

Establishing the Program of Origin Firing during S Phase in Fission Yeast

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SUMMARY

Initiation of eukaryotic DNA synthesis occurs at origins of replication that are utilized with characteristic times and frequencies during S phase. We have investigated origin usage by evaluating the kinetics of replication factor binding in fission yeast and show that similar to metazoa, ORC binding is periodic during the cell cycle, increasing during mitosis and peaking at M/G1. At an origin, the timing of ORC binding in M and pre-RC assembly in G1 correlates with the timing of firing during S, and the level of pre-IC formation reflects origin efficiency. Extending mitosis allows ORC to become more equally associated with origins and leads to genome-wide changes in origin usage, while overproduction of pre-IC factors increases replication of both efficient and inefficient origins. We propose that differential recruitment of ORC to origins during mitosis followed by competition among origins for limiting replication factors establishes the timing and efficiency of origin firing.

INTRODUCTION

In eukaryotic cells, DNA synthesis begins at origins of replication throughout the genome. During each S phase (S), a cell utilizes a subset of its origins that fire at characteristic times, indicative of a program of origin usage (Aladjem, 2007). Origin efficiency is defined as the frequency of origin usage in a population. Origins of replication have been identified from yeast to human, but little is known about the regulation of their timing and efficiency, in particular how the steps in replication initiation are controlled to generate the replication program. In this paper, we investigate the timing of origin firing and the efficiency of origin usage in a unicellular eukaryote, the fission yeast *Schizosaccharomyces pombe*.

Initiation of DNA replication requires the coordinated assembly of a number of components (reviewed in Bell and Dutta, 2002). First, the origin recognition complex (ORC) selects the sites for initiation by directly binding to origins. Next, in G1, Cdc6, Cdt1, and ORC recruit the MCM helicase, completing pre-replicative complex (pre-RC) formation. Finally, Cdc45 binding is necessary for loading the enzymes required for DNA

synthesis, forming the pre-initiation complex (pre-IC) and bringing about replication initiation. The basic mechanism for initiation is conserved among organisms, but one major difference is the regulation of ORC. In budding yeast, ORC association with origins is constant during the cell cycle (Aparicio et al., 1997; Diffley, 1994), and rereplication is prevented by phosphorylation of specific subunits (Nguyen et al., 2001; Remus et al., 2005). In contrast, in *Xenopus* egg extracts, chromatin binding of ORC is low upon entry into mitosis, increasing at anaphase/telophase, and ORC is released from chromatin during S (Romanowski et al., 1996; Sun et al., 2002). In mammalian cells, Orc1 association with chromatin is low in mitosis, binding as cells exit mitosis (M) and enter G1 and dissociating from chromatin at the end of S phase (Li and DePamphilis, 2002); others find that Orc1 associates with chromatin as cells proceed through telophase (Okuno et al., 2001).

The DNA sequences that define origins vary more widely than the components required for replication. The most well-studied eukaryotic chromosomal replication origins are those of budding yeast, which were identified as autonomously replicating sequences (ARS) with an 11 bp ARS consensus sequence (ACS) essential for origin function (Broach et al., 1983; Stinchcomb et al., 1979). Most origins in budding yeast fire efficiently, on average once every two cell cycles (Friedman et al., 1997; Poloumienko et al., 2001; Yamashita et al., 1997). In contrast, origins in most other eukaryotes neither fire efficiently nor contain a strict consensus sequence (Aladjem, 2007). Metazoan origins have a more extended structure and may not have a specific sequence requirement, as replication occurs at a number of possible sites on DNA introduced into *Xenopus* eggs and egg extracts and in *Drosophila* early embryos (Hyrien and Mechali, 1992; Shinomiya and Ina, 1991). It has been difficult to define the elements that constitute a complex eukaryotic origin, although studies to map metazoan origins will help to clarify this issue (Lucas et al., 2007; Mesner et al., 2006).

The fission yeast is a useful system for studying eukaryotic origin usage. Nearly all potential origins have been identified, and, as in metazoan eukaryotes, there is no known consensus sequence for origins (Feng et al., 2006; Hayashi et al., 2007; Heichinger et al., 2006). Origins in fission yeast consist of asymmetric AT stretches of around 1 kb in length with multiple AT hook motifs that serve as targets for ORC (Bell and Dutta, 2002). The study from our laboratory identified 401 strong and 503 putative origins that fire throughout S and are used with a continuum of efficiencies (Heichinger et al., 2006). Only a few origins are used as frequently as once in every two cell cycles,

and most origins fire in less than one out of every ten cell cycles (Dai et al., 2005; Heichinger et al., 2006). Finally, there is a strong correlation between origin efficiency and timing: generally, efficient origins fire early in S, while inefficient origins are late firing (Heichinger et al., 2006).

In this paper, we investigate the control of origin usage by determining the kinetics of replication factor binding and their effects on the replication program in fission yeast. Our results indicate that the timing and efficiency of origin firing is established during the mitosis of the previous cell cycle and the G1 prior to S phase, followed by competition among origins for limiting replication factors.

RESULTS

Replication Initiation at an Efficient Origin

We investigated the steps leading to fission yeast origin activation to determine whether the timing of ORC binding, pre-RC formation, and pre-IC assembly influences origin firing. ChIP analysis by quantitative real-time PCR (qPCR) was used to monitor replication factor binding at three origins (Figure 1A): *ori2004*, an efficient and early-firing origin; *ori2060*, an inefficient and late-firing origin; and *ars727*, a cryptic origin that fires on a plasmid but not in its normal chromosomal context. Synchronized cultures were obtained by arresting *cdc25-22* cells at 36.5°C for 4 hr in late G2 before releasing at 25°C for entry into M (Figure 1B). Estimates for cell cycle phases by DAPI staining and fluorescence-activated cell sorting (FACS) analysis showed that M occurred by 30 min and that S began around 50 min and proceeded until 80 min after shift to 25°C.

ori2004, which has an AT content of 76% and contains two AT hook motifs in regions I and III, has an efficiency of 50% in mitotic S and is one of the earliest-firing origins (Okuno et al., 1997, 1999). To determine Orp1 (the ortholog of Orc1 in other organisms) binding at *ori2004*, we immunoprecipitated a fully functional *orp1-HA* fusion protein expressed from its endogenous locus (Grallert and Nurse, 1996). qPCR analysis showed that Orp1 bound specifically to *ori2004*, with a peak of binding at region III (Figure S1A available online). Orp1 binding to *ori2004* showed cell-cycle periodicity: in two successive cell cycles, levels of Orp1 binding reached a maximum around the M to G1 transition (Figure 1C), 20 min before the start of replication. To ascertain whether other subunits of ORC exhibit this periodicity, we monitored the binding of Orp2 and Orp4 at *ori2004*. Both proteins showed similar kinetics of recruitment as Orp1, suggesting that the ORC complex binds periodically to *ori2004*, with maximal association occurring at M/G1 and reduced levels bound at other cell cycle stages (Figure 1D, see Figure S1B for Orp2 binding). These results should be compared with earlier studies that reported no major changes in ORC association with chromatin (Lygerou and Nurse, 1999) or with origins (Ogawa et al., 1999) during the cell cycle. In our present experiments, we used closely spaced time points and qPCR assays, which are more sensitive than the ethidium bromide staining of agarose gels used previously to assay ORC association with origins. We conclude that ORC binding to an early, efficient origin in fission yeast is periodic during the cell cycle, increasing during

M and peaking at M/G1 before the start of S, behavior that is similar to metazoan eukaryotes (DePamphilis, 2005).

Next, we investigated the relationships between the periodicity of ORC binding, pre-RC formation, and pre-IC assembly. As a marker for pre-RC formation, recruitment of MCM was monitored with a polyclonal antibody to Mcm4 (Nishitani et al., 2000). Maximal binding of Mcm4 occurred in G1, after peak ORC binding and before S, and Mcm4 binding was sharply periodic during the cell cycle (Figure 1E, Figure S1C). Pre-IC assembly was assayed by the recruitment of a functional Cdc45-YFP fusion protein to *ori2004*. For the strain containing Cdc45-YFP, S phase was delayed by 10 min, beginning 60 min after release from *cdc25-22* arrest (data not shown). Cdc45 bound to region II of *ori2004* (Figure S1D), and its association was sharply periodic and delayed by 10 min compared with maximal Mcm4 binding (Figure 1F). The co-occupancy of Cdc45 and Mcm4 on region II supports the idea that Cdc45 and MCM form a complex and that Cdc45 primes the activity of the MCM helicase (Moyer et al., 2006; Zou and Stillman, 2000). Our data are consistent with previous work showing MCM and Cdc45 recruitment to *ori2004* (Ogawa et al., 1999; Yabuuchi et al., 2006), and our higher temporal resolution allows the more precise determination of timing for the recruitment of ORC, pre-RC, and pre-IC to origins. We conclude that there is a temporal separation between the three steps, with maximum *ori2004* binding of ORC at M/G1, of pre-RC in G1, and of pre-IC at G1/S.

Replication Initiation at Inefficient and Inactive Origins

To determine whether the kinetics of ORC, pre-RC, and pre-IC recruitment contribute to the differences in the timing of origin firing, we analyzed these steps at *ori2060*. A schematic of this origin is shown in Figure 1A; *ori2060* is 74% AT-rich and contains two strong AT hook motifs. It is used at 10% efficiency and fires later in S, around 10 min after *ori2004* (Heichinger et al., 2006); for reference, the length of S in fission yeast is around 30 min in a synchronous culture at 25°C (Figure 1B). We assessed Orp1 and Mcm4 binding at *ori2060* by ChIP, scanning a 2.5 kb region centered on *ori2060*. Orp1 bound periodically to a region between the two AT hook motifs (Figure 2A, Figure S1E). Mcm4 binding was also periodic, reaching a maximum after the peak of ORC binding (Figure 2A, Figure S1F). However, compared to the timing of their binding to *ori2004*, maximal binding of both Orp1 and Mcm4 was delayed by around 10 min (compare Figures 2A and 2B). In addition, we observed a 10 min delay in the recruitment of Cdc45, similar to the delay in Mcm4 binding (Figure 2C). These results suggest that a delay in ORC and pre-RC recruitment in mitosis and G1, respectively, correlates with a delay of origin firing during the subsequent S.

We also evaluated *ars727*, which functions as an autonomous replicating sequence on a plasmid (Maundrell et al., 1988). *ars727* is 76% AT rich and contains two AT hook motifs, but it is inactive in its chromosomal context (Kim and Huberman, 2001). Our results showed a delay in Orp1 binding to *ars727* compared with *ori2004*, similar to *ori2060* (Figure 2D). Mcm4 recruitment at *ars727* was clearly delayed, occurring near the start of S (Figure 2E). Cdc45 binding, which occurred halfway through S, showed a significant delay and a pronounced

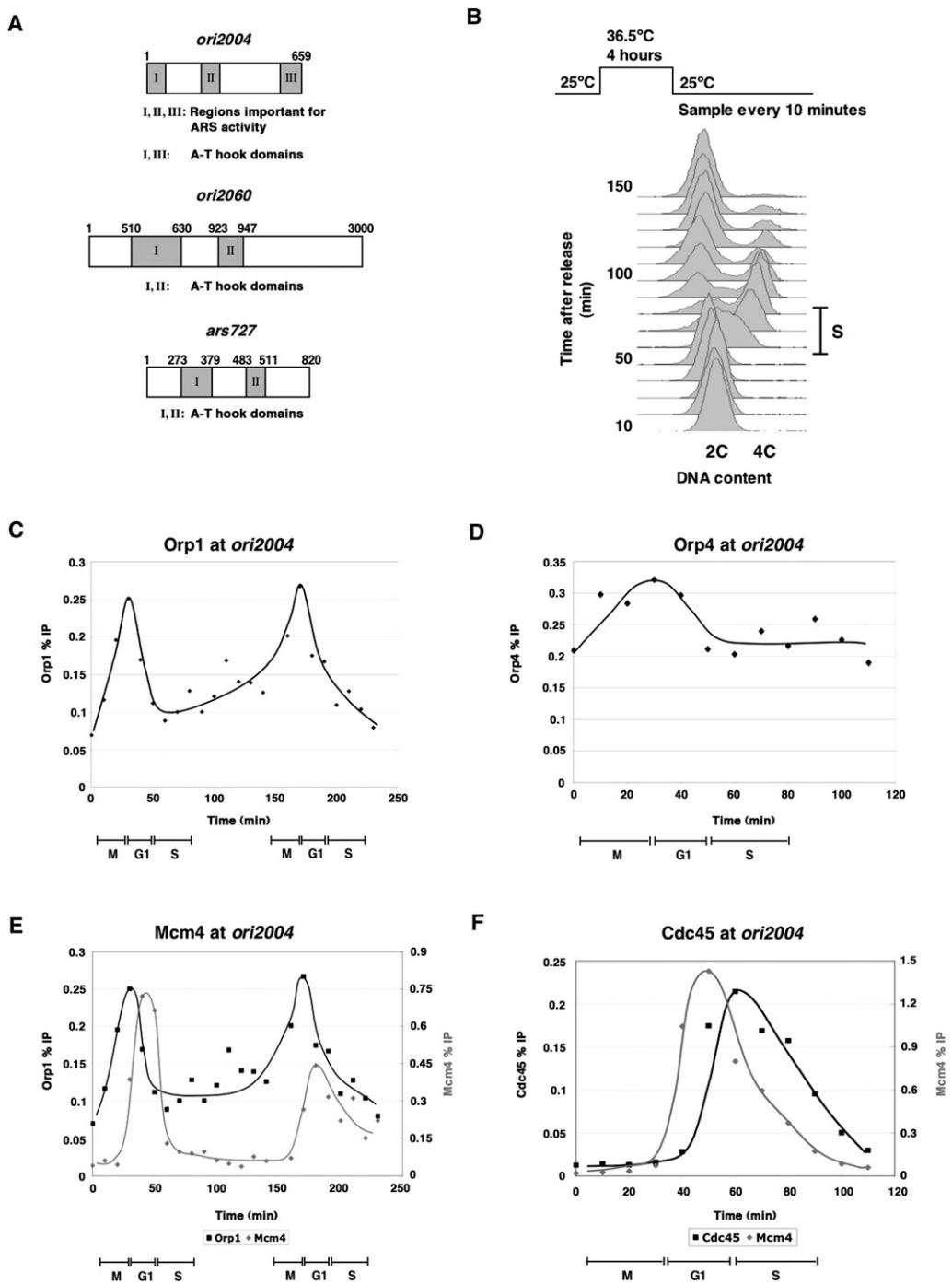


Figure 1. ChIP Analysis of ORC, Pre-RC, and Pre-IC Binding at *ori2004*

- (A) Schematic of *ori2004*, *ori2060*, and *ars727* (figure not drawn to scale).
- (B) FACS analysis indicates that S occurs between 50 and 80 min after release from *cdc25-22* arrest.
- (C) Orp1 binding to *ori2004* was assayed in a synchronous culture after release from *cdc25-22* arrest. Each point represents the result of qPCR using primers over region III. Orp1 binding to *ori2004* is periodic during the cell cycle, reaching a maximum at M/G1.
- (D) Association of an integrated, fully functional Orp4-HA fusion with *ori2004* as determined by ChIP. The same primers as in (C) were used. Orp4 shows similar timing and periodicity of binding at *ori2004* as Orp1.
- (E) Comparison of Orp1 and Mcm4 binding during the cell cycle. Each Mcm4 point represents the occupancy over region II of *ori2004*.
- (F) The peak of Cdc45 association with *ori2004* coincides with the start of replication, after maximal Mcm4 binding. IPs were performed from the same extracts, and each point represents the occupancy over region II of *ori2004*.

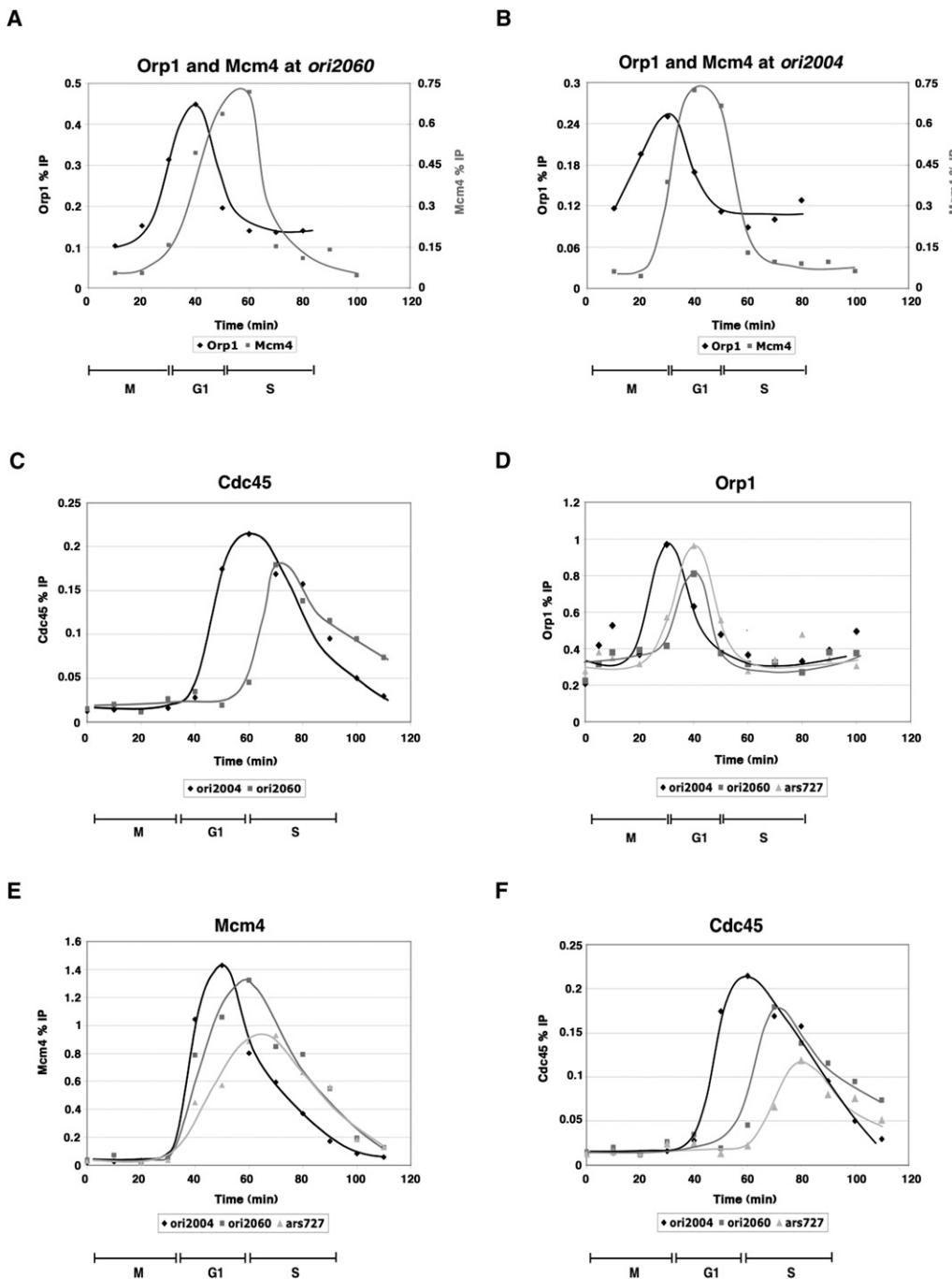


Figure 2. Pre-RC Formation at an Inefficient Origin, *ori2060*, and an Inactive Origin, *ars727*

(A and B) Time course of Orp1 and Mcm4 binding at *ori2060* and *ori2004*. For *ori2060*, each point represents the occupancy between the two AT hook regions. Orp1 binding at *ori2060* peaks around 40 min, while Mcm4 binding is maximal 50–60 min after release from *cdc25-22* arrest. ORC binding and pre-RC formation at *ori2060* occur approximately 10 min later than at *ori2004*.

(C) Comparison of Cdc45 binding at *ori2060* and *ori2004*. Maximal Cdc45 association at *ori2060* is delayed compared to *ori2004*.

(D–F) Time course of Orp1 (D), Mcm4 (E), and Cdc45 (F) at *ori2004*, *ori2060*, and *ars727* by ChIP shows a delay in ORC, pre-RC, and pre-IC formation at late-firing and inefficient or inactive origins.

decrease (Figure 2F). The relatively high level of Cdc45 binding at an inactive origin is likely due to passive replication (see below). We conclude that ORC and pre-RC can be assembled at nonfir-

ing origins and that the timing of pre-RC assembly is delayed. As there are efficient origins on the chromosome near *ars727*, it is likely that by the time that *ars727* is competent to recruit

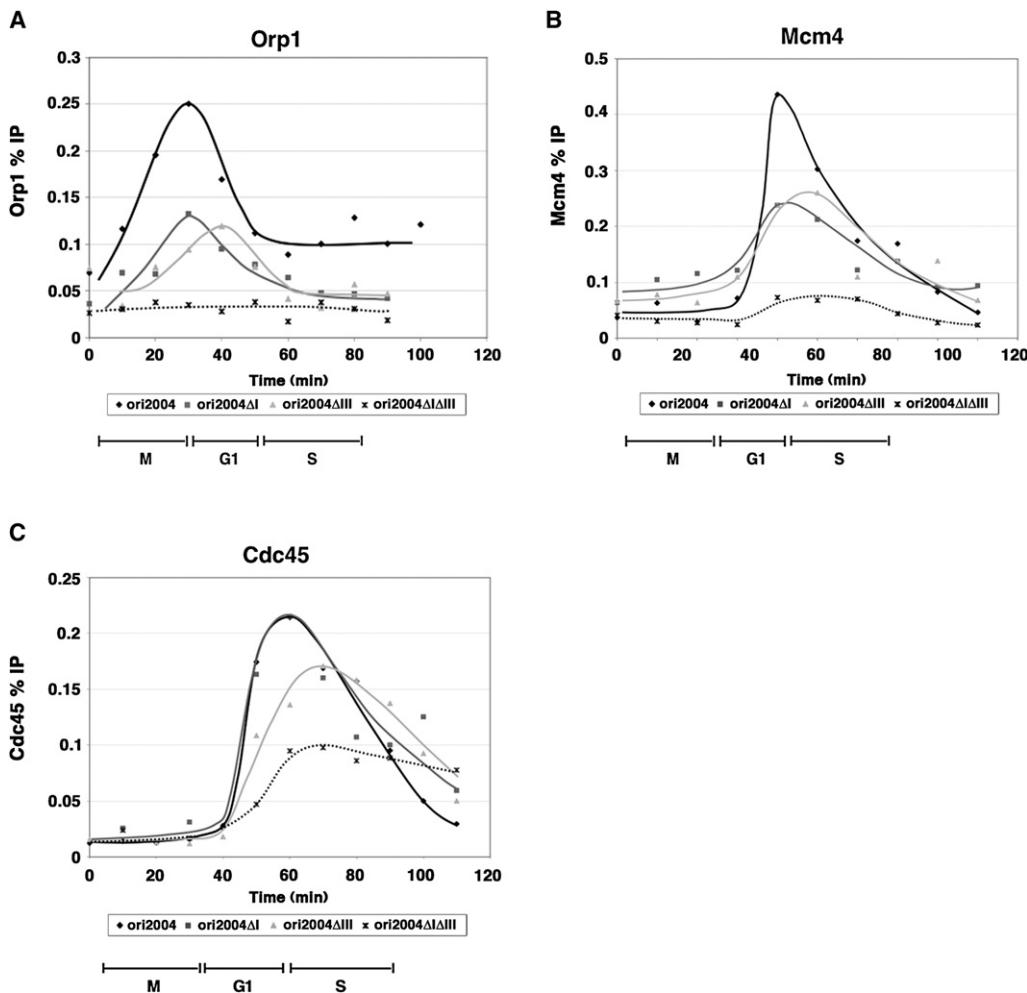


Figure 3. Reduction in ORC Binding results in Delays in Pre-RC and Pre-IC Assembly

(A) Time course of Orp1 binding at wild-type *ori2004* and three mutant alleles: *ori2004ΔI*, *ori2004ΔIII*, and *ori2004ΔIΔIII* (see Figure 1A). For the wild-type and *ori2004ΔI*, each point represents the occupancy of Orp1 over region III; *ori2004ΔIII* occupancy was quantified with primers over region I, while *ori2004ΔIΔIII* was assayed with primers over region II. Orp1 binding is reduced in the single *ori2004* mutants, absent in the double mutant, and specifically delayed in *ori2004ΔIII*. (B and C) Comparison of Mcm4 and Cdc45 binding in wild-type and *ori2004* deletions. Primers over region II were used to determine occupancy for both Mcm4 (B) and Cdc45 (C). Mcm4 occupancy is reduced in the *ori2004* mutants, and *ori2004ΔIII* shows a small delay in Mcm4 binding. Cdc45 binding is delayed in *ori2004ΔIII* and *ori2004ΔIΔIII*.

Cdc45, the region has already been passively replicated, preventing firing of the origin.

Reduction in ORC Binding Results in Delayed Pre-RC and Pre-IC Formation

Our results suggest that the timing of ORC binding to origins is correlated with the timing of pre-RC and pre-IC binding and the timing of origin firing. We next asked whether changing the affinity of an origin for ORC affects its binding dynamics and pre-RC assembly. We utilized three deletions of *ori2004*: *ori2004ΔI* and *ori2004ΔIII*, lacking AT hook motifs in regions I and III, respectively, and *ori2004ΔIΔIII*, lacking both AT hook motifs. While deletion of either AT hook motif alone does not dramatically affect origin activity, deletion of both motifs abolishes origin activity (Takahashi et al., 2003). Similar to intact

ori2004, we find that Orp1 binding is periodic at both *ori2004ΔI* and *ori2004ΔIII*, peaking around M/G1 (Figure 3A), suggesting that the increase in ORC binding does not require the presence of both AT hook motifs. *ori2004ΔI* has no effect on the timing of the increase, but there is a delay in the peak of ORC binding at *ori2004ΔIII*. Consistent with published results, we observed little or no ORC binding in the doubly deleted *ori2004ΔIΔIII* mutant (Takahashi et al., 2003).

Next, we assessed Mcm4 and Cdc45 binding in these mutants. There is little effect on the timing of Mcm4 binding at *ori2004ΔI*, although the peak level was significantly reduced. In contrast, *ori2004ΔIII* exhibited both delayed and reduced Mcm4 binding, and Mcm4 binding at *ori2004ΔIΔIII* was barely detectable (Figure 3B). Similar to these results, the timing of Cdc45 binding was unaffected at *ori2004ΔI*, while it was delayed

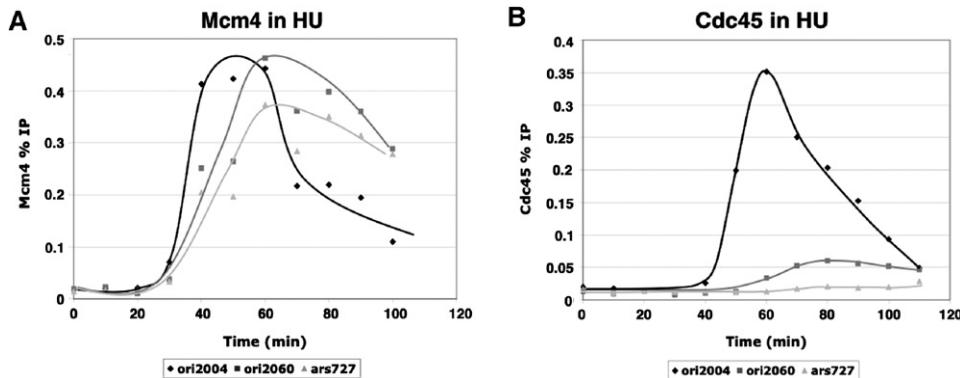


Figure 4. Mcm4 and Cdc45 Binding in Hydroxyurea-Treated Cells

(A) *cdc25-22* synchronized cells were released into hydroxyurea (HU) at 25°C for time course analysis. ChIP of Mcm4 at *ori2004*, *ori2060*, and *ars727* shows that pre-RC formation is generally not affected by HU treatment.

(B) HU treatment significantly reduces the maximal levels of Cdc45 binding at late-firing inefficient origins.

at *ori2004ΔIII* and *ori2004ΔIΔIII* (Figure 3C). Moreover, Cdc45 levels were greatly reduced at *ori2004ΔIΔIII* (Figure 3C). Analogous to *ori2060* and *ars727*, the Cdc45 binding that we observe at *ori2004ΔIΔIII* is likely due to passive replication, as this mutant origin does not fire by 2D gel analysis and binds little or no Orp1 and Mcm4 (Takahashi et al., 2003). The delay in Cdc45 binding to specific *ori2004* deletions is reflected in the timing of replication at these origins. Replication at *ori2004ΔIII* and *ori2004ΔIΔIII* is delayed by 10 and 15 min, respectively (Figure S2). These results indicate that *ori2004* deletion mutants that reduce ORC binding can delay ORC binding, delay and reduce pre-RC and pre-IC formation, and delay the timing of replication.

Cdc45 Recruitment Correlates with Origin Efficiency

In the experiments described above, we observed high levels of Cdc45 binding at *ori2060* and *ars727* despite their inefficient usage, and we detected Cdc45 binding above background levels in regions adjacent to both *ori2060* and *ars727*, suggesting that they are passively replicated (Figure S3). To eliminate this complication, we released cells from *cdc25-22* arrest into medium containing hydroxyurea (HU), which slows replication fork progression, limiting replication to a region around 10 kb centered on the origin (Heichinger et al., 2006; Patel et al., 2006). In contrast to budding yeast, the majority of origins are not subject to HU checkpoint repression in fission yeast (Hayashi et al., 2007; Heichinger et al., 2006; Mickle et al., 2007). Therefore, in our experiment, origin firing should still occur, but passive replication will be much reduced. We observed high levels of Mcm4 binding at all three origins, and the temporal sequence of pre-RC assembly was maintained (Figure 4A). In contrast, levels of Cdc45 binding were drastically reduced at the late-firing and inefficient origins *ori2060* and *ars727* (Figure 4B). The efficiencies of *ori2004*, *ori2060*, and *ars727* are 50%, 10%, and <5% (Heichinger et al., 2006); if we assume linearity of the ChIP data and set the Cdc45 binding at *ori2004* to 50%, then Cdc45 occupancy at *ori2060* and *ars727* is 10% and 4%, respectively. Therefore, we conclude that the level of Cdc45 bound at the origin in the presence of HU approximately reflects the efficiency of origin usage. We propose that in a normal

S phase, the delay in pre-RC formation at a late-firing origin results in competition for a smaller pool of available replication factors that constitute the pre-IC. Furthermore, passive replication through the region inhibits delayed pre-IC formation at such an origin, resulting in reduced firing efficiency.

Reprogramming Origin Activity

Our data have revealed a correlation between the timing of ORC and MCM binding to origins and the timing of replication. We hypothesized that origins bind ORC during M with varying affinities and that delays in ORC binding and pre-RC formation at late-firing origins result in low efficiencies due to these origins subsequently competing less effectively for limiting replication factors. To test this, we performed two sets of experiments. First, we investigated whether equalizing ORC binding results in changes in origin efficiencies. Because the increase in ORC binding occurs during M, we surmised that extending M might result in origins accumulating ORC more equally, leading to more equal distribution of pre-RC and pre-IC assembly among origins. As a consequence, early origins might become less efficient and late origins more efficient. Second, we tested whether there are limiting factors in replication by assessing whether overexpression of candidate genes would increase origin efficiency.

To extend M in cells, we used the drug MBC (Carbendazim), which prevents microtubule polymerization and disrupts the mitotic spindle. MBC treatment of cells after synchronization with *cdc25-22* prolonged mitosis and blocked S phase (Figure 5A, left panel). Orp1 accumulated at *ori2004*, *ori2060*, and *ars727* throughout the MBC treatment and reaches a maximum at the end of the extended M, confirming that ORC binding occurs during M (Figure 5A, right panel). We found no detectable increase in the total cellular level of Orp1 protein during MBC treatment by western analysis, suggesting that ORC is indeed redistributed among origins (data not shown). Mcm4 showed minimal binding (Figure S4A), confirming that cells must enter G1 before pre-RC formation can take place and establishing that the increase in ORC binding can be uncoupled from MCM recruitment and pre-RC assembly.

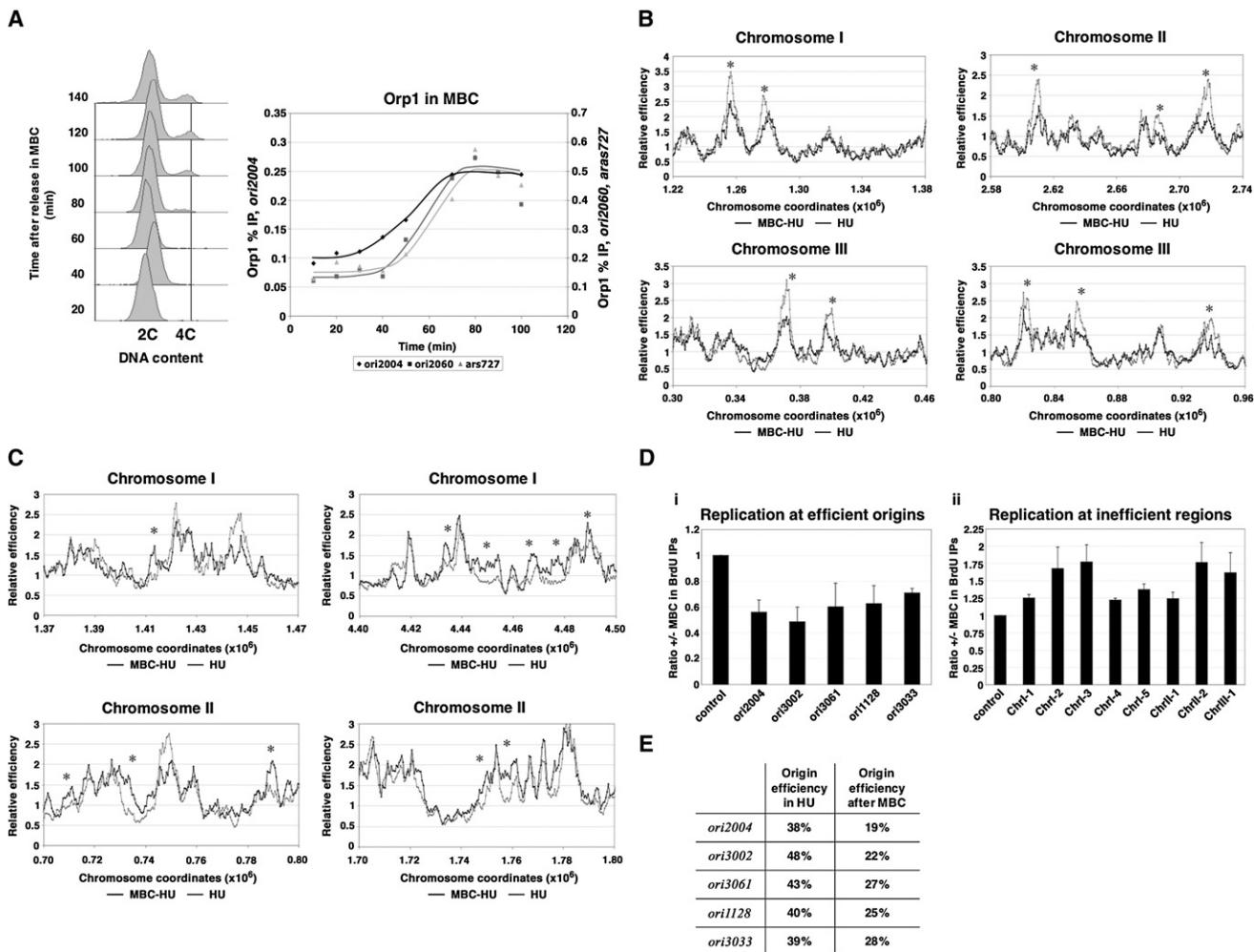


Figure 5. Extending Mitosis Alters Origin Efficiencies across the Genome

(A) Left: FACS analysis of cells synchronized with *cdc25-22* and released into MBC shows only a small subset of cells entering S after 70 min. Right: ChIP of O rp1 from MBC-treated cells. O rp1 accumulates at *ori2004*, *ori2060*, and *ars727* and remains bound during MBC treatment.

(B) Representative regions from array analyses showing reduction in the usage of early-firing efficient origins in cells treated with MBC and released into HU. MBC-treated and HU control cells are represented by black and gray lines, respectively. Asterisks mark reproducible decreases in three experiments.

(C) A subset of inefficient regions show increased BrdU incorporation after MBC treatment. Asterisks mark reproducible increases.

(D) Relative origin usage in MBC-treated samples and HU controls. BrdU IPs were assayed by qPCR. Bars represent the ratio of signal in MBC-treated to HU control samples, and a region that does not replicate in HU-treated cells was used for normalization. Values are averaged from three experiments, and error bars represent the standard deviation. (i) The five efficient origins tested display reduced signals in the MBC samples. (ii) Eight low-efficiency regions, labeled by chromosome, show reproducible increases in replication after MBC treatment.

(E) Origin efficiencies after MBC treatment. Replication efficiency was determined by qPCR of genomic DNA from both MBC-treated and HU control cells. Values are averaged from three experiments.

Next, we assayed whether equalization of ORC binding led to equalization in origin usage. Origin efficiencies across the genome were determined in cells delayed in M with MBC. *cdc25-22* cultures were arrested in G2 and treated with MBC 10 min before release into a synchronous cell cycle; after 65 min, MBC was washed out and cells were released into media containing HU and BrdU. A schematic of the experimental design is shown in Figure S4B, along with FACS profiles for the experiment. The efficiency of an origin can be assessed in HU-treated cultures, as origin firing can be measured in the absence of passive replication (Heichinger et al., 2006). BrdU-

labeled fragments were isolated by immunoprecipitation of genomic DNA and hybridized to Affymetrix tiling arrays that cover the fission yeast genome at high resolution. These results were compared to the replication profile of *cdc25-22*-synchronized cells treated with HU but not with MBC, providing a control for normal origin efficiencies across the genome. This method allows the relative efficiency of origin usage genome-wide to be determined on the basis of signal intensities from array hybridizations. Our results showed that many efficient early-firing origins were reduced in usage (Figure 5B); of the top 50 most efficient origins (from Heichinger et al. 2006) (Figure S5),

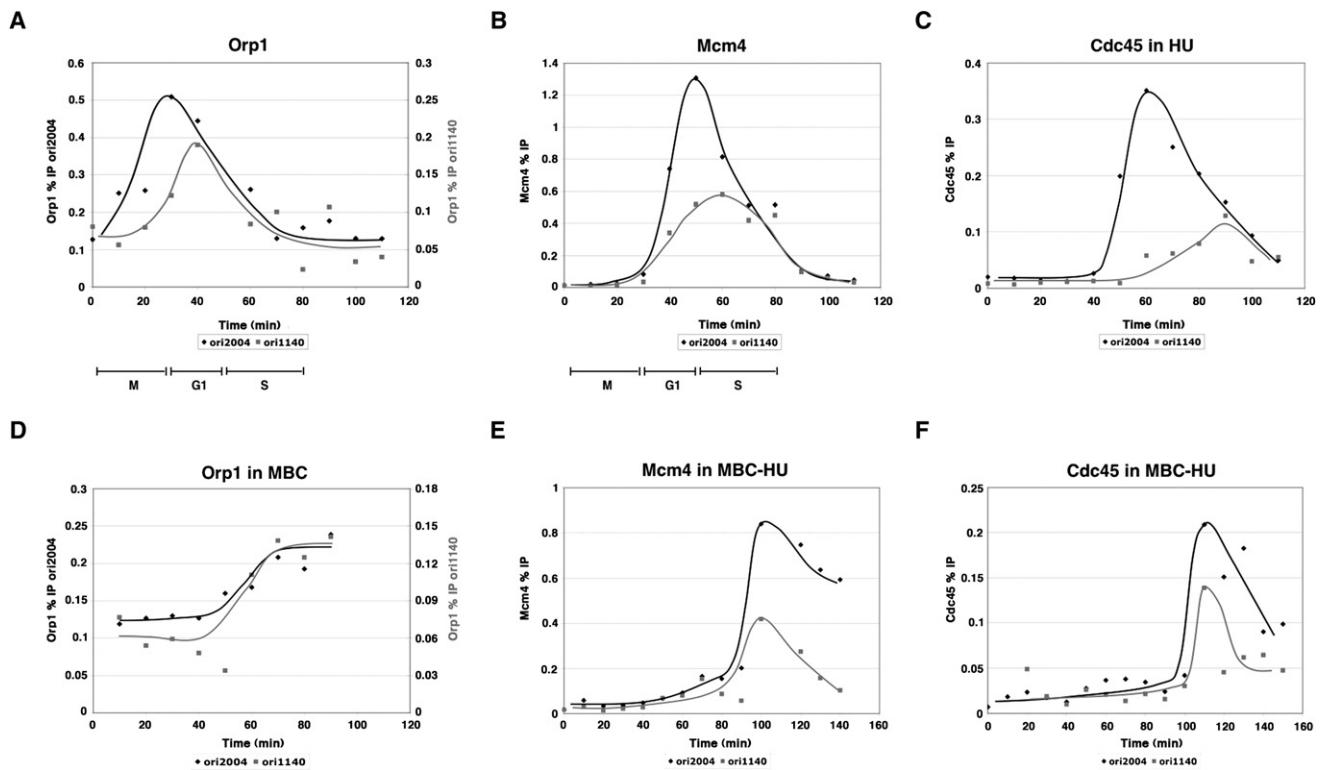


Figure 6. Prolonging Mitosis Advances the Timing of Pre-RC and Pre-IC Formation at an Induced Origin, *ori1140*

(A–C) Time course of Orp1, Mcm4, and Cdc45 binding by ChIP shows that all three steps are delayed at *ori1140* compared with *ori2004*.

(D) ChIP of Orp1 from MBC-treated cells. Orp1 accumulates at *ori2004* and *ori1140* and remains bound at the origin throughout the MBC treatment.

(E and F) The timing of Mcm4 and Cdc45 binding at *ori1140* after MBC treatment and growth in HU is advanced and coincides with these events at *ori2004*.

86% were reduced in efficiency as assessed in three independent experiments. To confirm the results of the array analysis, we performed qPCR of BrdU IPs, and a significant reduction in efficiency was observed for all five origins tested in MBC treated cells (Figure 5D). To determine actual origin efficiencies, we quantified unprocessed genomic DNA to compare the copy number of the five origins in MBC-treated cells and HU controls with qPCR. These data show that after treatment with MBC, efficient origins replicate at around half of their normal efficiencies (Figure 5E). In addition, some inefficient regions become replicated more efficiently (Figure 5C). Results from three biological repeat experiments identified approximately 50 regions that showed reproducible increases in efficiency of replication, with an additional 50 regions that showed less pronounced increases. qPCR analysis of immunoprecipitated BrdU-labeled DNA at eight loci confirmed the increased usage (Figure 5D). However, not all inefficient origins exhibited increased efficiencies; for example, *ori2060* and *ars727* efficiencies were largely unchanged (data not shown).

If the timing of ORC binding determines the timing and efficiency of origin firing, then pre-RC and pre-IC assembly should occur earlier at an origin whose efficiency has been increased as a result of MBC treatment. We characterized one such origin, *ori1140* (P.N., unpublished data), for Orp1, Mcm4, and Cdc45 binding. During a synchronous cell cycle, we found that the

binding of all three replication initiation factors occurred later at this origin than at *ori2004* (Figures 6A–6C). After MBC treatment, we observed that ORC binding increased and reached a maximum at *ori1140*, suggesting equalization of ORC recruitment (Figure 6D). We then assayed Mcm4 and Cdc45 binding in cells that undergo replication in the presence of HU after removal of MBC. Both Mcm4 and Cdc45 binding at *ori1140* were advanced in timing after a prolonged mitosis, reaching a peak around the same time as at *ori2004* (Figures 6E and 6F). Therefore, equalizing ORC binding can advance the timing of pre-RC and pre-IC formation at a normally late-firing and inefficient origin.

We have hypothesized that origin efficiencies are established by a combination of temporal regulation of ORC and pre-RC binding and limiting replication factors. If this is the case, then increasing the levels of these factors could lead to increased origin efficiency during S. We evaluated the effects of overproducing three factors involved in preinitiation complex formation: Cdc45, Hsk1, and Dfp1. Hsk1, the homolog of the Cdc7 kinase, acts along with its activator Dfp1 to phosphorylate MCMs and Cdc45 to promote pre-IC assembly (Brown and Kelly, 1998). We overexpressed *cdc45*, *hsk1*, and *dfp1* constitutively under the control of the thiamine-repressible *nmt1* promoter in media lacking thiamine and evaluated the effect in cells treated with HU during a synchronous cell cycle. Replication is increased at

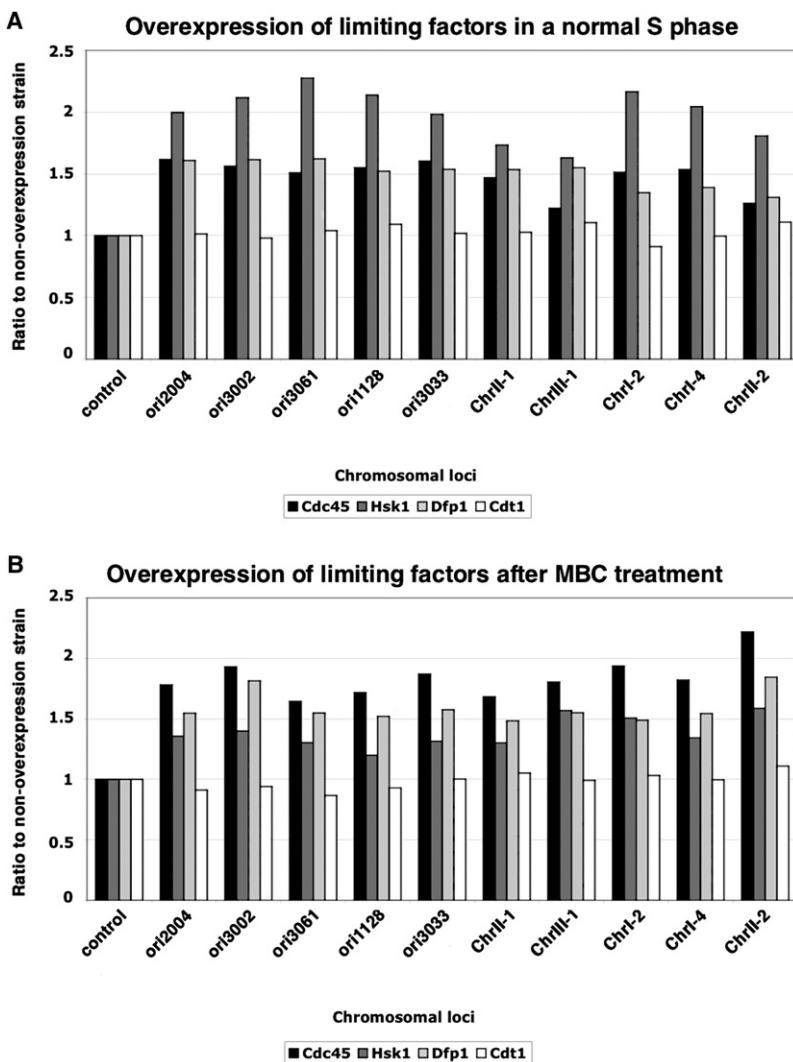


Figure 7. Overexpression of *cdc45*, *hsk1*, or *dfp1* Leads to Increased Replication

(A) BrdU signal is increased at efficient and inefficient origins in HU-treated synchronous cultures of strains overexpressing *cdc45*, *hsk1*, or *dfp1*. Bars represent the ratio of signal in overexpressing versus wild-type cells, and a region that does not replicate in HU was used for normalization. Values are averaged from at least three experiments.

(B) Relative origin usage in cells constitutively overproducing Cdc45, Hsk1, or Dfp1 after MBC treatment. qPCR of BrdU IPs showed increased signal in overexpressing strains at both efficient and inefficient origins.

a number of efficient and inefficient origins in strains overproducing Cdc45, Hsk1, or Dfp1 compared with a strain expressing wild-type levels of these factors (Figure 7A). In contrast, overexpression of the pre-RC component *cdt1* from the *nmt1* promoter does not increase replication from the tested origins (Figure 7A, white bars). We also assessed replication in cells overproducing Cdc45, Hsk1, or Dfp1 that have been synchronized with *cdc25-22* and treated with MBC followed by HU. This context allows us to determine whether origin efficiency can be similarly increased in an altered replication program. The results showed increased BrdU signal at a number of efficient and inefficient origins in strains overexpressing the candidate factors compared with nonoverexpressing cells (Figure 7B). Taken together, these data suggest that increasing levels of Cdc45, Hsk1, or Dfp1 result in increased replication from origins across the genome and therefore that their levels are normally limiting during S.

These results indicate that there is a redistribution of origin efficiencies among a number of origins after MBC treatment. We conclude that extending M so that ORC becomes more equally associated with a greater number of origins leads to an equilibra-

tion of origin efficiencies such that many efficient early origins are used less efficiently and a number of less efficient origins are used more efficiently. Moreover, we find that overproduction of potentially limiting factors that promote pre-IC formation results in increased replication from both efficient and inefficient origins, consistent with the hypothesis that competition among origins for these factors establishes origin efficiency.

DISCUSSION

Our main results can be summarized as follows: (1) ORC binding to fission yeast origins is periodic in the cell cycle, rising during M and peaking at the M/G1 transition. Pre-RC formation is also periodic, rising and peaking in G1. (2) Compared to an early efficient origin (*ori2004*), ORC binding and pre-RC formation are delayed at a late inefficient origin (*ori2060*) and at a cryptic origin not used in its normal chromosomal context (*ars727*). (3) The extent of pre-IC formation at these three origins reflects the efficiency of their usage. (4) Decreasing ORC binding through removal of AT hook motifs at *ori2004* results in delays in pre-RC and pre-IC formation and delayed replication. (5) Delaying cells in M equalizes ORC binding and leads to changes in origin usage across the genome for many origins such that early efficient origins are utilized less efficiently, and some late inefficient origins are used more efficiently. (6) Overproduction of pre-IC factors Cdc45, Hsk1, or Dfp1 results in increased origin efficiency across the genome.

Peak ORC association with fission yeast origins at M/G1 is similar to the timing of ORC binding to origins in metazoans (DePamphilis, 2005). Our results contrast with conclusions from earlier studies in both budding and fission yeasts, which have reported an unchanging association of ORC with chromatin (Lygerou and Nurse, 1999) and origins (Aparicio et al., 1997; Ogawa et al., 1999). The differences between the present and earlier fission yeast results concerning origin binding are likely due to the increased temporal resolution and quantitative sensitivity of the experiments reported here. Using ChIP, we cannot exclude the possibility that a conformational change at the origin

results in alterations in Orp1 ChIP signals. If this is the case, our results may reveal a specific event such as chromatin remodeling occurring at the origin during the M/G1 transition, prior to pre-RC formation. Our analysis also does not allow us to distinguish whether all cells exhibit periodic ORC behavior at a given origin or whether this occurs in a subpopulation of cells in which the origin is competent to fire.

Our work indicates that the timing of ORC and MCM binding during mitosis and G1 contributes to establishing the replication program during S. We show that pre-RCs are formed at origins regardless of their efficiencies, consistent with work from both budding and fission yeasts (Hayashi et al., 2007; Santocanale and Diffley, 1996), but that ORC binding and pre-RC formation are both delayed at a later firing origin. In contrast, no differences have been reported for MCM association with early- and late-firing origins in budding yeast (Aparicio et al., 1999). We also demonstrate that removal of AT hook motifs in *ori2004*, which reduces ORC binding, delays pre-RC and pre-IC formation as well as replication timing, suggesting that differences in affinity of origins for ORC during M play a role in the timing of origin firing during the S of the subsequent cell cycle.

We propose that delays in ORC and MCM binding during M and G1 lead to a nonequal association of Cdc45 and other replication factors among origins, thus establishing the replication timing program and determining the efficiency of origin usage. This can be understood if origins compete for a limited pool of replication factors such that origins at which ORC and pre-RC assemble late have a smaller number of these factors available to them. Consistently, we observe that the level of Cdc45 bound to an origin is closely related to origin efficiency; it should be noted that our results represent a population analysis and that Cdc45 may bind only to a subpopulation of active origins. Cdc45 has been suggested to be present in limiting quantities in human cells (Pollok et al., 2007), and the replication efficiency in *Xenopus* extracts correlates with the amount of Cdc45 on chromatin (Edwards et al., 2002). Moreover, a recent study using DNA combing has shown that Hsk1-Dfp1 in fission yeast regulates origin efficiency (Patel et al., 2008). Pre-IC formation at late origins may be restricted by decreasing levels of available factors as cells enter S and by passive replication through a region inactivating an origin so it cannot bind such factors. Therefore, the efficiency of an origin would be determined by both the timing of origin firing and its context in relation to other origins, with proximity to an early origin inactivating a late origin due to passive replication.

Our model proposes that the timing and therefore efficiency of origin firing are established by the recruitment of ORC to origins during M followed by competition among origins for limiting replication factors. ORC binding to origins may be determined by a combination of the primary sequence of an origin as well as its chromatin context. The timing of the increase in ORC binding during M and of pre-RC formation during G1 determines origin usage during the subsequent S. Our model could explain replication timing and efficiency as follows: ORC binding to early origins reaches a maximum at the exit of M, at which time ORC association with late origins is still low. At the beginning of G1, when MCM is competent to bind to origins, early origins have sufficient ORC to assemble a pre-RC, while late origins do not. Early

origins with pre-RCs can then bind Cdc45 and other replication factors, which we suggest are present in limiting amounts. At late origins, ORC binding increases and reaches a maximum only later in G1, when they can then recruit MCMs. These origins complete pre-RC assembly toward the end of G1 and will be delayed for Cdc45 binding compared to early efficient origins, and it is this delay that results in a difference in replication timing. Late origins will also have a smaller pool of available Cdc45 for pre-IC formation, which decreases the likelihood of pre-IC formation and origin usage. Support for this model comes from our experiments prolonging M, which allows time for ORC to bind fully at more origins, reducing the differences in usage of many efficient and some inefficient origins. If ORC is limiting, as has been shown in budding yeast, then the equilibration of ORC binding may reflect a dynamic on-off process for ORC at origins during the prolonged M (Rowley et al., 1995). Alternatively, ORC may simply accumulate to a maximal level at both efficient and inefficient origins, allowing them to compete more equally for limiting replication factors. When mitosis is extended, a decrease in efficiency of many of the most frequently used origins is observed, while some inefficiently replicated regions become more active. Moreover, overproducing pre-IC factors Cdc45, Hsk1, or Dfp1 increases replication in both efficient and inefficient regions, suggesting that these proteins, among others, are limiting for replication.

The fact that we observe partial equilibration of origin efficiencies after MBC treatment may be due to the inaccessibility of an origin in its chromatin context. Certain regions of the fission yeast genome are generally repressed for origin activity, as reported by Heichinger et al. (2006) and Hayashi et al. (2007), and, interestingly, the MBC-induced origins identified in this study are mostly located outside these regions (Figure S6). It is also possible that as some replication factors localize to nuclear foci (Meister et al., 2007), an origin may need to be positioned near a local pool for recruitment to occur. In addition, previous work investigating the program of origin firing in other organisms has implicated chromatin structure and histone modifications as important regulators. For example, acetylation status has been shown to change the pattern of origin firing, as deletion of the Rpd3 histone deacetylase in budding yeast results in increased histone acetylation and earlier firing of several origins (Aparicio et al., 2004; Vogelaar et al., 2002). These results are consistent with work in *Drosophila* follicle cells demonstrating that histones at active origins are hyperacetylated during the period of ORC binding and that RPD3 mutations result in redistribution of Orc2 in amplification stage cells (Aggarwal and Calvi, 2004). The timing of origin firing in fission yeast may likewise be regulated by chromatin modifications during the cell cycle, which may modulate the affinity of an origin for ORC. In our experiments, delaying cells in mitosis may lead to a subset of late origins acquiring the same modifications as early origins, thus altering the origin firing program by allowing more equivalent binding of ORC.

Our work demonstrates that origin efficiency and timing in fission yeast are regulated by events that occur during mitosis of the previous cell cycle and suggests that competition for limiting factors by origins during G1 and S determines their activity. Support for aspects of this model comes from experiments in budding yeast where late replication of origins at

telomeres is established between mitosis and G1 (Raghuraman et al., 1997), in CHO cells where ORC binds chromatin during M/G1, and in mammalian nuclei introduced into *Xenopus* extracts where replication timing is established by early G1 (Dimitrova and Gilbert, 1999; Okuno et al., 2001). In human cells, time-lapse imaging studies have shown that Orc1 exhibits a distinctive localization pattern during G1 resembling the pattern of DNA replication that occurs during S (S. Prasanth and B. Stillman, personal communication). Thus, it is possible that as in fission yeast, ORC binding and pre-RC assembly during M of the previous cell cycle and the G1 prior to S phase are key determinants for origin selection and the timing of origin firing in the cells of metazoan eukaryotes.

EXPERIMENTAL PROCEDURES

Strains and Growth Conditions

Standard media and methods were used (Hayles and Nurse, 1992; Moreno et al., 1991). Strains used in this study are listed in Table S1. *cdc25-22* cells were synchronized by growing in minimal medium plus supplements (EMM4S) at 25°C to 1–2 × 10⁶ cells/ml, shifting to 36.5°C for 4 hr to block cells in late G2, followed by release at 25°C. Samples for ChIP were taken every 10 min. For FACS, cells were fixed in 70% ethanol, and calcofluor staining was used to assess septation. Strains overexpressing *cdc45*, *hsk1*, and *dfp1* from the *nmt1* promoter were constructed by PCR integration (Bahler et al., 1998).

For MBC experiments, cells were synchronized with *cdc25-22* at the restrictive temperature for 3 hr and 45 min. MBC (Carbendazim, Sigma) was added at 50 µg/ml 10 min prior to the end of the arrest. For Figure 5A, cells were incubated with MBC after release to 25°C for the duration of the time course. For Figures 5B–5E, cells were incubated with MBC at 25°C for 65 min before filtering and washing with EMM4S + 12 mM HU. Cultures were resuspended into EMM4S + 12 mM HU + 300 mM BrdU to label newly synthesized DNA.

Chromatin Immunoprecipitation

ChIP was performed with some modifications to Martens and Winston (2002). Cells were fixed with 1% formaldehyde and lysed in a FastPrep cell disruptor; extracts were sonicated with a Misonix microtip to ~300–400 bp DNA. The following antibodies were used: anti-HA (12CA5, 1 µl per IP), anti-MCM4 (1 µl per IP, Nishitani et al. [2000]), and anti-GFP (1 µl per IP, gift of the Rout laboratory). For qPCR analysis, input DNA (at 1/100 dilution) and IP DNA were mixed with SYBR Green PCR mix (Applied Biosystems) and processed with an ABI 7900 or a Roche Lightcycler 480. The occupancy, or percent IP, was derived by relative quantification with the Ct or Cp values. Percent IP represents the ratio for a specific sequence of the amount of DNA associated with the immunoprecipitated protein to the total DNA used for the IP. For time course analysis, line graphs showing changes in percent IP were hand-drawn. Sequences of PCR primers are listed in Figure S7.

BrdU Immunoprecipitation and Array Hybridizations

Cells were treated with 0.01% sodium azide and harvested by centrifugation. After washing with 50 mM ethylenediaminetetraacetic acid (EDTA) and TE buffer (10 mM Tris [pH 8.0], 1 mM EDTA), genomic DNA was prepared (Hoffman and Winston, 1987), purified using the QIAGEN Genomic DNA kit, and sonicated to ~300–400 bp length. For IP, 4–5 µg DNA was mixed with 40 µl BrdU antibody (anti-mouse, BD Biosciences) followed by Protein G beads (GE Healthcare). Washes and elution were performed according to the above ChIP protocol. For amplification, DNA was blunted with T4 DNA polymerase, followed by linker ligation and PCR amplification for 22 cycles and purification on a QIAGEN PCR purification column (http://jura.wi.mit.edu/young_public/signaling/Protocols.html). Two micrograms of DNA was fragmented to 50–100 bp in One-Phor-All Buffer Plus (GE Healthcare) with Genechip fragmentation reagent (Affymetrix) and biotin labeled with the BioArray Terminal

Labeling Kit (Enzo Life Sciences) before hybridization to Affymetrix *S. pombe* tiling arrays.

Data Analysis

The tiling analysis software (TAS) package from Affymetrix was used to obtain linear signal intensity values. All poorly hybridized spots with values of 1.0 were removed from each data set. Signal intensities from BrdU IPs for experimental samples were compared to control IPs using genomic DNA from *cdc25-22* arrested cells by calculating the ratio of signal intensities for every spot with a value in both data sets. Smoothing of the dataset with the geometric mean was used over a window of 200 probes, or around 5 kb. The ratios were normalized to the median value of the smoothed data set to provide a baseline for comparison among the different hybridizations. Three sets of experiments for mitotic HU replication efficiencies as well as MBC-HU treatment were performed.

ACCESSION NUMBERS

The array data reported in this paper has been deposited in the NIH GEO database with the accession number GSE14864.

SUPPLEMENTAL DATA

Supplemental Data include one table and seven figures and can be found with this article online at [http://www.cell.com/supplemental/S0092-8674\(09\)00051-8](http://www.cell.com/supplemental/S0092-8674(09)00051-8).

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