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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. Chromatin immunoprecipitation (ChIP) analyzed 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 immmunoprecipitated 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

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