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Multiple ORC-binding sites are required for efficient MCM loading and origin firing in fission yeast

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In most eukaryotes, replication origins are composed of long chromosome regions, and the exact sequences required for origin recognition complex (ORC) and minichromosome maintenance (MCM) complex association remain elusive. Here, we show that two stretches of adenine/thymine residues are collectively essential for a fission yeast chromosomal origin. Chromatin immunoprecipitation assays revealed that the ORC subunits are located within a 1 kb region of *ori2004*. Analyses of deletion derivatives of *ori2004* showed that adenine stretches are required for ORC binding *in vivo*. Synergistic interaction between ORC and adenine stretches was observed. On the other hand, MCM subunits were localized preferentially to a region near the initiation site, which is distant from adenine stretches. This association was dependent on adenine stretches and stimulated by a non-adenine element. Our results suggest that association of multiple ORC molecules with a replication origin is required for efficient MCM loading and origin firing in fission yeast.

Keywords: adenine stretch/chromatin immunoprecipitation/pre-replicative complex/replication origin/*Schizosaccharomyces pombe*

Introduction

Eukaryotic DNA replication initiates from multiple replication origins distributed on chromosomes. Initiation of DNA replication is tightly regulated to occur with appropriate timing from specific regions. Since fixed regions are used as replication origins in almost all eukaryotic species, each replication origin is likely to contain information required to promote DNA replication (see review by Gilbert, 2001).

Structures of replication origins are well understood in the budding yeast, *Saccharomyces cerevisiae*. Budding yeast origins isolated as autonomously replicating sequences (ARSs) are composed of modular elements within a 100–200 bp region. A consensus sequence, designated as the ARS consensus sequence (ACS), is essential for origin function (Marahrens and Stillman, 1992). The ACS is the binding consensus for a six subunit origin recognition complex (ORC) (Bell and Stillman, 1992). ORC and two other factors, Cdc6 and Cdt1, are prerequisite for origin binding of the minichromosome maintenance (MCM) complex, consisting of Mcm2–7, resulting in a pre-replicative complex (pre-RC) (Diffley *et al*., 1994; Cocker *et al*., 1996; Aparicio *et al*., 1997). *In vivo* footprinting experiments have suggested that the MCM complex is loaded proximal to the ORC-binding site (Diffley *et al*., 1994).

Pre-RC assembly is a crucial step for initiation of DNA replication. ORC-, Cdc6/18-, Cdt1-dependent association of the MCM complex with chromatin prior to initiation is observed in many eukaryotes (Coleman *et al*., 1996; Ogawa *et al*., 1999; Nishitani *et al*., 2000). The MCM is essential for both initiation and elongation of DNA replication, probably functioning as a replicative DNA helicase (Ishimi, 1997). CDK and Cdc7–Dbf4 kinase activities are required for initiation of replication, presumably for activation of MCM helicase (Donaldson *et al*., 1998; Zou and Stillman, 2000). CDK activity also prevents re-activation of replication origins by repressing the assembly of pre-RC during the S and G_2 phases (see review by Diffley, 2001; Nguyen *et al*., 2001). Assembly of pre-RC is limited to the G_1 phase, and this regulation is crucial for 'once and only once' replication in a cell cycle.

In contrast to budding yeast, replication origins in most eukaryotes appear to require longer chromosome regions (see review by Gilbert, 2001). Short essential consensus sequences like the budding yeast ACS have not been found in such origins. For instance, several kilobase regions within the 8 kb region of the human β-globin origin are required for origin activity, when it is transferred to an ectopic locus (Aladjem *et al*., 1998). Amplification of the *Drosophila* chorion gene cluster requires a 440 bp *ACE3* element located ~1.5 kb from the amplification origin, and several amplification-enhancing elements (*AER*s) (Delidakis and Kafatos, 1989). Localization of ORC subunits to replication origins in *Drosophila* and human has been demonstrated previously (Austin *et al*., 1999; Ladenburger *et al*., 2002), and the results overall imply that specific sequences distributed over long stretches are important for origin function in metazoan cells. However, such sequence elements have not actually been identified, partly because methods available to analyze the origin structure are limited in metazoan cells due to their inability to maintain the origin region as an autonomously replicating plasmid.

Fission yeast, *Schizosaccharomyces pombe*, similar to higher eukaryotes, requires a long chromosome region as a replication origin, and studies with ARS fragments have shown that a several hundred base pair region is required for autonomous replication (Maundrell *et al*., 1988; Caddle and Calos, 1994; Dubey *et al*., 1994; Clyne and Kelly, 1995; Okuno *et al*., 1997). Short essential consensus sequences like the budding yeast ACS have not been found

among ARSs. Instead, stretches of asymmetrically placed adenine/thymine residues have been identified as important elements for ARS activity (Zhu *et al*., 1994; Clyne and Kelly, 1995; Dubey *et al*., 1996; Kim and Huberman, 1998; Okuno *et al*., 1999). We have identified three regions, regions I, II and III, essential for ARS activity of *ars2004* (Okuno *et al*., 1999). Regions I and III, 40 and 65 bp, respectively, consist of extensive adenines on one strand (Okuno *et al*., 1999). As shown by several groups, the fission yeast ORC interacts with adenine stretches *in vitro* (Kong and DePamphilis, 2001; Lee *et al*., 2001; Takahashi and Masukata, 2001). The *in vivo* ORC binding to AT-rich regions has been demonstrated recently by *in situ* footprinting (Kong and DePamphilis, 2002). In addition, it has been reported that Orc1 and Mcm6 associate with replication origins *in vivo* (Ogawa *et al*., 1999). The results thus suggest that fission yeast replication origins contain information to localize the ORC and MCM.

Considering the structural similarity of replication origins in fission yeast and metazoans, the reasons why long chromosomal regions are required for initiation of DNA replication may also be shared. To ascertain the sequence requirements for chromosomal replication origins in fission yeast, we deleted essential elements for ARS activity from the chromosomal *ori2004* locus and analyzed the origin activity and association with the ORC and MCM. Two adenine stretches in *ori2004* are collectively essential for initiation of replication. Adenine stretches are the ORC-binding sites *in vivo*. They are prerequisite for association of the MCM complex with a region that appears to coincide with the replication initiation site. The results further raise the possibility that multiple ORC molecules distributed within the long region are required for efficient firing of replication origins in organisms that require long chromosomal regions for initiation.

Results

Requirement of adenine stretches for initiation of chromosome DNA replication

Three essential regions, namely regions I, II and III, of 40, 125 and 65 bp, respectively, have been identified in *ars2004* by the ARS assay (Okuno *et al*., 1999). However, it has not been clear whether these elements are indeed functional on the chromosome. To examine this question, each of the three regions, individually or in combination, was deleted from the chromosomal *ori2004* locus. For synchronization of the cell cycle, *cdc25-22* cells and derivatives carrying the mutant *ori2004* were released from G_2/M block, and total genome DNA was isolated at 60, 75 and 90 min. Replication intermediates were analyzed by neutral/neutral two-dimensional gel electrophoresis followed by Southern hybridization with the *ori2004* probe. Progression through the cell cycle after release from the block was similar among deletion strains, as monitored by assessing populations of septum-containing cells (data not shown).

As shown in Figure 1B, two-dimensional gel electrophoresis of the *Eco*RV-digested DNA from wild-type cells at 60 min yielded a bubble-arc extending from the 1C position to near the 2C position, and a faint Y-arc (Figure 1B, top panel). At later time points, a Y-arc and a bubble-arc at a similar intensity were observed. These results indicate efficient firing of wild-type *ori2004* and some extent of passive replication of the *ori2004* locus from neighboring origins. In the cells lacking region I (ΔI), the amount of bubble-arc was reduced and the amount of complete Y-arc was increased at 75 and 90 min after release (Figure 1C, top panels), indicating the origin activity of *ori2004* to be reduced. In ΔII and ΔIII cells, the bubble-arc was reduced, with a concomitant increment of complete Y-arc as observed in ΔI cells (Figure 1C, middle and bottom panels). These results indicate that regions I, II and III are required for efficient initiation of DNA replication from *ori2004*.

Since regions I, II and III are not essential for chromosome replication origin activity, we next examined the effect of combined deletion. In ΔIΔII and ΔIIΔIII cells, a strong Y-arc accompanied by a faint bubble-arc was observed at 75 min (Figure 1D, top and middle panels), indicating the initiation from *ori2004* to be reduced to a very low level, although residual initiation activity remained. In contrast, in ΔIΔIII cells, no clear bubble-arc was evident (Figure 1D, bottom panels). These results suggest that regions I and III are collectively essential for origin activity of *ori2004*.

To exclude the possibility that the absence of a bubble-arc in the ΔIΔIII cells might be due to loss of the bubble-shaped intermediates during preparation of genome DNA, the same membranes were analyzed by hybridization with *ori2004* and other origin probes. *Hae*III-digested genome DNA prepared from wild-type and ΔIΔIII cells at 75 min after release was separated by two-dimensional gel electrophoresis and hybridized with the *ori2004* probe, then re-hybridized with the *ori3001* probe. As shown in Figure 1E, the wild-type cells yielded both bubble- and Y-arcs with *ori2004* and *ori3001* probes (Figure 1E, upper panels). In contrast, a bubble-arc was not detected in ΔIΔIII cells with the *ori2004* probe, whereas it was present with the *ori3001* probe, as in the wild-type cells (Figure 1E, lower panels). From these results, we concluded that regions I and III are collectively essential for activity of *ori2004*. Region II may not be essential, but is required for efficient origin function.

Cdc18 and Cdt1 promote chromatin association of MCM in G_1 phase

Since regions I, II and III are all required for efficient initiation of replication on chromosomes, we examined whether they function in association of ORC or assembly of pre-RC. To examine the association of ORC and MCM with the origin in G_1 phase, we used conditions in which MCM is loaded onto chromatin in G_1-arrested cells using a temperature-sensitive mutant for the *cdc10*$^+$ gene. Cdc10 is an essential component of a transcription complex regulating periodic transcription of several genes including *cdc18*$^+$ and *cdt1*$^+$, which are required for chromatin association of MCM proteins, and *cig2*$^+$, which encodes a B-type cyclin acting at the G_1–S transition (Kelly *et al*., 1993; Hofmann and Beach, 1994; Ayte *et al*., 2001). Thus, *cdc10-129* cells arrest in G_1 phase without forming pre-RC at the restrictive temperature, and it was expected that ectopic expression of Cdc18 and Cdt1 in *cdc10*-arrested cells would promote chromatin association of the MCM

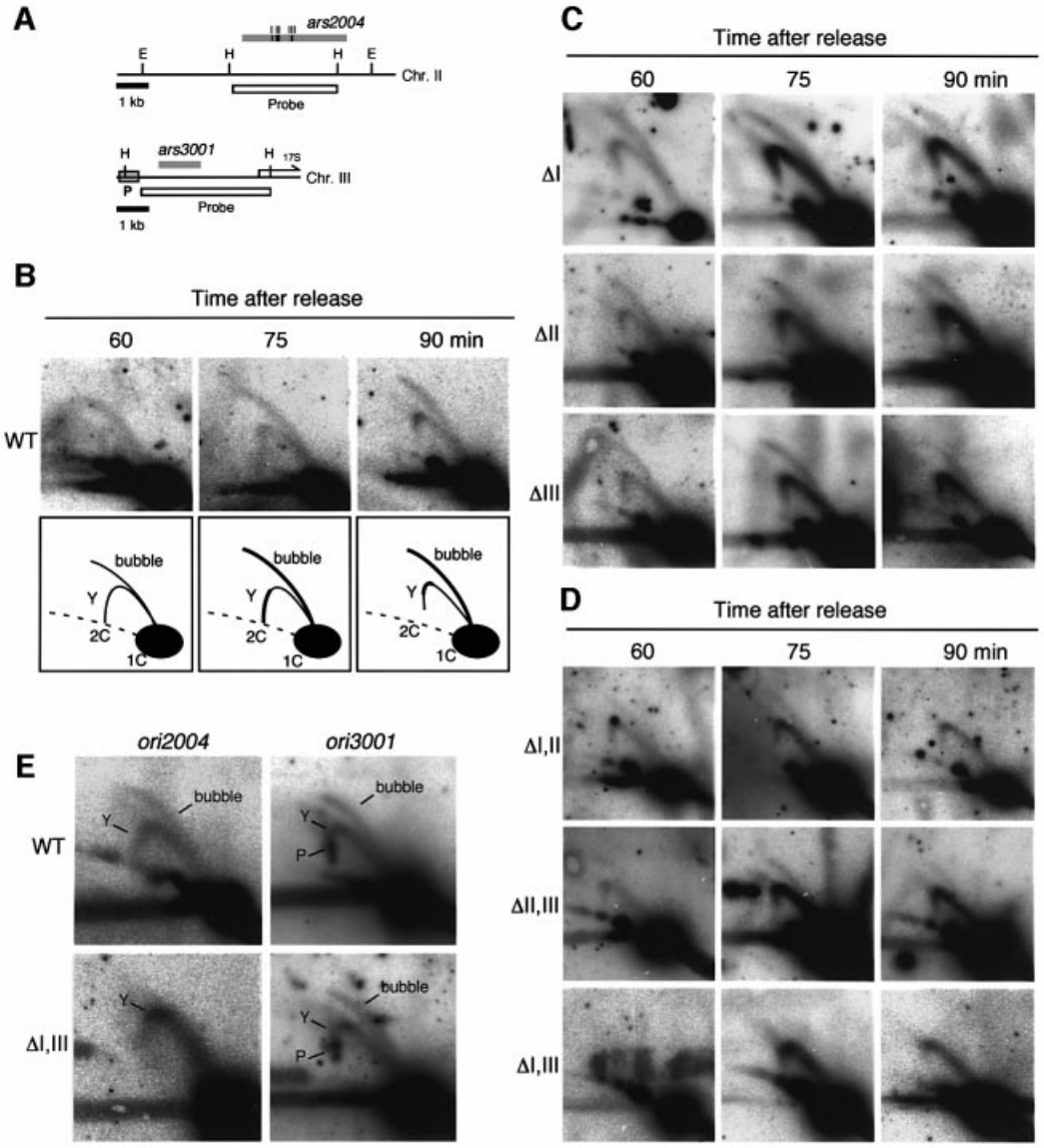

Fig. 1. Requirement for *cis*-acting elements for chromosomal *ori2004* activity. The *cdc25-22* derivatives with an altered *ori2004* locus were arrested at 36°C for 4 h and released at 25°C. Total genome DNA was isolated at 60, 75 and 90 min after release and digested with the appropriate restriction enzymes. Replication intermediates (RIs) were analyzed by neutral/neutral two-dimensional gel electrophoresis followed by Southern hybridization. (**A**) The positions of fragments and probes in *ori2004* (upper) and *ori3001* (lower) loci are presented. The restriction enzyme sites, *Eco*RV(E) and *Hae*III(H), are indicated. The replication fork pause region at the *ori3001* locus identified by Sanchez *et al*. (1998) is indicated by a gray box. Transcription of the 17S rDNA gene is indicated by an arrow. (**B**) The results of hybridization of the *Eco*RV–*Eco*RV (7.1 kb) *ori2004* fragment from *cdc25-22* cells at 60, 75 and 90 min after release from G_2/M block are presented together with schematic illustrations of bubble- and Y-arcs below the panels. (**C**) Genomic DNA was prepared from *ori2004* mutants lacking region I (ΔI), region II (ΔII) or region III (ΔIII). (**D**) Genomic DNA was prepared from a strain deleted of both regions I and II (ΔIΔII), II and III (ΔIIΔIII) and I and III (ΔIΔIII). (**E**) The results of hybridization of *Hae*III–*Hae*III-digested DNA from *cdc25-22* cells containing wild-type *ori2004* (WT) and Δregion I–Δregion III (ΔIΔIII), 75 min after release, are presented. The membranes were hybridized with an *ori2004* probe (left panels) and then re-hybridized with an *ori3001* probe (right panels). The replication pausing signal at the *ori3001* locus is indicated as 'P'.

complex without entrance into S phase. We used pre-synchronization at M phase by a cold-sensitive β-tubulin mutant, *nda3-KM311*, because it yielded better synchronization than *cdc10-129* alone.

When the *cdc10* mutant cells harboring plasmids expressing *cdc18*$^+$ and *cdt1*$^+$ genes under control of the inducible *nmt1* promoter or vectors alone were released from M phase block and shifted to the restrictive temperature for *cdc10-129*, the majority were arrested with a 1C DNA content as shown by flow cytometry (Figure 2A). It should be noted that re-replication was not observed with expression of Cdc18 and Cdt1, because we used a weak derivative of the *nmt1* promoter. Although *cdc18* cloned on a multicopy plasmid suppresses *cdc10-129* at the semi-restrictive temperature (Kelly *et al*., 1993), the cells expressing Cdc18 did not form colonies at 37°C (data not shown). Furthermore, we confirmed that cells expressing Cdc18 and Cdt1 remained in G_1 phase by the results showing that the Cdc2 kinase activity measured by phosphorylation of histone H1 was not affected by expression of Cdc18 and Cdt1 in the *cdc10*-arrested cells (data not shown).

To elucidate whether pre-RCs are formed under the above conditions, chromatin association of MCM proteins was analyzed. Cellular proteins (W) were separated into Triton-soluble (S) and -insoluble fractions after cell wall digestion. Chromatin-associated proteins (C) were solubilized by DNase I digestion and separated from the insoluble fraction (I). Orc1 was recovered mainly in the DNase I-insoluble fraction (I) and relative abundance in the fraction was not affected significantly by the cell cycle or by expression of Cdc18 and Cdt1 (Figure 2B, lanes 4 and 8). Mcm6 was not associated efficiently with chromatin in *cdc10*-arrested cells harboring vectors alone, as previously reported (Ogawa *et al*., 1999) (Figure 2B, bottom panel, lane 3). In contrast, about one-third of Mcm6 was found in the chromatin fraction in *cdc10*-arrested cells expressing Cdc18 and Cdt1 (Figure 2B, bottom panel, lane 7). Mcm2 and Mcm7, other subunits of the MCM complex, also accumulated in the chromatin-enriched fraction (data not shown). Such accumulation of MCM subunits was not observed in asynchronous cells with expression of Cdc18 and Cdt1 (Figure 2B; data not shown), excluding the possibility that MCM is loaded aberrantly onto the chromatin. These results indicate that Cdc18 and Cdt1 expressed in G_1-arrested cells promote chromatin association of the MCM complex. Below, we designate this condition as G_1 phase.

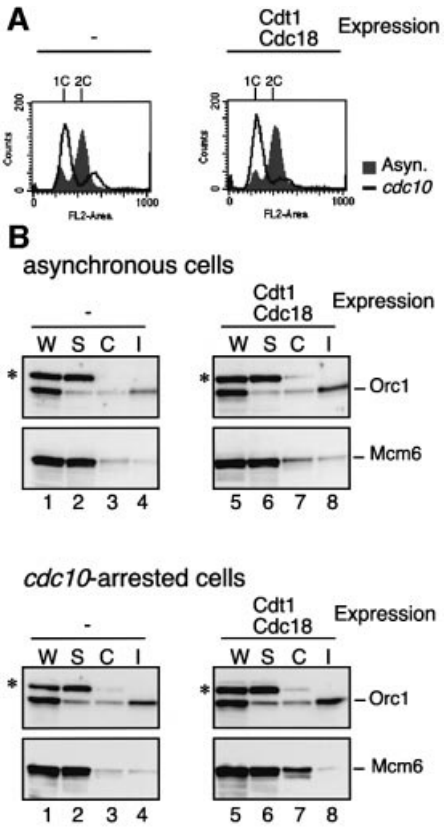

Fig. 2. G_1 cell cycle arrest and chromatin association of MCM. TTY44 cells carrying pREP81 and pREP82 vectors (lanes 1–4), or pREP81-cdt1 and pREP82-H6cdc18 (lanes 5–8) were cultured at 28°C in EMM without thiamine, shifted to 20°C for 4 h, and then arrested at G_1 phase at 37°C for 3 h. The *nda3-KM311* cold-sensitive mutation causing M phase arrest was used for pre-synchronization of cells prior to G_1 arrest. (**A**) The DNA contents of cells were analyzed by FACscan. The positions of 1C and 2C DNA contents are indicated. (**B**) Proteins in whole-cell extract (W), Triton-soluble fraction (S), chromatin-enriched fraction (C) and insoluble fraction (I) from asynchronous cells (upper panels) and G_1-arrested cells (lower panels) were separated by SDS–PAGE and immunoblotted with anti-FLAG (for Orc1) and anti-Mcm6 antibodies. The band marked with an asterisk reacts with anti-FLAG antibody, but is unrelated to Orc1.

ChIP scanning

Since regions required for initiation of DNA replication are distributed over ~1 kb, it is necessary to examine the localization of the pre-RC components within this stretch. For this purpose, we developed a chromatin immunoprecipitation (ChIP) scanning method as described below. DNA fragments associated with ORC or MCM were recovered by immunoprecipitation after *in vivo* cross-linking with formaldehyde (ChIP method). Recovery of specific regions within the origin was determined by PCR amplification from the immunoprecipitated and total cellular DNA after serial dilution. To minimize the error of PCR amplification, a linear range of PCR products amplified from eight serial dilutions was used for quantitation. For scanning the recovery of DNA fragments along the origin locus, 11 primer sets for the *ori2004* locus and a primer set for the *ori3002* locus as an internal control were designed.

ORC associates with adenine stretches in regions I and III

Using the G_1 cell extract of TTY44 (*h⁻ cdc10-129 nda3-KM311 ura4-D18 leu1-32 orc1-5FLAG*) cells expressing Cdc18 and Cdt1 from plasmids, we first examined the localization of ORC subunits. By immunoprecipitation with Orc1, segments within *ori2004*, *ori3002*, used as an internal control (see below), and *ori1* (data not shown) were amplified preferentially compared with the non-ARS region, suggesting localization of Orc1 at replication origins on fission yeast chromosomes. Figure 3A shows representative results of ChIP scanning for the *ori2004* locus from two independent immunoprecipitations. The relative recovery of each segment in *ori2004* compared with *ori3002*, an internal control, was similar in two experiments, although recovery of DNA differed slightly, supporting the reproducibility of the results. Segments 3–8 of *ori2004* were recovered by Orc1 immunoprecipitation (Orc1-IP) at a level several times greater than in the surrounding regions (Figure 3A). Furthermore, distribution of Orc4-immunoprecipitated DNA at this locus was similar to that of Orc1-immunoprecipitated DNA (Figure 3B). These results suggest that the fission yeast ORC associates with the region containing regions I, II and III of *ori2004*.

It has been shown that the ORC immunoprecipitate specifically interacts *in vitro* with adenine stretches in regions I and III of *ars2004* (Takahashi and Masukata, 2001). In order to determine whether ORC interacts with these regions *in vivo*, the effects of deletion of regions I and/or III on the ORC–*ori2004* association were examined. In ΔI cells, the recovery of DNA fragments around region I was greatly reduced (Figure 3C). Reciprocally, the recovery of DNA fragments around region III was reduced in ΔIII cells (Figure 3D). Furthermore, when both adenine stretches were deleted, no specific region within *ori2004* was recovered by Orc1-IP, indicating that Orc1 association

with *ori2004* depends on adenine stretches in regions I and III (Figure 3E). A similar amount of the *ori3002* locus, an internal control, was recovered, except in Figure 3D, where recovery by immunoprecipitation was slightly lower than in the other experiments. These results strongly suggest that the fission yeast ORC binds to adenine stretches in regions I and III *in vivo*. Interestingly, deletion of either adenine stretch reduced recovery of the remaining adenine stretch region (Figure 3C and D). These results suggest that ORC synergistically interacts with two adenine stretches in *ori2004*.

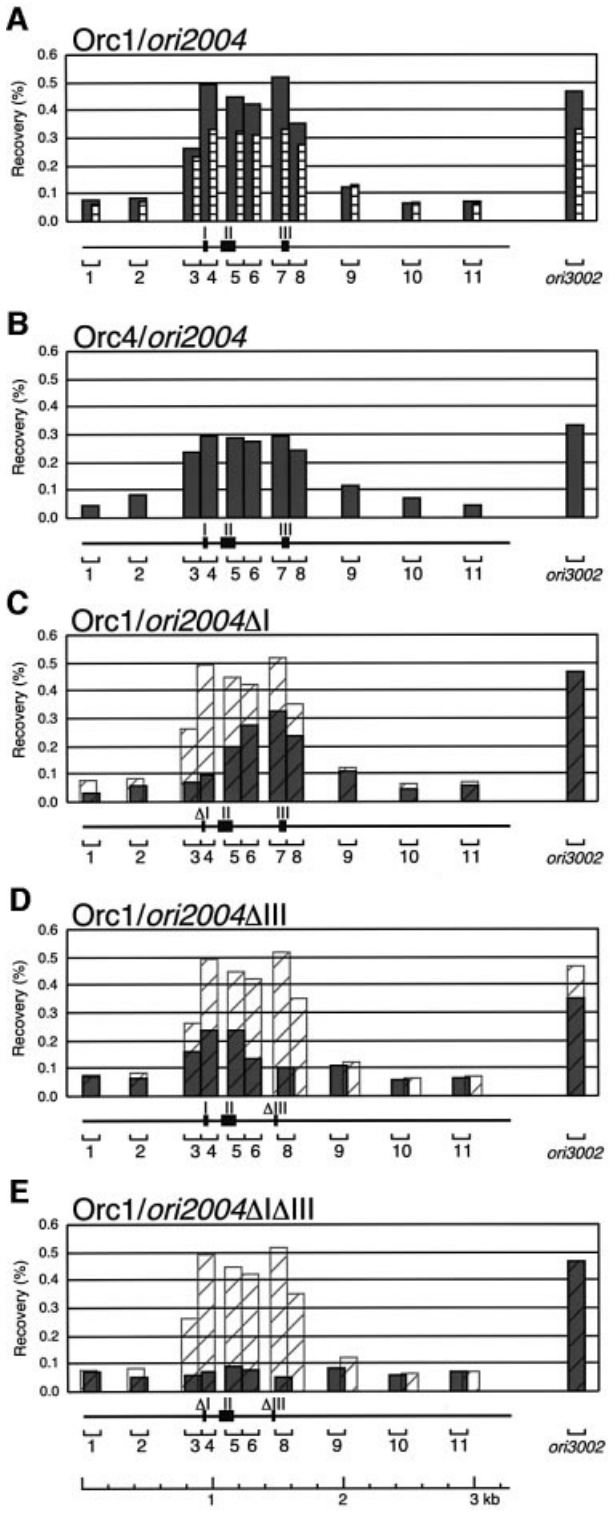

Fig. 3. Effect of deletion of adenine stretches on Orc1 localization. TTY44 derivatives carrying the wild-type *ori2004* locus (**A** and **B**), *ori2004*ΔI (**C**), *ori2004*ΔIII (**D**) or *ori2004*ΔIΔIII (**E**), harboring pREP81-cdt1 and pREP82-H6cdc18, were arrested in G_1 phase as described in Figure 2. Total cellular DNA and DNA immunoprecipitated with anti-FLAG for FLAG-Orc1 (A, C, D and E) or anti-Orc4 (B) antibodies, after serial dilution as templates for PCR amplification. Primers used were 11 sets for the *ori2004* locus, and one for the *ori3002* locus as an internal control. PCR products were analyzed as described in Materials and methods. The recovery of immunoprecipitated against total DNA calculated using a linear range of PCR amplification is presented in histogram form. Shaded and striped histograms in (A) show the results of two independent Orc1-IPs. Hatched histograms in (C–E) show superimposed wild-type *ori2004* DNA immunoprecipitated with Orc1. The positions of regions I, II and III in *ori2004* are indicated by filled boxes.

MCM is localized to a region distinct from the adenine stretches

Next, association of MCM subunits with *ori2004* was examined by ChIP scanning. In contrast to the localization of ORC subunits, DNA fragments recovered by Mcm6- and Mcm2-IP showed an apparent peak (Figure 4). Segments 5 and 6 were recovered preferentially by Mcm6-IP at 2- or 3-fold higher levels than segments 3 and 4 around region I, and segments 7 and 8 around region III (Figure 4A). The distribution of DNA fragments recovered by Mcm2-IP was very similar to that with Mcm6-IP (Figure 4B), suggesting that the MCM complex preferentially associates with a region in or near segments 5 and 6 in the *ori2004* locus. Taking these results together with those in Figure 3, MCM appears to associate with a region distinct from ORC association sites in *ori2004*.

Two adenine stretches in ori2004 are required for efficient association of MCM

Because association of ORC with the replication origin is prerequisite for loading of MCM (Aparicio *et al*., 1997), we examined how two adenine stretches contribute to MCM association. By deleting region I, recovery of segments 5 and 6 by Mcm6-IP was reduced to about one-third of the value with the wild-type strain (Figure 4C). Deletion of region III reduced recovery of segments 5 and 6 by about a quarter (Figure 4D). By combination of two deletions (ΔIΔIII, Figure 4E), recovery of the segments within the *ori2004* locus was drastically reduced to about one-tenth of the wild type. Recovery at the *ori3002* locus was not affected significantly by these deletions, indicating that the loss of MCM association was specific to *ori2004*. These results confirmed that ORC association with regions I and III is collectively essential for loading of MCM onto *ori2004*. The results also show that both adenine stretches at the *ori2004* locus are required for efficient association of MCM. However, their contribution to MCM loading appears to differ, the MCM association being more severely impaired by deletion of region I.

Region II functions for association of MCM with ori2004

Region II, which was found not to interact with ORC in our previous *in vitro* study (Takahashi and Masukata,

2001), nevertheless is important for the origin function on the chromosome. To elucidate its roles, we examined effects of its deletion on association of ORC and MCM using the ChIP scanning assay. As shown in Figure 5, while a lack of region II only slightly reduced the Orc1 association, recovery of DNA fragments of segment 6 by Mcm6-IP was reduced by about half (Figure 5A and B). These results suggest that region II is required for efficient association of MCM with *ori2004*.

To confirm the above results, the effects of region II deletion were examined in the ΔIII background, which eliminated the contribution of region III to ORC and MCM association. As shown in Figure 5C, association of Orc1 around region I was not reduced further in ΔIIΔIII cells compared with ΔIII cells (shown by hatched histograms). On the other hand, recovery of origin DNA by Mcm6-IP in ΔIIΔIII cells was about a half of that in ΔIII cells (Figure 5D). These results strongly suggest that region II functions in the association of MCM.

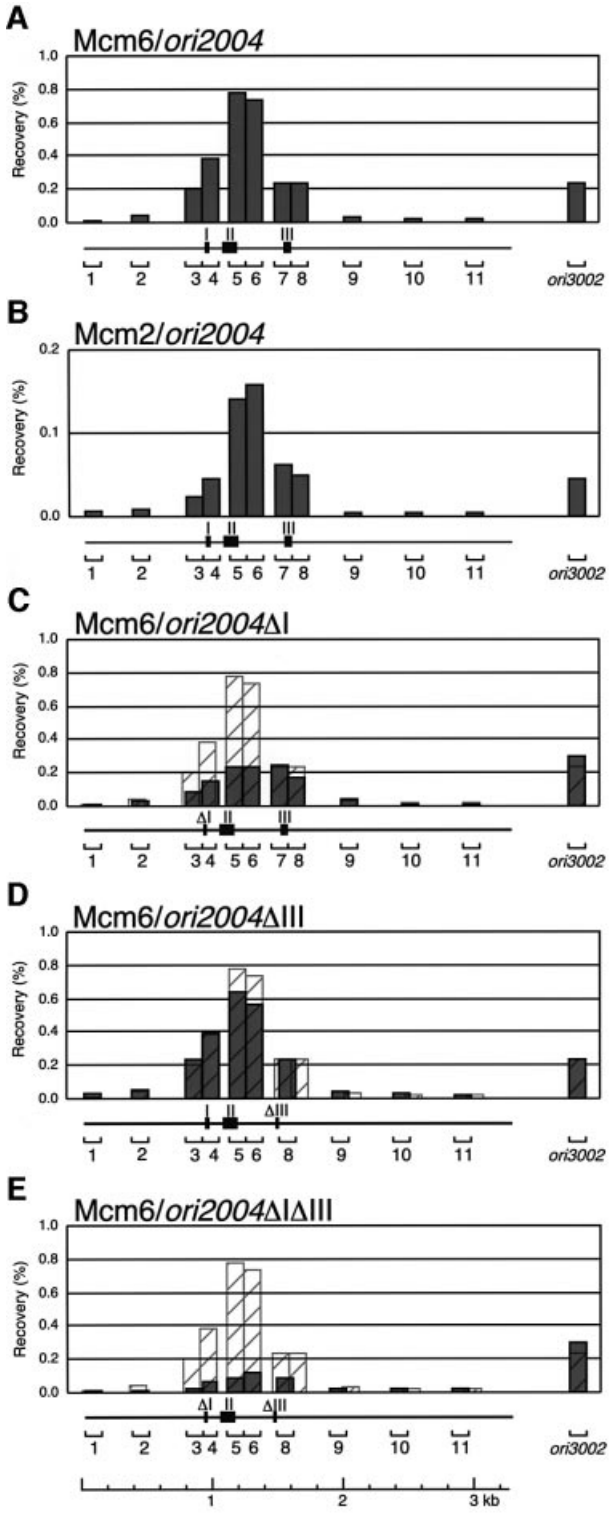

Fig. 4. Effects of deletion of adenine stretches on Mcm6 localization. ChIP scanning experiments were carried out as described in Figure 3, except that DNA was immunoprecipitated with anti-Mcm6 (**A**, **C**, **D** and **E**) or anti-Mcm2 (**B**) antibodies. The recovery of the immunoprecipitated DNA relative to total cellular DNA is shown by histograms with relevance to the positions of primer sets. The hatched histograms in (C–E) show superimposed recovery of wild-type *ori2004* DNA immunoprecipitated with anti-Mcm6.

Localization of the MCM complex is not determined by the orientation of the adenine stretch

Because the MCM-binding region lies 3′ to the adenine-rich strands of regions I and III, the direction of adenine stretches, which would specify the polarity of the ORC complex, may determine the MCM localization site. To test this possibility, we made a strain carrying an inverted region I and analyzed the localization of ORC and MCM. Since two adenine stretches in *ori2004* had a redundant role for ORC binding, region III was deleted to eliminate its effect. In the plasmid ARS assay, a derivative of the 3.2 kb ars2004ΔIII plasmid carrying inverted region I formed transformants, suggesting that the inversion did not impair the ARS activity (data not shown). As shown in Figure 6, association of Orc1 in the region I-inverted cells (I:inv ΔIII) was essentially the same as in ΔIII cells. While recovery of segment 5 by Mcm6-IP in the I:inv ΔIII cells was reduced by about half, the peak position of Mcm6 localization was not significantly affected. These results suggest that the MCM localization site is not determined simply by the direction of the adenine stretch. They also imply that the context between the adenine stretch and the other regions of *ori2004* might be important for MCM loading.

Discussion

The nucleotide sequences required for initiation of chromosome DNA replication have not been identified in most eukaryotes except for budding yeast. Here, we demonstrated that replication origin activity on the fission yeast chromosome depends on specific sequence elements. The 40 and 65 bp adenine stretches in *ori2004* are collectively essential for the replication origin function. The ChIP assay with deletions of adenine stretches shows that the essential role of the adenine stretches is association with ORC. On the other hand, MCM association occurs in a region distinct from the adenine stretches. Efficient loading of MCM requires multiple adenine stretches, which are presumably associated with multiple ORC molecules.

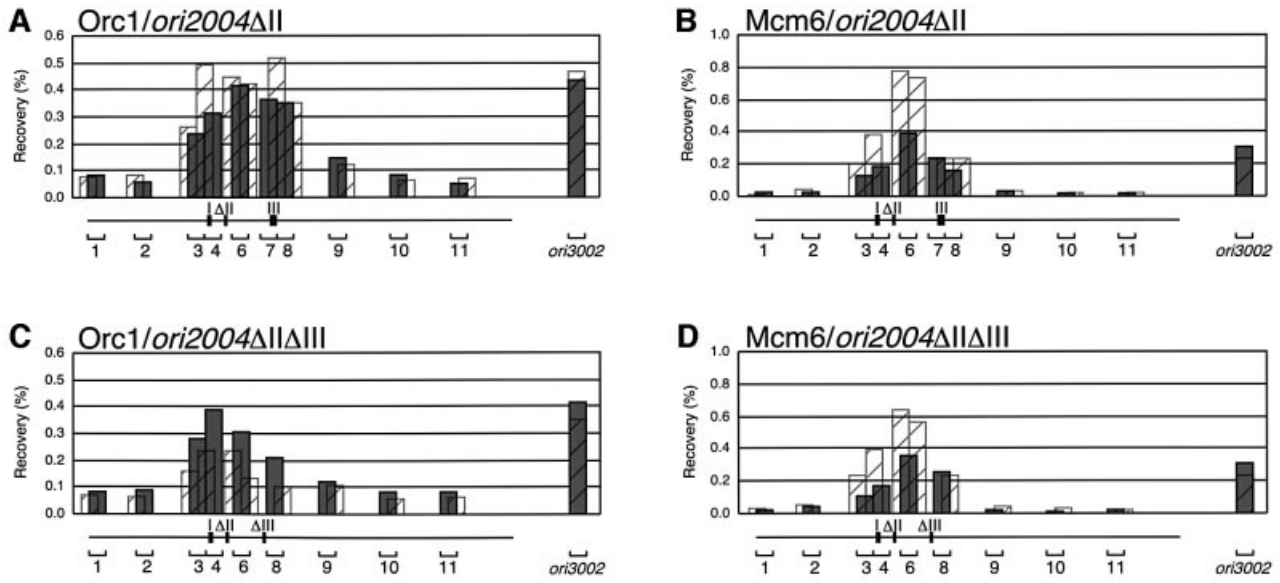

Fig. 5. Effects of deletion of region II on Orc1 and Mcm6 localization at the *ori2004* locus. ChIP scanning was performed as described in Figure 3 using *ori2004*ΔII (**A** and **B**) and *ori2004*ΔIIΔIII (**C** and **D**). DNA associated with Orc1 (A and C) and Mcm6 (B and D) was recovered by immunoprecipitation as shown in histogram form relative to total cellular DNA. Hatched histograms superimpose recovery of Orc1-IP DNA from wild-type *ori2004* (A) and that from *ori2004*ΔIII (C), or recovery of Mcm6-IP DNA from wild-type *ori2004* (B) and that from *ori2004*ΔIII (D).

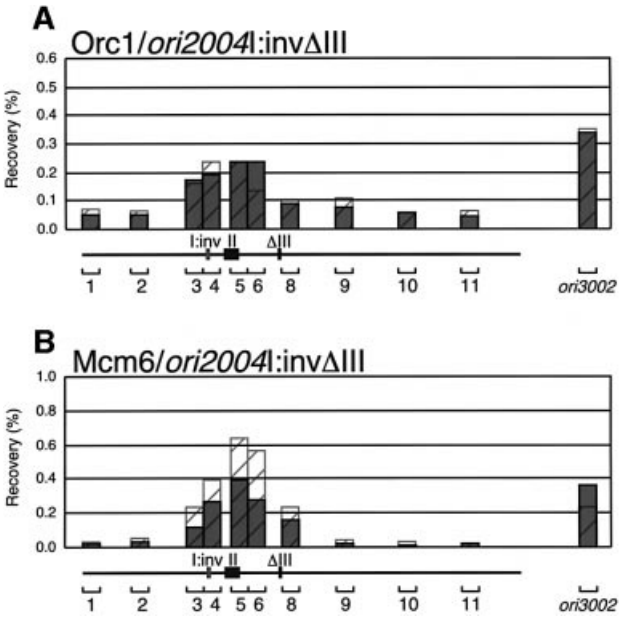

Fig. 6. Effects of inversion of region I on MCM localization. The results of ChIP scanning with a strain carrying an inverted region I for the *ori2004* locus are presented. Recovery of Orc1-IP DNA (**A**) and Mcm6-IP DNA (**B**) from the strain carrying the inverted region I in the ΔIII background is shown in histogram form. Hatched histograms in (A) and (B) superimpose recoveries from the ΔIII strain carrying region I in the natural orientation.

Multiple adenine stretches are required for efficient firing of chromosomal replication origins

Several groups have shown that asymmetrically aligned adenine/thymine stretches are essential for ARS activity in fission yeast (Zhu *et al*., 1994; Clyne and Kelly, 1995; Dubey *et al*., 1996; Kim and Huberman, 1998; Okuno *et al*., 1999). However, the dependence of the ARS activity on each adenine stretch becomes less clear in long chromosome fragments cloned on plasmids (Okuno *et al*., 1999). Here, we showed that both adenine stretches in *ori2004* are collectively important for efficient firing of the origin. Our results suggest that multiple adenine stretches, which are more widely distributed in some origins, are redundant to some extent, but collectively are essential components of the replication origin in fission yeast.

ORC binds to the adenine stretches

The results of ChIP experiments demonstrated that Orc1 and Orc4 are associated preferentially with replication origins such as *ori2004*, *ori3002* and *ori1*. We have carried out detailed ChIP scanning of the *ori2004* locus, partly because it is the most efficient origin both on the chromosome and on plasmids, and because deletion mutants of the chromosome locus are available. The results of ChIP scanning from independent Orc1-IPs yielded almost identical distributions of Orc1-associated segments (Figure 3A), although recovery of DNA differed slightly between experiments. Moreover, because the distributions of DNA immunoprecipitated with Orc1 and Orc4 were almost the same, localization of the ORC complex thereby would be detected. The requirement for regions I and III for the ORC association indicates that ORC binds to these regions. This conclusion is consistent with previous *in vitro* observations that ORC specifically interacts with regions I and III as well as with synthetic adenine stretches (Takahashi and Masukata, 2001). It has been shown that the N-terminal region of Orc4, which contains multiple AT-hook motifs, is responsible for ORC binding to the AT-rich tracts in *ars3002* and *ars1* (Chuang and Kelly, 1999; Kong and DePamphilis, 2001; Lee *et al*., 2001). In addition, a recent report has shown that *in vitro* binding sites of Orc4 are occupied *in vivo*, suggesting that Orc4 binds to AT-rich sites *in vivo* (Kong and DePamphilis, 2002). Thus, interaction of Orc4 with

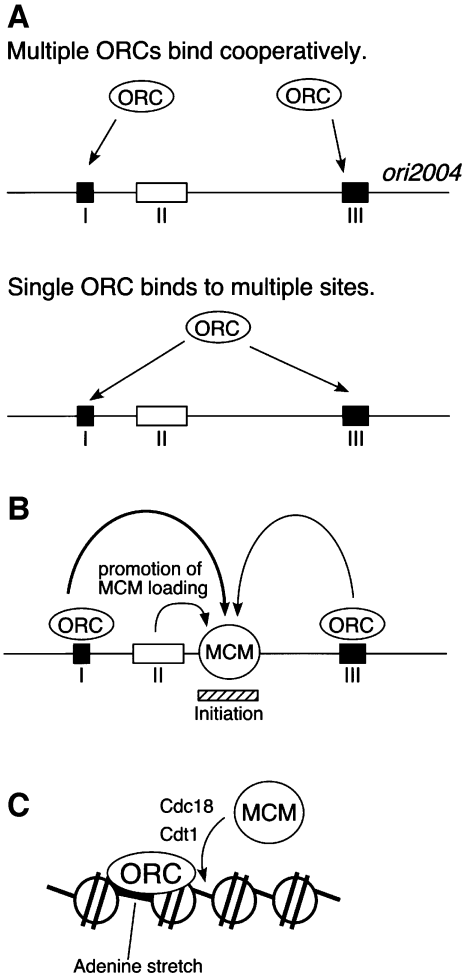

Fig. 7. Model of the structure of pre-RC in fission yeast. (**A**) Two alternative models for ORC association with *ori2004* are shown. Adenine stretches indicated by black boxes labeled with I and III are the ORC-binding sites. (**B**) Adenine stretches are collectively essential for loading of MCM onto a region distant from the adenine stretches. The non-adenine element shown by an open box (II) stimulates the MCM loading reaction. The initiation site mapped by two-dimensional gel electrophoresis is shown by a hatched box. (**C**) Regular nucleosomes appear to be formed within *ori2004*. ORC might associate with the MCM loading region across nucleosomes to load the MCM complex. Although multiple ORC and MCM molecules might be involved, one molecule of each is shown for illustration.

adenine stretches might be responsible for preferential association of ORC with replication origins *in vivo*.

The finding that ORC is localized at distantly located adenine stretches might be interpreted as association of ORC with one of alternative sites in different cells. For instance, ORC binds to region I of *ori2004* in one cell, while it associates with region III in another cell. However, this might not be the case, because the results of ChIP experiments with ΔI or ΔIII cells showed that ORC interacts synergistically with two adenine stretches (Figure 3). This would allow us to raise the following possibilities. First, multiple ORC molecules cooperatively interact with adenine stretches in the origin (Figure 7A). Alternatively, a single ORC molecule interacts with multiple adenine stretches (Figure 7A). We have shown that ORC immunoprecipitates interact with a 40 bp adenine/thymine stretch, which corresponds to region I of *ori2004* (Takahashi and Masukata, 2001). Thus, it seems more likely to us that at least two ORC molecules may bind cooperatively to regions I and III of *ori2004*, although we cannot exclude the latter possibility.

Non-adenine element enhances pre-RC formation

The results of two-dimensional gel analysis showed that region II, which is composed of non-adenine sequences, indeed is involved in chromosomal origin activity (Figure 1). However, residual firing was detected in ΔIΔII and ΔIIΔIII cells but not in ΔIΔIII cells, suggesting that region II is not essential. Consistent with this notion, experiments with ARS plasmid showed that region I or III fragments can functionally replace the two other regions, while the region II fragment cannot (Okuno *et al*., 1999). The results of ChIP experiments suggest that region II is important for MCM loading. Region II may be a stimulating element for pre-RC formation (Figure 7B).

It has been reported that a replication enhancer element derived from the *ura4*$^+$ locus origin cluster increases the stability of other ARS fragments in a distance- and orientation-independent manner, although it does not show ARS activity itself (Kim and Huberman, 1999). Because there is no significant sequence homology between region II and the enhancer element from the *ura4*$^+$ locus replication origins, it is not known whether there is any sequence specificity. However, the results of linker scanning analysis of region II suggested that the included sequence is crucial for function (Okuno *et al*., 1999). Region II may provide binding sites for certain DNA-binding proteins, possibly for factors involved in pre-RC formation, such as Cdt1, Cdc18 and the Noc3 homolog (Zhang *et al*., 2002). Alternatively, proteins involved in transcription might bind, since most fission yeast origins including *ori2004* are located at the intergenic promoter region upstream of two genes (Gomez and Antequera, 1999). Such proteins binding to region II might modulate chromatin structure at the origin locus and enhance accessibility of pre-RC components to the origin locus.

Localization of MCM subunits within the replication origins

ChIP experiments showed that two MCM subunits, Mcm2 and Mcm6, are localized preferentially to the region distant from the adenine stretches, suggesting distinct localization of the MCM complex and the ORC (Figure 4). Since MCM is considered to function as a DNA helicase for initiation of DNA replication, such a specific localization of the MCM complex might be involved in determination of the initiation site of DNA synthesis. Consistent with this notion, the initiation site at *ori2004* mapped by two-dimensional gel analyses appears to coincide with the peak of MCM distribution (Okuno *et al*., 1997). In addition, recent results have suggested that the initiation site and pre-RC site are almost the same in *ars3001* (Kong and DePamphilis, 2002). Thus, initiation of DNA synthesis may occur near the region where the MCM complex associates in the origin.

If the MCM is loaded onto the region where it is localized by ChIP analysis, how can it be loaded at the region distant from the adenine stretch where ORC binds? Although MCM is localized near region II, which appears to stimulate MCM loading, the MCM-binding site was not

Table I. *Schizosaccharomyces pombe* strains used in this study

Strain	Genotype	Source
TTY15	*h⁻ orp1::5FLAG-orp1*	Our stock[a]
HM288	*h⁺/h⁻ ade6-M216/ ade6-M210 ura4-D18/ura4-D18 leu1-32/leu1-32*	Our stock
EOY7	*h⁺ ade6-M210 ura4-D18 leu1-32 ori2004::ura4⁺*	This work
EOY68	*h⁺ cdc25-22 ori2004ΔI*	This work
EOY70	*h⁺ cdc25-22 ori2004ΔII*	This work
EOY72	*h⁺ cdc25-22 ori2004ΔIII*	This work
EOY85	*h⁻ cdc25-22 ori2004ΔIΔII*	This work
EOY87	*h⁺ cdc25-22 ori2004ΔIIΔIII*	This work
EOY90	*h⁻ cdc25-22 ori2004ΔIΔIII*	This work
TTY44	*h⁻ ura4-D18 leu1-32 nda3-KM311 cdc10-129 orp1::5FLAG-orp1*	This work
TTY78	*h⁺ ura4-D18 leu1-32 nda3-KM311 cdc10-129 orp1::5FLAG-orp1 ori2004ΔI*	This work
EOY75	*h⁻ ura4-D18 leu1-32 nda3-KM311 cdc10-129 orp1::5FLAG-orp1 ori2004ΔII*	This work
TTY81	*h⁻ ura4-D18 leu1-32 nda3-KM311 cdc10-129 orp1::5FLAG-orp1 ori2004ΔIII*	This work
TTY84	*h⁻ ura4-D18 leu1-32 nda3-KM311 cdc10-129 orp1::5FLAG-orp1 ori2004ΔIΔIII*	This work
EOY79	*h⁺ ura4-D18 leu1-32 nda3-KM311 cdc10-129 orp1::5FLAG-orp1 ori2004ΔIIΔIII*	This work
TTY95	*h⁻ ura4-D18 leu1-32 nda3-KM311 cdc10-129 orp1::5FLAG-orp1 ori2004I:inv*	This work
TTY97	*h⁻ ura4-D18 leu1-32 nda3-KM311 cdc10-129 orp1::5FLAG-orp1 ori2004I:inv,ΔIII*	This work

[a]Takahashi and Masukata (2001).

affected much by deletion of region II. On the other hand, the fact that deletion of region I has a more severe effect on MCM loading than region III deletion (Figure 4) implies that the ORC bound to region I is more proficient at MCM loading. However, the direction of the ORC binding sequence is not important for localization of MCM, because inversion of the adenine stretch of region I has little effect on the MCM-binding site (Figure 6). Since ORC, Cdc18 and Cdt1 may interact with MCM during pre-RC formation, it is possible that MCM is loaded onto a region that becomes proximal to the adenine stretches in the chromatin structure (Figure 7C). Consistent with this idea, micrococcal nuclease digestion of chromatin yields regular nucleosome ladders within *ori2004*, while region I appears to be nucleosome free (Y.Yamada and H.Masukata, unpublished results). At present, we cannot exclude other possibilities, e.g. that sequence- or structure-specific binding of pre-RC components determines MCM localization.

Origin structure in higher eukaryotes

This study demonstrated that two ORC-binding sites in *ori2004* are required for efficient ORC binding, MCM loading and, consequently, for efficient firing. Synergistic requirement of adenine stretches for ORC binding suggests cooperative binding of multiple ORC molecules. Because many fission yeast origins contain multiple adenine stretches that are important for ARS activity, a requirement for multiple ORC-binding sites in an origin seems to be a general feature in fission yeast. Thus, long chromosome regions in fission yeast replication origins are, at least in part, required for efficient ORC association. However, MCM localization in a narrow region may promote initiation of DNA replication from a limited region.

In metazoans, replication origins generally are also composed of long chromosome regions containing multiple redundant elements, except for some small replication origins such as the lamin B2 origin. Thus, binding of multiple ORC molecules to the origin might be required for origin function. One piece of evidence supporting this possibility is that the *Drosophila* ORC (DmORC) binds to four DNA fragments derived from the chorion gene amplification origin (Austin *et al*., 1999). DmORC binds to three fragments of *ACE3* and a fragment of *AER-d*. Interestingly, *ACE3*, which is required for efficient amplification of the locus, is located ~1.5 kb away from *AER-d*, where the majority of initiation occurs during amplification. It is possible that DmORC bound to the *ACE3* contributes to the initiation from *AER-d* by loading of MCM onto the *AER-d*. Long chromosome regions might be required for efficient ORC binding in most eukaryotes.

This work shows that fission yeast replication origins are a useful model system for analysis of functions of redundant and complex elements in eukaryotic replication origins. It will be extremely interesting to understand how multiple ORC binding acts to load MCM onto a distinct site by developing an *in vitro* system, in combination with *in vivo* study of the effects of chromatin structures on initiation of replication.

Materials and methods

Strains and media

The *S.pombe* strains used are listed in Table I. TTY44 was made by standard genetic methods as described earlier (Alfa *et al*., 1993). They were cultured in a complete medium, YE (0.5% yeast extract, 3% glucose), and an Edinburgh minimal medium, EMM (Moreno *et al*., 1991). TTY44, 78, 81, 84, 95, 97, EOY75 and 79 cells grown at 28°C were first incubated at 20°C for 4 h for arrest in M phase and then shifted to 37°C for 3 h for arrest in G_1 phase. Media containing 2% agar were used for plating. Transformation of *S.pombe* was performed by electroporation (Hood and Stachow, 1990).

Construction of ori2004 mutant strains

Construction of *ori2004* mutant strains was as described below. The 5′ and 3′ regions of *ars2004* in pXN289 (Okuno *et al*., 1997) were PCR amplified with *Hin*dIII site using primers 5′-GTAAAACGACGGCCA-GT-3′, 5′-TTTAAGCTTCACGGCATCTTTCTTC-3′, and 5′-ACAAAG-CTTTCTTCTGGAACTGCTG-3′, 5′-GGAAACAGCTATGACCATG-3′, digested with *Not*I–*Hin*dIII and *Hin*dIII–*Xba*I, respectively, and cloned into *Not*I–*Xba*I sites of a derivative of pBluescriptII SK+ lacking the *Hin*dIII site. Then, the 1.7 kb *Hin*dIII fragment of *ura4⁺* was inserted into the *Hin*dIII site. The resulting pARS2004Δ940 was digested with *Kpn*I and *Xba*I, and used for transformation of HM288. Ura⁺ transformants

Table II. Sequences of the primers used in this study

Name	Sequence (5′–3′)	Length of PCR fragment
ori2004 locus		
2004-1F	CCCTCACTAAAGGGATCATCCTC	
2004-1R	CCCTCCAAACCCTCCAAACCTCGC	140
2004-2F	GGATGCTTTCGAAATCCCTCGTG	
2004-2R	GGTGAGGTGGTAAAACGTACGC	143
2004-3F	GTTTGTCTCCGATAACGGGGG	
2004-3R	CACGGCATCTTTCTTCACGA	140
2004-4F	TCGTGAAGAAAGATGCCGTG	
2004-4R	GTGTGGTGTGTACTGAGTC	143
2004-5F	GCTAATTGAATGGAATGATTTGC	
2004-5R	GGGATTACGGATCCGAAAACTACCC	145
2004-6F	GGGTAGTTTTCGGATCCGTAATCCC	
2004-6R	CATACCAACCCTTACAAC	144
2004-7F	GAAACTTGTATATTATTTCTGCGTATAACC	
2004-7R	GAGTAATTAGCAATGTATGAATAGATCCAGCAGTTCC	142
2004-8F	CGTAGGTCTTCTGGAACTGCTGG	
2004-8R	GAAGCTAAATCGTTGCGTG	146
2004-9F	GTATCGTCTTGCTCGGTTTATTCCAGACCC	
2004-9R	CCTAGTGCCACACTTCCACC	140
2004-10F	GAGCGTGATAAAAACGTGACGGG	
2004-10R	CAACCCCATAACGATCAGTG	141
2004-11F	CTCTACGCTTGACTGTACTCTCTC	
2004-11R	GGGATGTTCCTACTCTTTGTTC	143
ori3002 locus		
3002-F	CCTGTTGAAATATGTATTTGGCGC	
3002-R	GATAGCTTTTGGATAAGTTATGACTTTTACG	141

were selected, and integration of the Δ*ars2004* fragment was confirmed by Southern hybridization. Haploid strain EOY7 carrying Δ*ori2004*::*ura4*$^+$ was obtained by tetrad dissection of the resulting strain. The *ori2004*ΔI, ΔII, ΔIII, ΔIΔII, ΔIIΔIII, ΔIΔIII, I:inv and I:invΔIII strains were obtained by transformation of EOY7 with *Kpn*I–*Xba*I-digested pARS2004ΔI, ΔII, ΔIII, ΔIΔII, ΔIIΔIII, ΔIΔIII (Okuno *et al*., 1999), p2004I:inv and p2004I:invΔIII (see below) plasmids, and *ura*$^-$ transformants were selected on 5-fluoro-orotic acid (5-FOA)-containing plates. In every case, integration was confirmed by Southern hybridization. p2004I:inv and p2004I:invΔIII were constructed as described below. The 3 kb *Kpn*I–*Xba*I fragment of pARS2004ΔI was cloned into pBluescriptII SK+. Region I oligonucleotides 5′-GTTAAAAAAAATTAAAAATTAACAAAAAAAAAAAAAAAAAACCTGCA-3′ and 5′-GGTTTTTTTTTTTTTTTTTTGTTAATTTTTAATTTTTTTAACTGCA-3′ were annealed, and inserted into the *Sse*8387I site in the plasmid, resulting in p2004I:inv. For construction of p2004I:invΔIII, a 0.5 kb *Bam*HI–*Hin*dIII fragment of pARS2004ΔIII was cloned into *Bam*HI–*Hin*dIII sites of p2004I:inv. EOY68, 70, 72, 75, 79, 85, 87, 90, TTY78, 81, 84, 95 and 97 were obtained by a standard genetic cross of the *ori2004* mutant strains with *cdc25-22* (Fantes, 1979), *cdc10-129* (Nurse *et al*., 1976) and/or *nda3-KM311* (Hiraoka *et al*., 1984) strains.

Construction of plasmids

Construction of the Cdc18 expression vector pREP82-H6cdc18 was as described below. The N-terminal region of Cdc18 on pREP3x-cdc18 (Kelly *et al*., 1993) was PCR amplified with a His tag and the *Nde*I site using primers 5′-AAGGATCCATATGCACCACCACCACCACCACTGTGAAACTCCAATAGGT-3′ and 5′-TGTGGGTGTTTGGAAATG-3′, and the products were digested with *Nde*I and *Pst*I. The resulting *Nde*I–*Pst*I fragment containing the N-terminal region of Cdc18 together with the *Pst*I–*Bam*HI fragment containing the C-terminal region of *cdc18* derived from pREP3x-cdc18 was inserted into *Nde*I–*Bam*HI sites of pREP82 (Basi *et al*., 1993), resulting in pREP82-H6cdc18. H6Cdc18 is fully functional because ectopic expression of H6Cdc18 complemented *cdc18-K46* mutation at 37°C. Construction of the Cdt1 expression vector pREP81-cdt1 will be described elsewhere.

Preparation of chromatin-enriched fractions and immunoblotting

Chromatin-enriched fractions were prepared as described previously, with some modification (Ogawa *et al*., 1999). Spheroplasts resuspended in HNG buffer [10 mM HEPES–NaOH pH 7.2, 50 mM NaAc, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 μg/ml leupeptin, 50 μg/ml TLCK, 2 μg/ml aprotinin] at a concentration of 10^8 cells/ml were lysed by addition of Triton X-100 at a final concentration of 1% for 5 min on ice, and the insoluble fraction was recovered by centrifugation for 15 min at 20 000 *g*, washed and incubated at 20°C for 20 min in HNG buffer containing 150 mM NaCl, 1.5 mM $Mg(Ac)_2$ and 2800 U/ml DNase I (TaKaRa). The DNase I-soluble fraction was separated from the insoluble fraction by centrifugation for 15 min at 20 000 *g*. Immunoblotting was performed as described previously (Takahashi and Masukata, 2001), with affinity-purified rabbit anti-Mcm6, anti-Mcm2 antibodies or mouse anti-FLAG M2 antibody (Kodak) at 1:1000, 1:1000 and 1:3000 dilutions, respectively.

ChIP

ChIP was accomplished as described previously (Ogawa *et al*., 1999).

PCR amplification with ampli-Taq Gold (Perkin-Elmer) was performed in 30 μl of PCR buffer containing 1.5 mM $MgCl_2$ (Perkin-Elmer) with each set of primers, and immunoprecipitated DNA or total DNA. Immunoprecipitated DNA was used at dilutions of 1/1500–1/192 000, while total DNA was at dilutions of 1/800 000–1/102 400 000 as templates. The nucleotide sequences of the primers used are listed in Table II. All were used at a concentration of 0.2 μM. PCR products were separated in 3% agarose gels and visualized with 0.5 μg/ml ethidium bromide. The gel images obtained with a charge-coupled device camera (LAS1000+; Fujifilm) were processed using Image Gauge software (Fujifilm). Recovery (%) was calculated from the linear range of PCR products with the equation:

$$\text{Recovery (\%)} = \text{immunoprecipitated DNA/total DNA} \times 100$$

Neutral/neutral two-dimensional gel electrophoresis

Neutral/neutral two-demensional gel electrophoresis was performed as described previously (Okuno *et al*., 1997).

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