC6Δ2-49* under the control of an inducible promoter and assayed for re-replication in strains containing constitutively nuclear MCM proteins and various mutations in *ORC6*. To test for inappropriate DNA replication, we arrested these cells with nocodazole, induced Cdc6Δ2-49 with galactose and examined the DNA content by FACS. We tested each of the three alleles of ORC6 that influence the Orc6-Clb5 interaction in this assay. We found that the *ORC6-ps,rxl* strain showed a reproducible gain in DNA content beyond 2N, whereas strains with either individual ORC6 allele (*-rxl* or *-ps*) maintained 2N DNA content (Figure 7A).

Figure 7


A.
+ Gal (Cdc6ΔN ON)
4 hr
3 hr
2 hr
1 hr
Nocodazole (M phase)
ORC6 WT
ORC6-ps
ORC6-rxl
ORC6-ps,rxl
MCM7NLS
GalCDC6ΔN
B.
+ Gal (Cdc6ΔN ON)
4 hr
3 hr
2 hr
1 hr
Nocodazole (M phase)
clb5-
ORC6-ps ORC2-ps
ORC6-ps,rxl ORC2-ps


C.
3
1
0
-Cdc6N
+Cdc6N
0 1 3 0 1 3
MCMs
Cdc6ΔN
ORC6-ps,rxl


140%
120%
100%
80%
60%
40%
20%
0%
Viability
0
1
2
3
Hours in Galactose
Orc6
Orc6-ps
Orc6-rxl
Orc6-ps,rxl

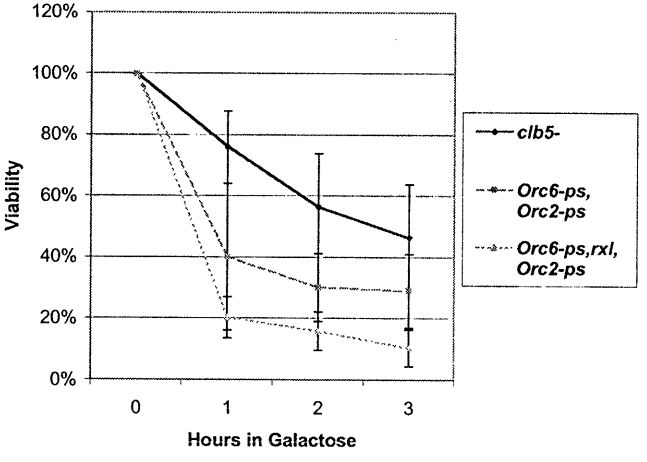

120%
100%
80%
60%
40%
20%
0%
Viability
0
1
2
3
Hours in Galactose
clb5-
Orc6-ps, Orc2-ps
Orc6-ps,rxl, Orc2-ps

Figure 7

D.

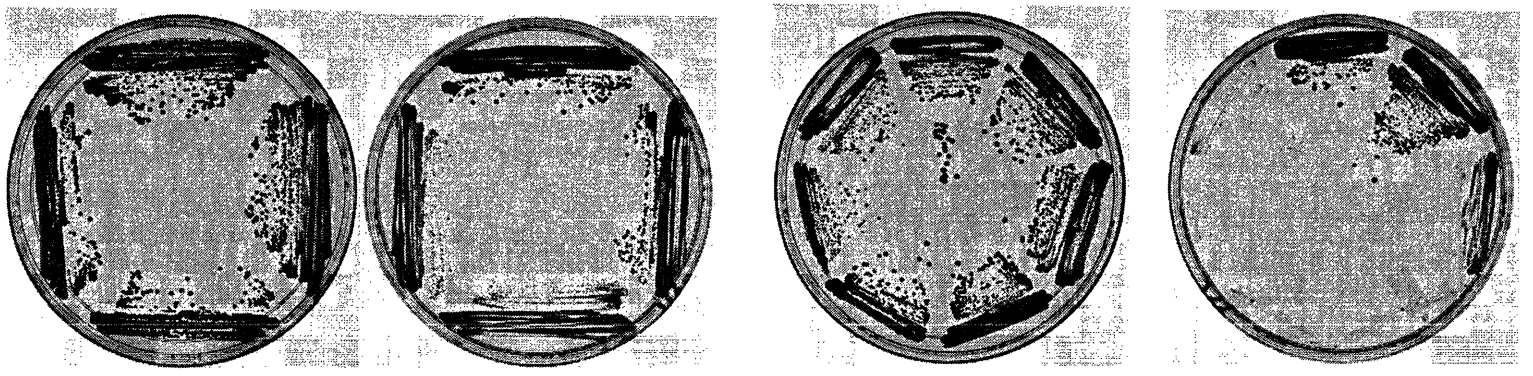

Glu
WT MCMs
Gal
Glu
MCM7NLS
Gal
GalCdc6ΔN
GalCdc6ΔN
ORC6WT
ORC6-ps
ORC6-rxl
ORC6-ps,rxl
ORC6WT
clb5Δ
ORC6-ps
ORC6-ps,rxl
ORC2-ps
ORC6-rxl
ORC6-ps
ORC2-ps
ORC6-ps,rxl

Figure 7. The ORC6 RXL motif helps protect cells from re-replicating in M phase.

(A) *ORC6-ps,rxl MCM7NLS, GalCDC6Δ2-4* cells can be induced to rereplicate. *ORC6* wild-type and mutant strains containaing *MCM7NLS* and *GalCDC6Δ2-49* were arrested with nocodazole in media containing 2% raffinose, and then induced after the zero hour timepoint with 2% galactose. Samples were taken every hour for FACS, viability, and western analysis of CDC6Δ2-49 levels (data not shown). For the viability assays, 500 cells were counted at the zero hour, and the same volume of sonicated cells was plated onto glucose media at each timepoint. The number of colonies was counted after two days, and the number of colonies at each timepoint was divided by the zero hour timepoint to determine a percentage viability. Standard deviations represent the average of at least three independent experiments.

(B) *Clb5Δ* cells can also be induced to rereplicate in the sensitized strain background, and *ORC2-ps* exacerbates the rereplication phenotype. Same as in A.

(C) Pre-RCs form on the chromatin in the *ORC6-ps,rxl MCM7NLS, GalCDC6Δ2-4* rereplicating cells. *ORC6-ps,rxl MCM7NLS* cells with or without *GalCdc6ΔN* were arrested as above and then the chromatin pellet was isolated and the DNAse I and Salt sensitive proteins extracted and visualized by western blotting.

(D) Cdc6ΔN dependent viability defects of strains sensitive to re-replication plated on galactose. *ORC6-rxl* strains have a slight viability defect when GalCdc6ΔN is induced (left). *ORC6-rxl strains, ORC6-ps,ORC2-ps* strains, and *clb5Δ* strains with the MCM proteins nuclearly localized all have a severe viability defect when Cdc6ΔN is induced by galactose.

The inappropriate DNA replication in these multiply mutated cells resulted in lethality. The *ORC6-ps-rxl, GAL1-CDC6D2-49, MCM7-nls* strain showed an immediate drop in viability when returned to growth on glucose media (Figure 7A). In contrast, mutation of the phosphorylation sites or the RXL motif of Orc6 alone resulted in only a slight drop in viability. Strains with wild type ORC6 showed no loss of viability. Consistent with this drop in viability when cells were exposed to galactose briefly, the *ORC6-ps,rxl* strain in this sensitized background failed to grow when grown on galactose containing media (Figure 7D). Interestingly, under these conditions the *ORC6-rxl* strain also had a significant growth defect. This growth defect did not require the Cdc6D2-49 protein to be highly over-expressed or the presence of the MCM7-NLS allele. When the mutant Cdc6 was driven by its own promoter we observed highly penetrant dominant lethality in triply heterozygous *CDC6D2-49/CDC6 ORC6-ps,rxl/ORC6 MCM7-NLS/MCM7* diploids, and a less extreme but still strong phenotype in triply heterozygous CDC6D2-49/CDC6 ORC6-rxl/ORC6 MCM7-NLS/MCM7 diploids (Figure 6D). As described above, growth defects were also observed in the absence of the modified Mcm7 protein and *ORC6-rxl GalCDC6Δ2-49* and *ORC6-ps,rxl GalCDC6* strains both grew slowly on galactose media. Although these altered strains showed lethality, re-replication was only reproducibly observed in the *ORC6-ps,rxl GalCDC6Δ2-49 MCM7-NLS* strain.

Since the ORC6 RXL motif and phosphorylation sites are important for ORC's interaction with Clb5, we deleted *CLB5* in the same sensitized strain background (*MCM7-NLS GalCDC6Δ2-49*) and assayed for re-replication (Figure 7B). Although the peaks were broader, this strain appears to show some replication beyond 2C DNA

content, and this is accompanied by a drop in viability. This strain is also dead when grown onto galactose, again in agreement with the earlier genetic results (Figure 7B).

Since the phosphorylation sites on *ORC2* were also implicated in control of re-replication (Nguyen et al. 2001), we were interested in how they interacted with the *ORC6* mutations. To this end we mutated the six predicted *ORC2* CDK phosphorylation sites in the strain we saw the most re-replication (*ORC6-ps-rxl, GAL1-CDC6D2-49, MCM7-nls*) and tested for re-replication in nocodazole arrested cells. These cells showed robust re-replication with many of the cells showing greater than 4N DNA content (Figure 7B). These cells also showed a rapid loss of viability. The importance of the Clb5-Orc6 interaction for the observed re-replication is emphasized by the comparison with the same strain lacking the Orc6-rxl mutation. Mutating all of the phosphorylation sites on *ORC2* as well as those on *ORC6* in these sensitized strains led to little re-replication in our W303 strain background when Cdc6Δ2-49 was induced as measured by FACS, and only a partial viability defect when these cells were plated back onto glucose.

To confirm that the increase in DNA content in these experiments was due to re-initiation, we assayed for the presence of pre-RCs in *ORC6-ps,rxl, MCM7NLS, GalCDC6Δ2-49* cells arrested in M phase (Figure 7C). We found that MCM proteins accumulated in the chromatin bound fraction of the cells in the *ORC6-ps,rxl* strain arrested in nocodazole, with the MCMs nuclearly localized and Gal Cdc6Δ2-49 expressed, but not in a similar strain lacking *GAL-Cdc6Δ2-49*, indicating that the increase in DNA content that we see may be due to re-initiation from inappropriately re-formed pre-RCs.

Discussion

We have identified a stable association between the S phase cyclin Clb5 and the origin recognition complex. The molecular basis for this interaction involves a hydrophobic patch on Clb5 interacting with an RXL motif on Orc6, and secondarily the CDK consensus phosphorylation sites on Orc6. This interaction recruits Clb5 to origins of replication at the time that they initiate, and Clb5 stays at the origin after initiation to play a role in preventing re-replication. This interaction is specific to Clb5, and not Clb2, and therefore suggests a specific role for the activating S phase cyclin in preventing re-initiation of replication.

Taken together, our data suggest a model whereby Clb5 acts locally at origins to prevent origin re-initiation as soon as the pre-RC is dismantled. We propose that Clb5 marks which origins have been replicated by binding to any Orc6 molecule that is exposed upon pre-RC disassembly. This includes ORC molecules that are exposed both through origin activation and replication fork passage through an origin. Clb5 is therefore part of the post-RC with ORC.

Global and Local Replication Control

Kinases act both globally and locally to initiate replication, and these same two levels of control appear to prevent re-initiation of replication. In initiation, CDK activity acts globally, phosphorylating Sld2 and perhaps other targets, to allow all origins to initiate (Pasero et al. 1999; Masumoto et al. 2002). In contrast, DDK (Cdc7/Dbf4) activity is recruited to individual origins through an interaction between Dbf4 and ORC to act locally at each individual origin. Consistent with this local function, DDK activity

is required throughout S phase to initiate origins, probably by phosphorylating the MCMs at each origin (reviewed in (Sclafani 2000)). In prevention of re-initiation, all CDKs are probably able to act globally to phosphorylate the MCMs, Cdc6, and ORC. In contrast, only Clb5/Cdc28 appears to act at a local level, binding ORC at each origin after it initiates to prevent reformation or reactivation of the pre-RC. Interestingly, our studies suggest that this interaction is unnecessary for origin activation (Figure 5). Although it would be elegant for the cyclin to activate replication locally, and remain at the origin to prevent re-initiation, this type of mechanism might not protect origins that were replicated by a replication fork emanating from outside the origin. In contrast, tying Clb5 origin association to pre-RC disassembly can protect any origin that is replicated, regardless of whether it initiates replication.

The recruitment of a CDK directly to origins of replication to act locally has been observed in other organisms. In *Xenopus* egg extracts, cyclin E is recruited to origins of replication through Cdc6 to activate replication (Furstenthal et al. 2001a; Furstenthal et al. 2001b). Despite the dissimilarities in outcome between this interaction and that between Clb5 and ORC, the molecular basis for this interaction is also through an RXL motif. In fission yeast, the mitotic cyclin Cdc13 binds to replication origins in M phase to prevent endoreduplication (Wuarin et al. 2002). This interaction may function similarly to the interaction between Clb5 and Orc6, although it is not clear whether the multiple complete rounds of endoreduplication induced in *S. pombe* are regulated by the same pathways that prevent re-initiation of individual origins within a single *S. cerevisiae* cell cycle. The molecular domains responsible for this interaction have not been mapped and it is not known whether they involve the cyclin targeting domain.

Cyclins also interact with ORC in vertebrates, although whether this function prevents re-replication is unknown. Interactions have been described between *Xenopus* ORC and Cdc2/Cyclin A1, and between human ORC and Cyclin A/Cdk2(Leatherwood et al. 1996; Romanowski et al. 2000). Overexpression of Cyclin A/Cdk2 in a human cell line induces re-replication instead of protecting against it (Vaziri et al. 2003). Although this could reflect a perturbation in the balance between the repressive and activating roles of the S phase kinase, this could also indicate that human Cyclin A/Cdk2 does not protect origins in the same way as Clb5. Although we have found that deletion of Clb5 sensitizes cells to re-replication, we have not assayed the effect of Clb5 overexpression.

Multiple roles for ORC phosphorylation in prevention of re-replication.

It was previously observed that there are at least three levels of CDK control over DNA re-replication in *S. cerevisiae:* phosphorylation of ORC, Cdc6, and the MCM2-7 protein complex (Nguyen et al. 2001). All three of these mechanisms must be mutated to observe re-replication. Cdc6 phosphorylation targets Cdc6 for degradation, and MCM phosphorylation targets the MCMs for nuclear export. The role of ORC phosphorylation is unknown.

Our data suggest that one role for the ORC phosphorylation sites in preventing re-replication is recruitment of Clb5 to the origin. We find that the Orc6 phosphorylation sites contribute to Clb5 association with the origin, and so this recruitment may be their sole function. An ORC complex lacking Orc6 but with a wild-type Orc2 appears to fully abrogate stable association with Clb5. This suggests that Orc2 does not contribute significantly to the interaction with Clb5 (Figure 2).

The Orc2 phosphorylation sites may have a distinct role in preventing re-replication. Mutating the Orc2 CDK phosphorylation sites appears to have a synergistic effect on the capability of cells to re-replicate even in cells that have no Clb5 associated with the origin. One possibility is that phosphorylated Orc2 interferes directly with formation of the pre-RC. ORC phosphorylation is not likely to be incompatible with maintenance of the pre-RC or with initiation, because both Orc2 and Orc6 are fully shifted on a gel to a form representing at least partial phosphorylation in a *cdc7-4* arrest prior to initiation, from which cells can recover (Nguyen et al. 2001).

Redundancy in Mechanisms Controlling Re-replication

Local recruitment of Clb5 to origins of replication may represent a fourth level of control over re-replication in *S. cerevisiae*, implying a surprising redundancy in the prevention of re-replication. Indeed, at least three of these mechanisms must be mutated simultaneously to induce enough re-replication to be visible by FACS analysis. However, our data suggests that there are severe repercussions for the cell when only two of these mechanisms are mutated simultaneously (Figure 6). These results suggest that lethal amounts of re-replication occur long before significant amounts of replication can be seen by FACS. This lethality underscores the importance of cellular control over the initiation machinery in maintaining genomic fidelity.

There may be still more mechanisms restraining M phase re-replication. Additional mechanisms are suggested by the observation that even with all of the known mechanisms to control re-initiation deleted, the entire genome is still not re-replicated (Figure 7). Previous studies have indicated that some origins are more vulnerable than

others to re-replication during M phase(Nguyen et al. 2001; Vaziri et al. 2003). The re-replication that we observe may well be a result of repeated re-firing of only a subset of origins, consistent with the large distribution of DNA content that we observe in the FACS profiles of re-replicating cells. Even initiation from a subset of origins should be sufficient to replicate the entire genome, suggesting that there may be mechanisms inhibiting efficient elongation as well. The limiting amount of DNA replication could also be due to limiting pools of resources such as dNTPs that are normally tightly regulated in S phase to allow replication (reviewed in (Elledge et al. 1992)). If replication forks are collapsing due to lack of resources in mitotic cells, checkpoints may be activated (Tercero and Diffley 2001). Indeed, a recent report from human cells suggests that the DNA damage checkpoint prevents cells from re-replicating their DNA (Vaziri et al. 2003). It is possible that checkpoints are triggered in *S. cerevisiae* as well, limiting the extent of re-replication. Consistent with this idea, we find that mutations sensitizing cells to re-replication are synthetically lethal with a *mec1Δ* (data not shown).

Cyclin Specificity-Why does Clb5 act locally?

Our data suggests a unique role for Clb5 in associating with ORC at the origin to prevent re-replication. The interaction between Orc6 and Clb5 was originally identified in a screen for proteins that interacted with Clb5 through a hydrophobic patch which is proposed to help target the cyclin to specific partners(Cross and Jacobson 2000). Consistent with this indeed being the case, Orc6 interacts only weakly with Clb2 by two hybrid or by CoIP, and this interaction is not dependent on the RXL targeting motif

(Figure 1B, 3B). We have been unable to detect a direct interaction between either Clb2 or Clb3 and the origin by ChIP (data not shown).

A specific role among the cyclins for Clb5 in locally preventing re-replication provides a means to prevent re-initiation when the most potent CDK activator of replication is present. Clb5 is a highly potent CDK activator of DNA replication, as evidenced by its role in firing both early and late origins of replication, and by the inability of Clb2 to perform Clb5's role when expressed at comparable levels (Donaldson et al. 1998; Cross et al. 1999; Donaldson 2000). Therefore, if cells lose Clb5 activity and therefore local protection of the origin, they will by necessity also be less susceptible to activation of reformed pre-RCs. Consistent with this, even though both a *clb5Δ* and an *ORC6-ps,rxl* strain no longer have Clb5 bound protectively at the origin, we find that the amount of re-replication in *clb5Δ* cells is much less than in *ORC6-ps,rxl* cells in the same sensitized strain background (Figure 7).

A specific role for Clb5 in local origin protection would leave the origin unprotected after Clb5 is degraded at the metaphase to anaphase transition. Interestingly, the activating subunit of the S phase kinase Cdc7 is degraded at the metaphase to anaphase transition concomitant with Clb5 degradation (Ferreira et al. 2000). Therefore, although the other cyclins are present at high enough levels to be capable of activating origins, local protection of the origin may be less important because the second activating kinase is not present. It is also possible that the pool of Clb5 associated with the origin is protected from degradation. Indeed, we see some evidence of Clb5 origin association after the bulk of Clb5 is degraded (Figure 4C). A third possibility is that since the M

phase cyclins are extremely abundant at this period in the cell cycle they may act globally in a strong enough manner as to eliminate the need for Clb5 to act locally.

Mechanism for Clb5 Function

There are two possible mechanisms for Clb5 protection of origins from re-initiation. Clb5 association with the origin could act catalytically to phosphorylate targets, or it could act physically to prevent pre-RC formation.

Clb5 binding could, of itself, be sufficient to prevent pre-RC reformation. Clb5 could act as a localized switch, preventing pre-RC reformation by physically blocking the origin after the pre-RC is dismantled. If the kinase performs a physical role at the origin to prevent pre-RC formation, then association of the kinase with the origin and association of the pre-RC with the origin should be mutually exclusive. This is consistent with our observation that Clb5 binds to origins only at the time that they initiate, despite high overall Clb5 abundance before then (Figure 4C). A physical model for Clb5 action at the origin could represent a co-opting of the domains usually used to increase catalytic efficiency.

If the kinase performs a catalytic role at the origin to prevent pre-RC formation, then an obvious candidate protein to be continually phosphorylated by local catalytic activity is ORC. However, our data suggests that the stable interaction between Orc6 and Clb5 is unnecessary for ORC phosphorylation. Although a Clb5 hydrophobic patch mutation does decrease phosphorylation efficiency of both Orc6 and Orc2 *in vitro*, this decrease is not dependent on the presence of Orc6 in the complex (Figure 2C,D). Furthermore, the patch appears unnecessary for ORC phosphorylation *in vivo*. Orc6 is

fully shifted to a phosphorylated form on a gel in an Orc6 RXL mutant and a Clb5 hydrophobic patch mutant in asynchronous cells (Figure 3A). It remains possible that this shifted species does not represent full ORC phosphorylation, or that the interaction is necessary for ORC phosphorylation at certain points in the cell cycle. More strikingly, the Orc6 RXL domain has an important role in blocking replication even when all of the known ORC phosphorylation sites are mutated (Figure 7).

Local catalytic activity could also phosphorylate any MCMs, Cdc6, or other replication proteins that approached the origin any time after initiation and before the next G1. The association of Clb5 with ORC could be a means to increase the local concentration of the kinase. The Orc6 RXL domain has an important role in blocking re-replication even when the MCM proteins are nuclearly localized, and the N terminal Cdc6 phosphorylation sites are mutated (Figure 7). Therefore, local Clb5 catalytic activity could act through a novel mechanism of inhibiting MCM function by phosphorylation, through phosphorylation of the C-terminal Cdc6 phosphorylation sites to further destabilize Cdc6(Perkins et al. 2001), or through phosphorylation of a novel target. However, Cdc6 does not appear to be further stabilized in the mutant Orc6 strains by western blotting (data not shown).

The current data do not differentiate between a physical or a catalytic role for Clb5 at origins of replication. Indeed, it is also possible that both modes of protection act synergistically. Further experiments will be necessary to confirm whether the catalytic activity of Cdc28 is required for, or contributes to Clb5's protective role at origins of DNA replication.

An origin-localized replication control switch.

We propose that Clb5/Cdc28 binds to ORC only after replication initiation has occurred (Fig. 5). We speculate that this could be due to steric hindrance of Clb5 association with ORC by pre-RC components. Such a mechanism would allow Clb5/Cdc28 to locally block initiation from origins that have initiated or been passively replicated by a replication fork derived from an adjacent origin (and therefore lack a pre-RC) but not at unreplicated origins in the same cell (which would have a pre-RC). This model provides an economical mechanism allowing Clb5 to globally promote replication initiation (by a mechanism that may not require origin localization), and at the same time to locally block re-initiation at origins that have already been replicated. The specialization of Clb5 to mediate such a function (rather than Clb1-4) is consistent with an important role for Clb5 during S phase. Such a locally acting mechanism is distinct from the more global mechanisms that affect Cdc6 and Mcm2-7 complex function and has the advantage that it could rapidly act at origins as soon as they have initiated.

Experimental Procedures

Plasmids and Strains

Strains (W303 background) and plasmids were prepared using standard laboratory methods (Ausubel 1992). Plasmid pSPB65 contained *ORC6* in pRS315, and pSB6-21 contained the *ORC6* phosphorylation site mutations S106A, S116A, S123A, T146A. FC606 and FC608 were identical to SPB65 and SPB6-21 but contained the *ORC6-rxl* mutation R178A,L180A. The *ORC6* coding sequence in SPB65, SPB6-21, FC606 and FC608 was tagged with Protein A as described (Aitchison et al., 1995; Wach et al., 1997) to produce SPB65-A, SPB6-21-A, FC606-A and FC608-A. The wild-type chromosomal *ORC6* locus was tagged similarly. Plasmids pRS406*ORC6-wt*, pRS406*ORC6-rxl*, pRS406*ORC6-ps*, were made by cloning a NotI/ XhoI fragment of ORC6 from pSB65, pFC606, and pSPB6.21, respectively, into pRS406WT cut with the same enzymes. pRS406*ORC6-ps, rxl* was made by amplifying the RXL mutation from pRS406*ORC6-rxl* with oligos SB831, TCTATTACTAGGGCAAAGGCAGCA and SB795, CACACACTAATTGCCATGGGC, and then using this megaprimer to amplify the *ORC6* Cdk phosphorylation site mutations from pRS406*ORC6-ps* with the oligo SB795, CACACACTAATTGCCATGGGC. This product was cut with NdeI/ NcoI and cloned into pRS406*ORC6-wt* cut with the same enzymes. *ORC6* mutant strains were constructed by cutting plasmids pRS406*ORC6-wt*, pRS406*ORC6-rxl*, pRS406*ORC6-ps*, and pRS406*ORC6-ps-,rxl* with BsmI and integrating into ySB1028 (*orc6::HisMX*, pSPB66 (*ORC6 , URA3*)) and then streaking onto 5-FOA to lose the pSB66*ORC6* plasmid. *GAL-Cdc6Δ2-48-HA* strains were constructed as described (Klemm and Bell

2001). Strains containing mutations in *ARS1* were made as described previously (Wilmes and Bell, 2002). *ORC2-ps* strains and *MCM7-NLS* strains were constructed as described with plasmids pJL1095 and pJL1206, respectively, generous gifts from Joachim Li (Nguyen et al. 2001). See Table S1 for strain genotypes.

2-hybrid analysis.

Full-length *CLB5*, *clb5-hpm*, *clb5-Q241A* ('*qa*') and *CLB2* were fused to the *GAL4* DNA-binding domain in the vector pBDU-C1 (James et al., 1996) as described previously (Cross and Jacobson 2000). *GAL4* activation domain (AD) fusion libraries (James et al., 1996) were transformed into PJ69-4A (James et al., 1996) carrying *CLB5-DBD*, and transformants selected for a His+ Leu+ Ura+ phenotype and subsequently screened for an Ade+ phenotype, essentially as described (James et al., 1996). The His+ and Ade+ phenotypes result from activation of different reporters present in PJ69-4A, eliminating a high background from selecting for the His+ phenotype alone (James et al., 1996). Following *CLB5-DBD* plasmid loss selected by 5-FOA, the GAD plasmid transformants were mated to PJ69-4α carrying *CLB5-DBD* or *clb5-hpm-DBD* plasmids, to identify interactors specifically dependent on the Clb5 hydrophobic patch. Transformants were tested similarly with *CLB5-qa-DBD* and *CLB2-DBD*. The *ORC6-AD* clone (C5I-13) identified in this screen contained the Gal4 AD fused to *ORC6* coding sequence starting from I38 and extending to about 25 codons from the *ORC6* C-terminus. (This clone was extended to contain the complete *ORC6* C-terminus with similar results to those presented; data not shown). The *ORC6* phosphorylation site mutations (S106A, S116A, S123A, T146A) present in plasmid SPB6-21 were transferred into C5I-13 by

substitution of an NcoI-NdeI fragment. The R178A, L180A mutation was introduced by PCR mutagenesis into either wild-type or phosphorylation-site-mutated *ORC6*, and the desired coding sequences in the entire mutagenized regions confirmed.

Clb5/Cdc28 Expression and Purification

His-HA-Clb5-wt and His-HA-Clb5-hpm were expressed in FastBac baculo transfer vectors (pSB1024 and pSB1023) as was GST-Cdc28-HA (pFBCdc28). Baculovirus expression of wt and mutant Clb5/Cdc28 was carried out as described previously (Klemm et al, 1997). The proteins were purified from the nuclear extracts by binding and elution from Glutathione Sepharose 4 Fast Flow resin (Amersham Pharmacia, Piscataway, NJ), as directed by the manufacturer, except that the binding buffer was 12.5 mM HEPES-KOH(pH7.5), 0.4M KCl, 2.5mM magnesium acetate, 1 mM EDTA, 1 mM EGTA, 0.01%NP40, 1 mM dithiothreitol, and the elution buffer was the same plus 10 mM reduced glutathione (Sigma- Aldrich, St. Louis, MO).

Kinase assays

The proteins were mixed together on ice in the indicated concentrations in 12.5 mM HEPES-KOH(pH7.5), 0.4M KCl, 2.5mM magnesium acetate, 1 mM EDTA, 1 mM EGTA, 0.01%NP40, 1 mM dithiothreitol, with 10 μCuries gamma ^{32}P, 5 μM ATP, and 5 μM magnesium chloride. Reactions were incubated at room temperature for 20 minutes, and then run on 10% SDS-PAGE, and examined by autoradiography.

Electrophoretic mobility shift assays.

ORC/origin EMSA's were performed as described previously (Lee and Bell, 1997), except that the level of ATP present in all reactions was 0.5 mM, and the competitor DNA was 50 mg/ml dGdC.

Protein A affinity purifications and Western blotting

Protein A affinity purifications of Orc6-PrA were performed as described previously (Archambault et al., 2003). *MYC-CLB5* (wt and mutant) was expressed from the chromosomal locus. Orc6-PrA, wt and mutants, were expressed from low-copy plasmids SPB65-A, SPB6-21-A, FC606-A and FC608-A (see above). These plasmids expressed a somewhat lower level of Orc6-PrA than was observed with chromosomally tagged *ORC6*; hydrophobic patch-dependent Clb5-Orc6 interaction was also observed in the latter context (data not shown).

Chromatin Immunoprecipitation

ChIP was performed as described (Aparicio and Bell, 1997), with minor modifications. Cells were crosslinked on ice overnight before washing. Cells were broken with a 45 second pulse with an equal volume of glass beads at setting 6.5 in a FastPrep FP120 machine (Bio 101/ Savant, Holbrook, NY). Myc-Clb5 was immunoprecipitated overnight with a 1:250 dilution of 9E11 monoclonal antibody (Genetex, San Antonio, TX). Quantitation was performed by running samples in triplicate on a 7000 Sequence Detection System (Applied Biosystems, Branchberg, New Jersey) and comparing to tenfold dilutions of similarly prepared genomic DNA with TaqMan Universal PCR Mix (Applied Biosystems, Branchberg, New Jersey) and 0.5x Sybr Green (Molecular Probes,

Eugene, OR). The indicated origin/non-origin DNA was plotted, with the standard deviations of the three reactions.

Re-replication and Viability Assays

Strains were arrested with 15 μg/ml nocodazole (Sigma- Aldrich, St. Louis, MO) in media containing 2% raffinose for four hours at room temperature, and then induced after the zero hour timepoint with 2% galactose. Samples were taken every hour for FACS, viability, and Western analysis of Cdc6Δ2-48-HA levels (data not shown). For the viability assays, 500 cells were counted at the zero hour, and the same volume of sonicated cells was plated onto glucose media at each timepoint. The number of colonies was counted after two days, and the number of colonies at each timepoint was divided by the zero hour time-point to determine a percentage viability. DNA content was measured by flow cytometry as described previously (Bell et al, 1993).

Chromatin Association Assays

Chromatin association was assayed as described previously (James et al., 1996). Westerns were performed as described above. Mcm2-7 were detected with a 1:2000 dilution of a polyclonal antibody, UMU185. Orc2 and Orc6 were detected with 1:1000 dilutions of the monoclonal antibodies SB61 and SB49, respectively. Cdc6Δ2-48-HA was detected with a 1:1000 dilution of Ha.11 (Babco, Richmond, CA).

References

Adams, P.D., X. Li, W.R. Sellers, K.B. Baker, X. Leng, J.W. Harper, Y. Taya, and W.G. Kaelin, Jr. 1999. Retinoblastoma protein contains a C-terminal motif that targets it for phosphorylation by cyclin-cdk complexes. *Mol Cell Biol* **19**: 1068-80.

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

Ausubel, F.M. 1992. *Current protocols in molecular biology*. Published by Greene Pub. Associates and Wiley-Interscience : J. Wiley, New York.

Bell, S.P. and A. Dutta. 2002. DNA replication in eukaryotic cells. *Annu Rev Biochem* **71**: 333-74.

Brown, N.R., M.E. Noble, J.A. Endicott, and L.N. Johnson. 1999. The structural basis for specificity of substrate and recruitment peptides for cyclin-dependent kinases. *Nat Cell Biol* **1**: 438-43.

Calzada, A., M. Sanchez, E. Sanchez, and A. Bueno. 2000. The stability of the Cdc6 protein is regulated by cyclin-dependent kinase/cyclin B complexes in Saccharomyces cerevisiae. *J Biol Chem* **275**: 9734-41.

Cliften, P., P. Sudarsanam, A. Desikan, L. Fulton, B. Fulton, J. Majors, R. Waterston, B.A. Cohen, and M. Johnston. 2003. Finding functional features in Saccharomyces genomes by phylogenetic footprinting. *Science* **301**: 71-6.

Cross, F.R. and M.D. Jacobson. 2000. Conservation and function of a potential substrate-binding domain in the yeast Clb5 B-type cyclin. *Mol Cell Biol* **20**: 4782-90.

Cross, F.R., M. Yuste-Rojas, S. Gray, and M.D. Jacobson. 1999. Specialization and targeting of B-type cyclins. *Mol Cell* **4**: 11-9.

Devault, A., E.A. Vallen, T. Yuan, S. Green, A. Bensimon, and E. Schwob. 2002. Identification of Tah11/Sid2 as the ortholog of the replication licensing factor Cdt1 in Saccharomyces cerevisiae. *Curr Biol* **12**: 689-94.

Diffley, J.F., J.H. Cocker, S.J. Dowell, and A. Rowley. 1994. Two steps in the assembly of complexes at yeast replication origins in vivo. *Cell* **78**: 303-16.

Donaldson, A.D. 2000. The yeast mitotic cyclin Clb2 cannot substitute for S phase cyclins in replication origin firing. *EMBO Rep* **1**: 507-12.

Donaldson, A.D., M.K. Raghuraman, K.L. Friedman, F.R. Cross, B.J. Brewer, and W.L. Fangman. 1998. CLB5-dependent activation of late replication origins in S. cerevisiae. *Mol Cell* **2**: 173-82.

Donovan, S., J. Harwood, L.S. Drury, and J.F. Diffley. 1997. Cdc6p-dependent loading of Mcm proteins onto pre-replicative chromatin in budding yeast. *Proc Natl Acad Sci U S A* **94**: 5611-6.

Drury, L.S., G. Perkins, and J.F. Diffley. 1997. The Cdc4/34/53 pathway targets Cdc6p for proteolysis in budding yeast. *Embo J* **16**: 5966-76.

-. 2000. The cyclin-dependent kinase Cdc28p regulates distinct modes of Cdc6p proteolysis during the budding yeast cell cycle. *Curr Biol* **10**: 231-40.

Elledge, S.J., Z. Zhou, and J.B. Allen. 1992. Ribonucleotide reductase: regulation, regulation, regulation. *Trends Biochem Sci* **17**: 119-23.

Ferreira, M.F., C. Santocanale, L.S. Drury, and J.F. Diffley. 2000. Dbf4p, an essential S phase-promoting factor, is targeted for degradation by the anaphase-promoting complex. *Mol Cell Biol* **20**: 242-8.

Furstenthal, L., B.K. Kaiser, C. Swanson, and P.K. Jackson. 2001a. Cyclin E uses Cdc6 as a chromatin-associated receptor required for DNA replication. *J Cell Biol* **152**: 1267-78.

Furstenthal, L., C. Swanson, B.K. Kaiser, A.G. Eldridge, and P.K. Jackson. 2001b. Triggering ubiquitination of a CDK inhibitor at origins of DNA replication. *Nat Cell Biol* **3**: 715-22.

James, P., J. Halladay, and E.A. Craig. 1996. Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast. *Genetics* **144**: 1425-36.

Jang, S.W., S. Elsasser, J.L. Campbell, and J. Kim. 2001. Identification of Cdc6 protein domains involved in interaction with Mcm2 protein and Cdc4 protein in budding yeast cells. *Biochem J* **354**: 655-61.

Kellis, M., N. Patterson, M. Endrizzi, B. Birren, and E.S. Lander. 2003. Sequencing and comparison of yeast species to identify genes and regulatory elements. *Nature* **423**: 241-54.

Klemm, R.D. and S.P. Bell. 2001. ATP bound to the origin recognition complex is important for preRC formation. *Proc Natl Acad Sci U S A* **98**: 8361-7.

Labib, K., J.F. Diffley, and S.E. Kearsey. 1999. G1-phase and B-type cyclins exclude the DNA-replication factor Mcm4 from the nucleus. *Nat Cell Biol* **1**: 415-22.

Leatherwood, J., G.A. Lopez, and P. Russell. 1996. Interaction of Cdc2 and Cdc18 with a fission yeast ORC2-like protein. *Nature* **379**: 360-363.

Lee, D.G. and S.P. Bell. 1997. Architecture of the yeast origin recognition complex bound to origins of DNA replication. *Mol Cell Biol* **17**: 7159-68.

Liang, C. and B. Stillman. 1997. Persistent initiation of DNA replication and chromatin-bound MCM proteins during the cell cycle in cdc6 mutants. *Genes Dev* **11**: 3375-86.

Masumoto, H., S. Muramatsu, Y. Kamimura, and H. Araki. 2002. S-Cdk-dependent phosphorylation of Sld2 essential for chromosomal DNA replication in budding yeast. *Nature* **415**: 651-5.

Miller, M.E. and F.R. Cross. 2001. Cyclin specificity: how many wheels do you need on a unicycle? *J Cell Sci* **114**: 1811-20.

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

Nguyen, V.Q., C. Co, and J.J. Li. 2001. Cyclin-dependent kinases prevent DNA re-replication through multiple mechanisms. *Nature* **411**: 1068-73.

Pasero, P., B.P. Duncker, E. Schwob, and S.M. Gasser. 1999. A role for the Cdc7 kinase regulatory subunit Dbf4p in the formation of initiation-competent origins of replication. *Genes Dev* **13**: 2159-76.

Perkins, G., L.S. Drury, and J.F. Diffley. 2001. Separate SCF(CDC4) recognition elements target Cdc6 for proteolysis in S phase and mitosis. *Embo J* **20**: 4836-45.

Romanowski, P., J. Marr, M.A. Madine, A. Rowles, J.J. Blow, J. Gautier, and R.A. Laskey. 2000. Interaction of Xenopus Cdc2 x cyclin A1 with the origin recognition complex. *J Biol Chem* **275**: 4239-43.

Russo, A.A., P.D. Jeffrey, A.K. Patten, J. Massague, and N.P. Pavletich. 1996. Crystal structure of the p27Kip1 cyclin-dependent-kinase inhibitor bound to the cyclin A-Cdk2 complex. *Nature* **382**: 325-31.

Schulman, B.A., D.L. Lindstrom, and E. Harlow. 1998. Substrate recruitment to cyclin-dependent kinase 2 by a multipurpose docking site on cyclin A. *Proc Natl Acad Sci U S A* **95**: 10453-8.

Schwob, E., T. Bohm, M.D. Mendenhall, and K. Nasmyth. 1994. The B-type cyclin kinase inhibitor p40SIC1 controls the G1 to S transition in S. cerevisiae. *Cell* **79**: 233-44.

Schwob, E. and K. Nasmyth. 1993. CLB5 and CLB6, a new pair of B cyclins involved in DNA replication in Saccharomyces cerevisiae. *Genes Dev* **7**: 1160-75.

Sclafani, R.A. 2000. Cdc7p-Dbf4p becomes famous in the cell cycle. *J Cell Sci* **113 (Pt 12)**: 2111-7.

Takeda, D.Y., J.A. Wohlschlegel, and A. Dutta. 2001. A bipartite substrate recognition motif for cyclin-dependent kinases. *J Biol Chem* **276**: 1993-7.

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

Tanaka, T., D. Knapp, and K. Nasmyth. 1997. Loading of an Mcm protein onto DNA replication origins is regulated by Cdc6p and CDKs. *Cell* **90**: 649-60.

Tercero, J.A. and J.F. Diffley. 2001. Regulation of DNA replication fork progression through damaged DNA by the Mec1/Rad53 checkpoint. *Nature* **412**: 553-7.

Vaziri, C., S. Saxena, Y. Jeon, C. Lee, K. Murata, Y. Machida, N. Wagle, D.S. Hwang, and A. Dutta. 2003. A p53-dependent checkpoint pathway prevents rereplication. *Mol Cell* **11**: 997-1008.

Weinreich, M., C. Liang, H.H. Chen, and B. Stillman. 2001. Binding of cyclin-dependent kinases to ORC and Cdc6p regulates the chromosome replication cycle. *Proc Natl Acad Sci U S A* **98**: 11211-7.

Wilmes, G.M. and S.P. Bell. 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**: 101-6.

Wohlschlegel, J.A., B.T. Dwyer, D.Y. Takeda, and A. Dutta. 2001. Mutational analysis of the Cy motif from p21 reveals sequence degeneracy and specificity for different cyclin-dependent kinases. *Mol Cell Biol* **21**: 4868-74.

Wuarin, J., V. Buck, P. Nurse, and J.B. Millar. 2002. Stable association of mitotic cyclin B/Cdc2 to replication origins prevents endoreduplication. *Cell* **111**: 419-31.

Chapter IV

Discussion

Key Conclusions

The studies that I have described in this thesis investigate the formation of protein complexes at origins of replication throughout the cell cycle. In chapter two, I described the dissection of a *cis*-acting element required for efficient function of the well-characterized yeast origin *ARS1*. These studies suggest that an A-rich sequence similar to ORC's binding site is necessary at the B2 element of the origin. ORC does not bind these alternate sequences well *in vitro*. Since this sequence is necessary for efficient MCM chromatin association *in vivo*, it is likely that it interacts with the Orc1 homolog Cdc6 or with Cdt1 or the MCMs during MCM loading. In chapter three, I described chromatin Immunoprecipitation experiments suggesting that the S phase cyclin Clb5 is part of the post-RC at origins after initiation. Clb5 associates with origins through a hydrophobic patch on Clb5 that interacts with an RXL motif on Orc6, and secondarily with the consensus CDK phosphorylation sites on Orc6, as determined by two hybrid, co-immunoprecipitation, and chromatin immunoprecipitation. Interruption of this interaction has no effect on initiation of replication. In contrast, interrupting this interaction makes cells more susceptible to DNA re-replication in M phase. These studies underline the importance of strict regulation over complex formation at origins as a manner to regulate DNA replication.

Origin Structure

The recent localization of the majority of yeast origins to within 500 base pairs can provide a foundation for expanding our understanding of the sequence requirements at origins of replication (Raghuraman et al. 2001; Wyrick et al. 2001). For these data sets

to be useful, however, will first require a better understanding of the requirements for ORC binding. Combining studies delineating in more detail the residues required for ORC binding *in vitro* with sequence comparisons of the known essential ORC binding sites may allow the definition of a longer and more specific consensus. This consensus might give us the ability to predict the essential A element at newly identified origins without mutational analysis. Knowledge of both the sequences of many origins and the precise locations of the ORC binding sites will facilitate analysis of the auxiliary sequences necessary for origin function. This would then allow the alignment of the B regions of many origins, and possibly the determination of more specific sequence elements necessary for origin function. It is noteworthy that all of the well-characterized yeast origins contain many extra weak ORC binding sites in the important B region of the origin. *ARS1* is the only origin containing only a single extra ACS match, and that site appears to be necessary for pre-RC function. Large scale comparisons may allow determination of whether this sequence specificity that I identified in chapter two as important for *ARS1* function is important at other yeast origins.

Pre-RC architecture

An understanding of the architecture of the pre-RC is central to beginning to understand the mechanisms of initiation. The architecture of the pre-RC encompasses where the pre-RC proteins contact the origin, where they contact each other, and the stoichiometry of the different members of the complex. This information will further analysis of the important transitions that take place at the origin. These include origin

unwinding, the recruitment of the replication machinery, the synthesis of DNA, and the regulation of all of these processes throughout the cell cycle.

The experiments described in chapter two suggest the possibility that an alternate conformation of ORC multimerizes at the origin during G1 to help form the pre-RC. This model would predict that ORC could be co-immunoprecipitated with itself at some point in the cell cycle if the same ORC subunit was marked with two different tags. However, because the ORC epitopes are masked by the pre-RC, it might be difficult to detect a multimerization even if one occurred.

Even less is known about the association of the other pre-RC components with the origin, although we can make some guesses about the stoichiometry of these complexes. Since the MCM2-7 complex is thought to travel with the bidirectional replication fork, there are probably at least two complexes at each origin. However, there may be even greater than two MCM complexes per origin. This is suggested by experiments in *Xenopus* demonstrating that many copies of the MCMs can bind to the origin spread out around ORC (Edwards et al. 2002), although initiation in Xenopus egg extracts is nonspecific. In *S. cerevisiae*, the length of DNA protected by MCMs differs at some origins, suggesting that some origins may associate with more MCM complexes than others (Labib et al. 2001). As Cdc6 and Cdt1 probably load the MCMs onto the DNA, their stoichiometry may mirror that of the MCMs, or they may each load multiple MCM complexes. Indeed, it is unclear whether Cdc6 and Cdt1 are stable components of the pre-RC, or whether they associate only briefly as they load the MCMs.

Although the sequences necessary at the B2 element resemble ORC's binding site, they could also be binding sites for any of the members of the pre-RC. Cdc6 is

particularly likely, as it is highly homologous to Orc1, one of the major ORC subunits implicated in DNA binding. Furthermore, Cdc6 contains a possible DNA binding motif, a winged helix, that appears important for function in *S. pombe*. However, no specific DNA binding has been implicated for Cdc6. It is likely recruited to the origin through interactions with Orc1, but could well be recruited through joint interactions with Orc1 and the B2 element. The effect of a mutation in the B2 element at *ARS1* on Cdc6 origin association could be tested *in vivo* in cells isolated early in G1 via elutriation. Cdt1 is relatively newly discovered and not well-characterized and could interact with the DNA, although origin association has not been detected in *S. cerevisiae* (Tanaka and Diffley 2002). There has been a report of DNA binding activity by mouse Cdt1 (Yanagi et al. 2002). Likewise, other newly identified putative pre-RC proteins, such as Mcm10 or Noc3, could interact with specific DNA sequences (Homesley et al. 2000; Zhang et al. 2002). However, they would only be candidates for B2 element binding if they acted upstream of MCM association with the origin, and this is not thought to be the case for MCM10 in higher organisms (Wohlschlegel et al. 2002).

By analogy with prokaryotic origins of replication, it is likely that the initiation complexes interact with specific sequences in the DUE area of the origin to unwind the DNA. Although ORC binding to DNA does not cause any origin unwinding, it is possible that ORC, Cdc6, and Cdt1 may cooperate to open origins. The MCMs are stably associated with the origin after they are loaded, even if ORC, Cdc6, and Cdt1 are salt-extracted from the chromatin. This suggests that they are topologically associated with the DNA, but does not differentiate between association around single-stranded or double-stranded DNA. More substantial origin unwinding does not occur until DDK and

CDK activity initiates DNA replication, as measured by RPA association with the origin, supercoiling state of a plasmid in a Xenopus extract, or chemically probed unwinding at the origin *in vivo* (Tanaka and Nasmyth 1998; Geraghty et al. 2000; Walter and Newport 2000). The B2 element overlaps with the DUE, and the origin of bi-directional replication, where DNA synthesis begins, is between the B2 element and the A element. Therefore, interactions with the B2 element are probably central to the initial unwinding of the origin.

The recent development of an *in vitro* system for pre-RC assembly will facilitate more direct probing of the architecture of the pre-RC (Seki and Diffley 2000). Experiments that are very difficult to perform *in vivo* such as probing for DNA unwinding may be more informative on reconstituted pre-RCs. Similarly, purification of the pre-RC will also facilitate counting of the approximate stoichiometry of the proteins, and identification of the sequence elements needed for association of the pre-RC proteins with the origin. All of these experiments would be facilitated by the reconstitution of the pre-RC entirely from purified proteins.

What is the role of ORC phosphorylation in preventing re-replication?

The experiments described in chapter three suggest that ORC phosphorylation might prevent origin activation by multiple mechanisms. The Orc6 phosphorylation sites contribute to recruitment of Clb5 to the origin. In contrast, phosphorylated Orc2 is not sufficient to support stable association of Clb5 as ORC lacking Orc6 can be phosphorylated by Clb5 but does not associate stably with Clb5. Experiments are underway to determine whether Clb5 associates with the origin in an *ORC2* CDK

phosphorylation site mutant to address whether Orc2 phosphorylation also modulates Clb5 association with the origin.

Another possibility is that Orc2 phosphorylation prevents reformation of the pre-RC directly. This is consistent with our observation that Clb5 does not associate with the origin in an ORC6-ps,rxl strain, but mutating the Orc2 phosphorylation sites still causes a considerable increase in re-replication. This hypothesis could be tested both *in vivo* and *in vitro*. *In vivo*, MCM chromatin association during M phase could be tested in combinations of mutants affecting re-replication. This would assess whether the Orc2 phosphorylation sites contribute to prevention of pre-RC formation or a downstream step in origin activation. If Orc2 phosphorylation prevents pre-RC assembly, then mutants of ORC with amino acids simulating phosphorylation (Glu/Asp) would not be expected to complement the replication defect and lack of viability of an *orc2Δ* strain. Similar experiments could be performed in an *in vitro* pre-RC assembly system. *In vitro*, ORC could be phosphorylated, and then removed from physical association with Clb5/Cdc28 to more definitively separate the role of ORC phosphorylation from the role of Clb5 association.

How does Clb5 Origin association Prevent Re-replication?

Clb5 may act at the origin by physically blocking re-replication. The timing of Clb5 origin association, after an origin has fired, but not before, suggests the possibility that pre-RC formation is incompatible with Clb5 association with the origin. This could be tested by preventing pre-RC formation by depleting cells of Cdc6. When Cdc6 is turned off, pre-RCs are not formed, and origins do not initiate. However, cells still travel

through the cell cycle, eventually undergoing a reductional anaphase, and Clb5 is still induced (Piatti et al. 1996). This would allow testing of whether Clb5 associates with origins that do not have pre-RCs, but have also not initiated. In contrast, arresting cells directly before they fire their origins, with a *cdc7* temperature sensitive allele, would test whether Clb5 associates at the time that origins fire, or directly after. Again, these *in vivo* studies could be complemented with studies of pre-RC assembly *in vitro,* where it would be possible to see if Clb5 could associate with origin DNA containing pre-formed pre-RCs.

If Clb5 does not physically block pre-RC formation, it may be acting catalytically to phosphorylate local substrates in the vicinity of the origin. A mutation in the kinase interacting domain in Clb5 that completely abolishes Clb5 associated kinase activity by preventing association with Cdc28 could be used to test whether kinase activity is necessary for origin protection by Clb5 (Cross et al. 1999). If this mutant did not protect cells from re-replication, it would suggest a catalytic block to re-replication. However, this mutation could also prevent a physical block to re-replication if the physical presence of Cdc28 were necessary to provide enough bulk to prevent the pre-RC from reforming at the origin, even though Cdc28 is approximately half the molecular weight of Clb5. Therefore, a more definitive experiment might be to test whether Clb5 fused to catalytically inactive Cdc28 could still protect cells from re-replicating, assuming that this protein could still associate with the origin.

Why Clb5? Specialization of Clb function.

Since Clb5 shares sequence similarity with Clb6, it might seem likely that Clb6 could also associate with origins. Clb5 and 6 are the first B-type cyclins to be synthesized, and are the Clbs that normally initiate replication. It would be elegant if their association with origins were to immediately prevent re-initiation after an origin initiated. To our surprise, mutating Clb6 in a *Clb5Δ, Cdc6ΔN* or *Clb5Δ, ORC6-ps,rxl, Cdc6ΔN* background rescued the lethality of these cells, instead of worsening the phenotype. When Clb5 and 6 are both deleted, replication is delayed for approximately half an hour until Clb3 and 4 levels rise (Donaldson et al. 1998). One possibility is that the delay before initiation of replication, allows very little time after origins have initiated and before cells divide before lethal re-replication to occur. This model predicts that although these cells have no defects in a normal cell cycle, they would still have a defect when arrested in mitosis.

The increase in the levels of the other B-type cyclins could help protect origins after initiation. We found that by two hybrid (Clb2) and CoIP (Clb 2 and Clb3) Orc6 is capable of interacting with the other Clbs. These interactions are weaker, and are dependent only on the phosphorylation sites, not the RXL domain. We have not been able to detect any association between Clb2 or Clb3 and the origin by ChIP (data not shown). It is possible, though, that in the absence of Clb5 this interaction is stronger. To that end, it would be interesting to assess the association of the other B-type cyclins in a *Clb5Δ* background to see if they can associate with the origin.

Cell Cycle Regulation of Re-replication

Local association of Clbs with origins is likely to protect cells from re-replication during most, but not all of the cell cycle. Clb5 is present during more of the cell cycle than any of the other Clbs, but the bulk of Clb5 is still degraded at the metaphase to anaphase transition. After anaphase, the levels of the other Clb kinases are still extremely high and may be capable of activating origins. Interestingly, Dbf4, the activating subunit of the S phase kinase Cdc7, is degraded coincidentally with Clb5 (Ferreira et al. 2000), which could provide a mechanism to prevent re-initiation of any pre-RCs that do re-form during the absence of Clb5 protection after metaphase. This model predicts that arresting cells at the end of mitosis with mutations in *CDC14* or *CDC15* would allow pre-RCs to re-form, but not re-initiate, in a sensitized strain background containing nuclearly localized MCMs and *Cdc6ΔN*. The importance of Dbf4 degradation could be tested by expressing a stabilized version of Dbf4 to see if this then allows re-replication in this strain background. Similarly, bypassing the requirement for Cdc7 in initiation with a *bob1* allele of *MCM5* (Hardy et al. 1997) would be predicted to allow re-replication in this strain background if Cdc7 degradation normally represses re-initiation.

Cells may be vulnerable to re-replication from different mechanisms at different times during the cell cycle, or in different cell types. All of the studies to date of re-replication phenomena in *S. cerevisiae* have focused on re-replication during M phase. Our genetic interactions between re-replication mutants suggest that these mechanisms are also important in a normal cell cycle, since these cells have problems with re-replication but are never arrested in M phase. Further experiments are necessary to assess the roles of different mechanisms at different times in the cell cycle. It is likely to be much easier to induce re-replication in embryonic or developing cells. Embryonic

cells must be poised to replicate their DNA very quickly and therefore do so more promiscuously than differentiated cells. Many of the mutations studied in higher eukaryotes cause multiple rounds of endoreduplication in some cell types. Although not ideal, it may be far preferential to completely rereplicate an extra copy of the genome than to replicate 10 extra copies of a few discrete parts of the genome.

References

Cross, F.R., M. Yuste-Rojas, S. Gray, and M.D. Jacobson. 1999. Specialization and targeting of B-type cyclins. *Mol Cell* **4**: 11-9.

Donaldson, A.D., M.K. Raghuraman, K.L. Friedman, F.R. Cross, B.J. Brewer, and W.L. Fangman. 1998. CLB5-dependent activation of late replication origins in S. cerevisiae. *Mol Cell* **2**: 173-82.

Edwards, M.C., A.V. Tutter, C. Cvetic, C.H. Gilbert, T.A. Prokhorova, and J.C. Walter. 2002. MCM2-7 complexes bind chromatin in a distributed pattern surrounding the origin recognition complex in Xenopus egg extracts. *J Biol Chem* **277**: 33049-57.

Ferreira, M.F., C. Santocanale, L.S. Drury, and J.F. Diffley. 2000. Dbf4p, an essential S phase-promoting factor, is targeted for degradation by the anaphase-promoting complex. *Mol Cell Biol* **20**: 242-8.

Geraghty, D.S., M. Ding, N.H. Heintz, and D.S. Pederson. 2000. Premature structural changes at replication origins in a yeast minichromosome maintenance (MCM) mutant. *J Biol Chem* **275**: 18011-21.

Hardy, C.F., O. Dryga, S. Seematter, P.M. Pahl, and R.A. Sclafani. 1997. mcm5/cdc46-bob1 bypasses the requirement for the S phase activator Cdc7p. *Proc Natl Acad Sci U S A* **94**: 3151-5.

Homesley, L., M. Lei, Y. Kawasaki, S. Sawyer, T. Christensen, and B.K. Tye. 2000. Mcm10 and the MCM2-7 complex interact to initiate DNA synthesis and to release replication factors from origins. *Genes Dev* **14**: 913-26.

Labib, K., S.E. Kearsey, and J.F. Diffley. 2001. MCM2-7 proteins are essential components of prereplicative complexes that accumulate cooperatively in the nucleus during G1-phase and are required to establish, but not maintain, the S-phase checkpoint. *Mol Biol Cell* **12**: 3658-67.

Piatti, S., T. Bohm, J.H. Cocker, J.F. Diffley, and K. Nasmyth. 1996. Activation of S-phase-promoting CDKs in late G1 defines a "point of no return" after which Cdc6 synthesis cannot promote DNA replication in yeast. *Genes Dev* **10**: 1516-31.

Raghuraman, M.K., E.A. Winzeler, D. Collingwood, S. Hunt, L. Wodicka, A. Conway, D.J. Lockhart, R.W. Davis, B.J. Brewer, and W.L. Fangman. 2001. Replication dynamics of the yeast genome. *Science* **294**: 115-21.

Seki, T. and J.F. Diffley. 2000. Stepwise assembly of initiation proteins at budding yeast replication origins in vitro. *Proc Natl Acad Sci U S A* **97**: 14115-20.

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

Tanaka, T. and K. Nasmyth. 1998. Association of RPA with chromosomal replication origins requires an Mcm protein, and is regulated by Rad53, and cyclin- and Dbf4-dependent kinases. *Embo J* **17**: 5182-91.

Walter, J. and J. Newport. 2000. Initiation of eukaryotic DNA replication: origin unwinding and sequential chromatin association of Cdc45, RPA, and DNA polymerase alpha. *Mol Cell* **5**: 617-27.

Wohlschlegel, J.A., S.K. Dhar, T.A. Prokhorova, A. Dutta, and J.C. Walter. 2002. Xenopus Mcm10 binds to origins of DNA replication after Mcm2-7 and stimulates origin binding of Cdc45. *Mol Cell* **9**: 233-40.

Wyrick, J.J., J.G. Aparicio, T. Chen, J.D. Barnett, E.G. Jennings, R.A. Young, S.P. Bell, and O.M. Aparicio. 2001. Genome-wide distribution of ORC and MCM proteins in S. cerevisiae: high-resolution mapping of replication origins. *Science* **294**: 2357-60.

Yanagi, K., T. Mizuno, Z. You, and F. Hanaoka. 2002. Mouse geminin inhibits not only Cdt1-MCM6 interactions but also a novel intrinsic Cdt1 DNA binding activity. *J Biol Chem* **277**: 40871-80.

Zhang, Y., Z. Yu, X. Fu, and C. Liang. 2002. Noc3p, a bHLH protein, plays an integral role in the initiation of DNA replication in budding yeast. *Cell* **109**: 849-60.