Cell Cycle Regulation of Complex Formation at Origins of DNA Replication

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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 auxiliary 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.
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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
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 is 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
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 | Mechanism
<table>
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<tr>
<td>S. pombe</td>
<td>CDK-independent: WCE2-7</td>
</tr>
<tr>
<td>S. cerevisiae</td>
<td>ORC: ORC-7</td>
</tr>
<tr>
<td>C. elegans</td>
<td>melanogaster D</td>
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<tr>
<td>X. laevis</td>
<td>ORE1/2-7</td>
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<td>C. elegans</td>
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<tr>
<td>H. sapiens</td>
<td>Melezona: ORE1/2-7</td>
</tr>
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</table>

Table 1: 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 cyclins 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.

\textit{S. pombe}

Re-replication in \textit{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 \textit{S. pombe} over all of the factors regulated in \textit{S. cerevisiae}: ORC, Cdc6, Cdt1, and the MCMs. The role of ORC phosphorylation in preventing re-replication is also unclear in \textit{S. pombe}. Orc2 is phosphorylated by the G2/M cyclin Cdc13/Cdc2 \textit{in vitro} and \textit{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 \textit{S. cerevisiae}. Cdt1 abundance parallels that of Cdc18 in \textit{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 \textit{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
organisms. 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


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. 


Chapter II

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

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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.


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. XhoI PstI

B. Random Library Sequences

C. B2- Sequences

D. B2+ Sequences

E. B2+ Sequences Moved 0/1 Bases
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 ARS (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 X 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 ARS 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
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.

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<td>0.27 2.9E-11 6.6E-08 3.3E-07</td>
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</table>

**Average** of Randomly Generated A-rich Sequences:

| 6.2 ± 1.7 kcal/mol |
| 8.2 ± 3.3 kcal/mol |
| 7.9 ± 3.6 kcal/mol |
| 5.3 ± 1.6 kcal/mol |

**Average** of B2+ Sequences:

| 6.4 ± 1.7 kcal/mol |
| 4.6 ± 1.1 kcal/mol |
| 4.5 ± 1.2 kcal/mol |
| 7.4 ± 0.9 kcal/mol |

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Figure 2

The figure shows a scatter plot with the horizontal axis representing % Plasmid Loss/Generation and the vertical axis representing Helical Stability (Kcal/Mol). Two distinct clusters are observed: B2:WT and B2:802-810. The data points for B2:802-810 are more concentrated near the upper region of the plot, while those for B2:WT are more spread out. A regression line with an R² value of 0.04 indicates a weak correlation between the variables.
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 ARSI 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 x 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.
% Plasmid

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<tr>
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<td>AAACAAATATA</td>
<td>8</td>
<td>3.4</td>
<td>8</td>
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<td>B2-M2</td>
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<tr>
<td>B2-M3</td>
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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.

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- B1
- A

Protein:

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- M1
- M2
- M3
- M4

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 ARS\(1\) DNA (lanes 1-3), while Orc1-5p protects the A element and the B2 element of wild-type ARS\(1\) DNA (lanes 4-6), with characteristic hypersensitive sites in between. In contrast, Orc1-5p does not protect the B2 elements of the B2\(^+\) ARS\(1\) DNAs (lanes 7-18). Lanes 1, 4, 7, 10, 13, and 16 contain no protein. Lanes 2, 5, 8, 11, 14, and 17 contain 50 ng of 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 *Gall-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
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. [Graph showing % Plasmid Loss/Generation for different conditions: Control, Gal Cdc6p, Gal Cdc6 K114Ep, and B2-(802-810).]

B. [Graph showing % Plasmid Loss/Generation for different conditions: Control and Gal Cdc6p across various mutants (WT, B2-, m5, m6, m8, m10 (802-810)).]
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 ARS I 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 initiation 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 ARSI B2 mutant library was created by primer extension of the primers B2MUT1 (TATTACCTCGAGGTATTTNNNNNNNNGTTTGTGCACTTGCCTGCAGGCC TTTT) and B2MUT2 (AAAAGGCCCTGCAGGCAAGTGC), 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

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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 1/50th of the IP or 1/10,000th 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


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.

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**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

A. DBD

AD

CDC28
p27
ORC6
orc6-ps
orc6-rxl
orc6-ps,rxl

-leu-ura
-his
-ade

B. DBD

AD

ORC6
orc6-ps
orc6-rxl
orc6-ps,rxl

-leu-ura
-his+AT
-ade

C.

S. cer. --IPELPPMQTNESSPSITRRKLAFFEDEDEDEDEEPPGND--
S. bay. --IPELPPAQNPETPSARRKLAFEEDDEEEDDEEEDNDL--
S. mik. --IPVLPPMQTNIPVTRRKLAFEEEETHDEELGSKC--
S. par. --IPELPPMQTPITRRKLAFEEDDEEDEESENSN--
S. cas. --VKLSPGNLNTDSPIKARRKLFEDSDSEQDDEDSH--

* * * * * * * * *

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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 (RRKL A) 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.

Crb5/Cdc28:

| WT ORC | WT ORC | Orc1-5
|--------|--------|--------|

B.

Antibody: Orc2 | Orc4 | None | None | Orc2 | Orc4 | None | Ha | GST | His | None | None | Ha | GST | His

Crb5/Cdc28: | | | | ORC: | | | | | | | | | |
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 hpm hpm WT hpm
Cdc6: | Cdc6 WT | Cdc6 WT | Cdc6ΔN | Cdc6ΔN |
Clb5/Cdc28: WT hpm hpm WT hpm
Cdc6: | Cdc6ΔC | Cdc6ΔC | 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-phosphorylated 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
Figure 3

- Myc
- PLA AP
- PLA
- Myc
- Extract

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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.

B.
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-rx1 or Orc6-ps-rx1 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. 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 +;+ +;m +;+ +;+

- **ORC6-rxl**
  - +;+ +;m +;+ m;m m;+
  - +;tm +;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 +;+

- **ORC6-ps,rxl**
  - +;+ 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

Ctb5Δ clb6Δ

ORC6-RXL cdc6ΔN

ORC6-RXL cdc6Δ2-49

Ctb5Δ clb6Δ

D.

leu-, his-, LYS+

LEU+, HIS+, lys-

CDC6-wt

CDC6ΔN

ORC6-wt

ORC6-ps

ORC6-rlx

ORC6-ps,rlx

ORC6-wt

ORC6-ps

ORC6-rlx

ORC6-ps,rlx

Non-selective medium (YEPD)

Diploid-selective medium (-leu-his-lys)

+ MCM7-NLS
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 clb5A. clb5A 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
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 containing 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 \textit{ORC6-ps-rxl}, \textit{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 \textit{ORC6-ps,rxl} strain in this sensitized background failed to grow when grown on galactose containing media (Figure 7D). Interestingly, under these conditions the \textit{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 \textit{CDC6D2-49/CDC6 ORC6-ps,rxl/ORC6 MCM7-NLS/MCM7} diploids, and a less extreme but still strong phenotype in triply heterozygous \textit{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 \textit{ORC6-rxl GalCDC6Δ2-49} and \textit{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 \textit{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 \textit{CLB5} in the same sensitized strain background (\textit{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, MCM7-NLS, 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 \textit{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 \textit{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 meclΔ (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 pRS4060RC6-wt, pRS4060RC6-rxl, pRS4060RC6-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. pRS4060RC6-ps, rxl was made by amplifying the RXL mutation from pRS4060RC6-rxl with oligos SB831, TCTATTACTAGGGCAAAGGCAGCA and SB795, CACACACTAATTGCCATGGGC, and then using this megaprimer to amplify the ORC6 Cdk phosphorylation site mutations from pRS4060RC6-ps with the oligo SB795, CACACACTAATTGCCATGGGC. This product was cut with NdeI/ NcoI and cloned into pRS4060RC6-wt cut with the same enzymes. ORC6 mutant strains were constructed by cutting plasmids pRS4060RC6-wt, pRS4060RC6-rxl, pRS4060RC6-ps, and pRS4060RC6-ps-,rxl with BsmI and integrating into ySB1028 (orc6::HisMX, pSPB66 (ORC6, URA3)) and then streaking onto 5-FOA to lose the pSB66ORC6 plasmid. GAL-Cdc6A2-48-HA strains were constructed as described (Klemm and Bell
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 (C51-13) 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 C51-13 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, 143
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


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 $ARS_I$. 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 \textit{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. \textit{ARS}I 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 \textit{ARS}I function is important at other yeast origins.

\textbf{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 \textit{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 \textit{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 \textit{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 \textit{ORC2} CDK

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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 \textit{CDC14} or \textit{CDC15} would allow pre-RCs to re-form, but not re-initiate, in a sensitized strain background containing nuclearly localized MCMs and \textit{Cdc6\textdbl}N. 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 \textit{bob1} allele of \textit{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 \textit{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


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