Interaction of fission yeast ORC with essential adenine/thymine stretches in replication origins

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Abstract

Background: Eukaryotic DNA replication is initiated from distinct regions on the chromosome. However, the mechanism for recognition of replication origins is not known for most eukaryotes. In fission yeast, replication origins are isolated as autonomously replicating sequences (ARSs). Multiple adenine/thymine clusters are essential for replication, but no short consensus sequences are found. In this paper, we examined the interaction of adenine/thymine clusters with the replication initiation factor ORC.

Results: The SpOrc1 or SpOrc2 immunoprecipitates (IPs) containing at least four subunits of SpORC, interacted with the ars2004 fragment, which is derived from a predominant replication origin on the chromosome. SpORC-IPs preferentially interacted with two regions of the ars2004, which consist of consecutive adenines and AAAAT repeats and are essential for ARS activity. The nucleotide sequences required for the interaction with SpORC-IPs correspond closely to those necessary for in vivo ARS activity.

Conclusion: Our results suggest that the SpORC interacts with adenine/thymine stretches, which have been shown to be the most important component in the fission yeast replication origin. The presence of multiple SpORC-binding sites, with certain sequence variations, is characteristic for the fission yeast replication origins.

Introduction

DNA replication is initiated from specific sites on chromosomes called replication origins. The interaction of an initiator protein with a specific sequence near the replication origin is required for sequential assembly of other replication factors at the replication origins on chromosomes of bacteria, animal viruses and budding yeast.

In the budding yeast Saccharomyces cerevisiae, replication origins have been isolated as chromosome fragments that can replicate autonomously (ARSs). ARSs are composed of an 11 base pair (bp) ARS consensus sequence (ACS) and several additional elements within about 150 bp (Broach et al. 1983; Van Houten & Newlon 1990; Marahrens & Stillman 1992). The ACS is the site for ATP-dependent binding of a six-protein complex called the origin recognition complex (ORC) (Bell & Stillman 1992). A single base alteration in the ACS abolishes both the interaction with ORC and the ARS activity (Bell & Stillman 1992). Thus, a highly specific interaction between ORC and the ACS is essential for replication. This interaction is a prerequisite for the loading of Cdc6 and minichromosome maintenance (MCM) proteins, and the resulting complex on the origin is called the prereplicative complex (pre-RC) (Difflrey et al. 1994; Liang et al. 1995; Cocker et al. 1996; Aparicio et al. 1997; Donovan et al. 1997; Tanaka et al. 1997).

Establishment of pre-RCs during G1 and their absence in the G2 phase is thought to be crucial for the regulation of a single round of DNA replication in a cell cycle.

Replication factors, including ORC, Cdc6 and MCM, are conserved among eukaryotic species (Dutta & Bell 1997). However, the primary structures of the regions required for initiation of chromosome replication differ greatly. Short essential sequences like the budding yeast ACS have not been found in other species (DePamphilis 1996, 1999; Gilbert 1998). For initiation of replication from the human β-globin origin, the Locus Control Region (LCR), located about 50 kb away from the origin, is required, suggesting that distant chromosome regions are involved in the initiation of replication (Aladjem et al. 1992).
In Drosophila melanogaster, amplification of the chorion gene cluster requires the AER-d (orß), where the amplification is initiated (Delidakis & Kafatos 1989; Heck & Spradling 1990), and the 440 bp ACE3 element located about 1.5 kb from the AER-d (Orr-Weaver et al. 1989). The ACE3 and AER-d do not contain any short consensus sequence. Therefore, the structures of replication origins in metazoans are very different from those in budding yeast. Recently, it was shown that Drosophila ORC (DmORC) localizes at ACE3 and AER-d during amplification of the chorion gene cluster and that purified DmORC interacts with both ACE3 and AER-d in vitro (Austin et al. 1999). However, the sequence or structure required for the interaction with ORC has not been identified and the recognition of replication origins is still unclear.

In the fission yeast Schizosaccharomyces pombe, replication origins have been isolated as ARSs that are larger than several hundred base pairs (Maundrell et al. 1985, 1988; Johnston & Baker 1987; Olsson et al. 1993; Cadle & Calos 1994; Dubey et al. 1994; Clyne & Kelly 1995; Okuno et al. 1997). However, a short essential sequence, like the ACS in the budding yeast, has not been found in such ARSs. Instead, adenine or thymine clusters located asymmetrically on one strand are required for the ARS activity (Dubey et al. 1994; Zhu et al. 1994; Clyne & Kelly 1995; Kim & Huberman 1998; Okuno et al. 1999). We have previously identified three essential regions in ars2004 that functions as a predominant replication origin on the chromosome II (Okuno et al. 1997). Regions I and III, 40 and 65 bp, respectively, consisting of extensive adenines on one strand, are more important for ARS activity than the 125 bp region II (Okuno et al. 1999). Substitution of region I or region III with synthetic sequences has shown that consecutive adenines but not AT alternates are required for ARS activity (Okuno et al. 1999). Thus, it is likely that DNA sequence with adenine stretches may participate in interactions with initiation factors rather than in the destabilization of the DNA duplex at the replication origin.

We have previously shown, using chromatin DNA immunoprecipitation after in vivo crosslinking (ChIP method), that SpOrc1, a subunit of SpORC, is preferentially located at chromosomal replication origins (Ogawa et al. 1999). In ars2004, SpOrc1 has been shown to localize at the central 1 kb region containing three essential regions. SpORC, which is composed of six subunits, SpOrc1–6 (Moon et al. 1999), may interact with certain sequence elements in the replication origins. SpOrc1, 2, 4 and 5 have been shown to be essential for viability (Muzzi-Falconi & Kelly 1995; Leatherwood et al. 1996; Chuang & Kelly 1999; Lygerou & Nurse 1999; Kiely et al. 2000). The N-terminal region of SpOrc4, which contains AT-hook motifs, is not conserved in other species (Chuang & Kelly 1999). The AT-hook motifs in high mobility group (HMG) family proteins are thought to be responsible for interaction with AT-rich DNA (Maher & Nathans 1996). An N-terminal polypeptide of SpOrc4 has been shown to bind to the ars1 fragment in vitro (Chuang & Kelly 1999). These results suggest that the SpORC interacts with the replication origins. In order to understand the molecular mechanism of initiation of replication in fission yeast, it is important to determine the DNA sequence that interacts with the initiation factors, such as ORC.

To determine DNA elements that interact with SpORC, we developed an in vitro system using protein complexes containing ORC subunits prepared by a conventional immunoprecipitation from fission yeast cell extracts. The SpORC immunoprecipitates (IPs) preferentially interacted with ARS fragments including the ars2004. Interestingly, SpORC-IPs interacted with essential regions I and III of the ars2004 but not with region II or non-ARS DNA. The sequences required for in vitro interaction with SpORC-IPs are the same as those necessary for the in vivo ARS activity. These results imply that fission yeast replication origins are recognized through SpORC-interaction with adenine/thymine stretches.

**Results**

**Co-immunoprecipitation of SpORC subunits**

To prepare ORC complexes from fission yeast cells, the chromosomal *orp1* gene that encodes SpOrc1 was replaced with the *orp1* carrying five FLAG tags at the N-terminus. The *orp1*-FLAG strain grew normally, indicating that the FLAG-tagged SpOrc1, designated as SpOrc1F, was functional. We employed conventional immunoprecipitation to prepare protein complexes containing SpOrc1F, which may retain the activity of in vivo ORC complex.

We first examined whether SpOrc1F was associated with other subunits of SpORC. Immunoprecipitates prepared from the *orp1*-FLAG or an untagged wild-type strain with anti-FLAG antibody were separated by SDS-PAGE and analysed by immunoblotting with anti-FLAG antibody or polyclonal antibodies against SpOrc1, SpOrc2, SpOrc4 and SpOrc5. In the IPs from
the orp1-FLAG strain, anti-FLAG and anti-SpOrc1 antibodies detected the SpOrc1F with an apparent molecular mass of 93 kDa (Fig. 1B, lane 2), which was consistent with the predicted molecular weight of SpOrc1F. In addition, SpOrc2, SpOrc4 and SpOrc5 with apparent molecular masses of 63, 110 and 49 kDa, respectively, were detected in the IPs from the orp1-FLAG strain (Fig. 1, lane 2). None was found in the immunoprecipitates from the untagged strain (Fig. 1, lane 1), indicating that these ORC subunits were associated with SpOrc1F. We carried out a reciprocal experiment using a strain in which the native orp2+ gene that encodes SpOrc2 was replaced with orp2-FLAG. As expected, the IPs prepared from the orp2-FLAG strain contained all ORC subunits examined (Fig. 1, lane 3). These results show that our ORC-IPs contained at least SpOrc1, 2, 4 and 5.

Specific binding of SpORC-IPs to DNA replication origins

The scheme of an in vitro system to examine whether the fission yeast ORC interacts with replication origins is shown in Fig. 2A. The ORC-IPs prepared from the orp1-FLAG strain was incubated with a set of DNA fragments in the presence of ATP and Mg2+. The DNA substrates labelled with 32P at the 5' ends were prepared from plasmid pARS2004M by digestion with restriction enzymes generating 3.2, 1.2, 1.0 and 0.8 kb fragments. The 1.0 kb fragment contained a 940-bp minimum ARS fragment of the ars2004 (Okuno et al. 1999). As shown in Fig. 2B, the SpOrc1-IPs specifically bound to the 1.0 kb ars2004 fragment but not to the other fragments, which had been derived from the vector (Fig. 2B, lane 2). None of the fragments bound to the IPs from the untagged strain (Fig. 2B, lane 1), to the IPs that had been prepared in the absence of the anti-FLAG antibody (Fig. 2B, lane 3), or in the presence of FLAG peptide, which competed for the recovery of SpOrc1F (Fig. 2B, lane 4). In addition, the specific interaction of SpOrc2-IPs with the ars2004 was observed by using the orp2-FLAG strain instead of the orp1-FLAG strain (Fig. 2C). These results show that the ars2004 fragment selectively binds to the ORC-IPs.

We then examined the interaction of ORC-IPs with other ARS fragments. It has been shown that ars2002 and ars1 are utilized as replication origins less frequently than ars2004 (Okuno et al. 1997; Okuno et al. 1999). We employed competition experiments to detect the interaction of ORC-IPs with ars2004 or ars1. Unlabelled DNA fragments, either 1 kb ars2004, 1 kb ars2002, 1.2 kb ars1 or 1 kb non-ARS fragment derived from orp4+ ORF, together with the labelled pARS2004M fragments, were added to the binding reaction. By the addition of a 50-fold excess of unlabelled ars2004 fragment, the binding of labelled ars2004 fragment to SpORC-IPs was decreased to about 10% of the value in the absence of the competitor (Fig. 3A, lane 2 and Fig. 3B). The ars2004 fragment bound to the ORC-IPs reduced to
about 35% or 15% by the addition of a 50-fold excess of unlabelled \textit{ars}2002 or \textit{ars}1, respectively (Fig. 3A, lanes 5 and 8). On the other hand, binding was not significantly affected by a 250-fold excess of a non-ARS fragment (Fig. 3A, lane 12 and Fig. 3B). These results show that ORC-IPs interact preferentially with ARS fragments. The ORC-IPs appear to bind to \textit{ars}2004 more efficiently than \textit{ars}2002 or \textit{ars}1.

To examine whether the \textit{in vitro} activity to interact with the ARS fragment depends on SpORC, we constructed two deletion mutants, \textit{orp}1-N390 and \textit{orp}1-N226, lacking approximately one-half and two-thirds of SpOrc1 from the C-terminus, respectively. Only two viable spores were yielded by tetrad analysis of a diploid \textit{orp}1\textsuperscript{+}/\textit{orp}1\Delta strain carrying the \textit{orp}1-N390 or \textit{orp}1-N226 plasmid, indicating that these deletions impaired the essential function of \textit{orp}1 (data not shown). Plasmid carrying the full-length \textit{orp}1\textsuperscript{+}-FLAG or its deletion derivatives with FLAG tags at the N-terminus was introduced into the wild-type haploid strain and FLAG-IPs prepared from the extracts were used for the DNA binding reaction. The \textit{ars}2004 fragment specifically bound to the full-length Orc1F-IPs, while the Orc1N390-IPs or Orc1N226-IPs did not bind to the \textit{ars}2004 fragment (data not shown). These deletion derivatives of Orc1 were defective in ORC complex formation, because SpOrc4 or SpOrc5 was not detected by Western blotting of Orc1N390-IPs and Orc1N226-IPs (data not shown). These results show that the C-terminal deletions of SpOrc1 abolish the ORC complex formation and/or impair interaction with the ARS fragment.

**ATP stimulates binding of SpORC-IPs to the ARS fragment**

It has been shown that interaction of the budding yeast ORC with the ARS fragments requires ATP but not its hydrolysis (Klemm et al. 1997). We examined the requirement of ATP for the binding of SpORC-IPs to the \textit{ars}2004 fragment. SpOrc1-IPs were incubated with labelled DNA fragments in the presence or absence of ATP or ATP-γ-S, a poorly hydrolysable analogue of ATP. When ATP was omitted from the reaction mixture, the amount of the \textit{ars}2004 DNA bound to the ORC-IPs was reduced to about one-third of that

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**Figure 2** \textit{In vitro} binding of SpORC-IPs to the \textit{ars}2004. (A) Scheme of the \textit{in vitro} ORC-ARS binding assay. Fission yeast ORC was immunoprecipitated from \textit{orp}1-FLAG cell extracts using anti-FLAG antibody conjugated with magnetic beads. The \textsuperscript{32}P-labelled DNA fragments containing the minimum ARS fragment of the \textit{ars}2004 (1.0 kb) and vector fragments (3.2, 1.2 and 0.8 kb) were incubated with the IPs, and the DNA bound to IPs was analysed by agarose gel electrophoresis followed by autoradiography. Mag: Magnet F; FLAG-epitope. (B) Immunoprecipitates were prepared from untagged wild-type (lane 1), \textit{orp}1-FLAG (lanes 2, 3 and 4) strains with anti-FLAG antibody (lanes 1, 2 and 4), without the antibody (lane 3) or in the presence of FLAG-peptide (lane 4). The \textsuperscript{32}P-labelled pARS2004M fragments (lane M) were incubated with IPs and the DNA bound to IPs was analysed by agarose gel electrophoresis. The position of the \textit{ars}2004 fragment is shown by the arrow. The recovery of the ARS fragment by immunoprecipitation ranges 0.3–3.0% of the input in similar experiments. The lower panel shows immunoblots with the anti-FLAG antibody. (C) The same sets of experiments were carried out as in (B), except IPs from the \textit{orp}2-FLAG strain were used (lanes 2, 3 and 4).
obtained in the presence of ATP (Fig. 4A, lane 2 and
Fig. 4B). The ARS binding activity of ORC-IPs in the
absence of ATP is presumably not due to the
contamination of ATP from cell extract, because
preparation of SpORC-IPs in the presence of apyrase
that degrades ATP to ADP and AMP did not affect the
efficiency of binding without ATP (data not shown).
When ATPγS was added instead of ATP, binding of
the ars2004 fragment to the ORC-IPs was stimulated
to a level corresponding to about two-thirds of that in
the presence of ATP (Fig. 4A, lane 3). These results
suggest that the interaction of SpORC with the ARS
fragment is stimulated by the presence of adenine
nucleotide.

SpORC-IPs interacts with essential regions of
the ars2004

We have previously identified three regions essential for
autonomous replication of the ars2004 (Okuno et al.
1999). The 40 bp region I and 65 bp region III are
composed of highly clustered adenine/thymine
stretches, while the 125 bp region II does not contain
a long adenine stretch (Fig. 5A). To test the possibility
that the SpORC interacted with these essential regions,
unlabelled DNA fragments containing one of these
essential regions were added as competitors to the
reaction mixture for binding of SpORC-IPs to the
ars2004 fragment. By addition of the region I fragment
at a 500-fold excess to the labelled ars2004 fragment,
the amount of ars2004 DNA bound to the SpORC-
IPs was reduced to less than 10% of that in the absence
of the competitor (Fig. 5B, lanes 1–4 and Fig. 5C).
The addition of the region III fragment at 100-fold
excess abolished the binding of the ars2004 DNA to
SpORC-IPs (Fig. 5B, lanes 9–12). In contrast, the
region II fragment did not affect the binding reaction at
the concentrations tested (Fig. 5B, lanes 5–8). These
results show that SpORC-IPs interacts with regions I
and III but not region II of ars2004.

Sequence requirements for SpORC interaction

Although both regions I and III of the ars2004 contain
adenines and thymines asymmetrically present on one
strand, their sequences are significantly different from
each other (Fig. 5A). Region I contains long adenine/
thymine tracts, while region III is composed of repeats
of TTTTA/AAAAT. We asked whether the SpORC-
IPs interacted with specific sequences or simple AT-
rich sequences, by testing the effects of various added
To study the interaction of fission yeast origin recognition complex (ORC) with its replication origins, double-stranded oligonucleotides were used as competitors in binding assays. The oligonucleotides used were A40/T40, (AAAT/TTTA)10, (AAT/TTA)13, and (AT/TA)20, which share the same AT content. These were tested for their ability to compete with the labeled ars2004 fragment. The results showed that the addition of A40/T40 at a 400-fold excess reduced the binding of the ARS fragment to SpORC-IPs to about 10% of the level in the absence of the competitor. In contrast, the addition of (AT/TA)20 fragment did not show significant competition, suggesting that interruptions with guanine or cytosine were inhibitory for the interaction. Furthermore, (AAAC/TTTG)10 addition did not show significant competition, indicating that guanine or cytosine interruptions were inhibitory. Addition of unlabelled single-stranded dA40 or dT40 at a 400-fold excess did not affect the binding reaction, showing that SpORC-IPs interacted preferentially with double-stranded DNA.

**Figure 4** Effects of ATP and ATP-γ-S on ARS binding to ORC-IPs. The ORC-IPs were incubated with the 32P-labelled pARS2004M fragments (lane M) in the presence of 2 mM ATP (lane 1), 2 mM ATP-γ-S (lane 3) or in the absence of nucleotide (lane 2). (B) Relative amounts of the ars2004 fragment bound to SpOrc1-IPs as determined in Fig. 3, are shown by columns.

In contrast, the (AT/TA)20 fragment did not compete for binding of the ars2004 fragment at the concentrations examined (Fig. 6A, lanes 10–12). These results showed that SpORC-IPs interacted with continuous adenines/thymines but not with AT alternates. Furthermore, (AAAT/TTTA)10 yielded competition to almost the same extent as with A40/T40 (Fig. 7A, lanes 4–6), while (AAT/TTA)13 reduced the binding to about 30% of the level in the absence of the competitor (Fig. 6A, lanes 7–9). Therefore, three or more consecutive adenines are required for efficient interaction with SpORC-IPs. The addition of (AAAC/TTTG)10 at a 400-fold excess did not show significant competition, suggesting that interruptions with guanine or cytosine were inhibitory for the interaction (data not shown). Addition of unlabelled single-stranded dA40 or dT40 at a 400-fold excess did not affect the binding reaction (data not shown), showing that SpORC-IPs interacted preferentially with double-stranded DNA. From the results described above, we concluded that specific sequences composed of Aₙ(A/T), where n ≥ 3, are required for interaction with SpORC-IPs.

**Discussion**

Using an *in vitro* assay system, we have demonstrated that fission yeast ORC-IPs interact with the ars2004 fragment, which is derived from a predominant replication origin on fission yeast chromosome. In addition, the SpORC-IPs interact with several other ARSs but not with non-ARS fragments, suggesting that the nucleotide sequence or the structure specific to the replication origins is recognized by SpORC. SpORC-IPs interact with two essential regions in the ars2004, composed of highly clustered adenine/thymine stretches. Competition experiments with a synthetic sequence reveal that SpORC-IPs interact with consecutive adenines/thymines with certain sequence variations. Our results provide a clue to understanding the recognition of replication origins by initiator proteins in most eukaryotes, which do not contain short consensus sequences.

We have previously shown that the SpOrc1 is associated with replication origins throughout the cell cycle (Ogawa *et al.* 1999). Thus, the SpOrc1 is considered as a component of the complex that interacts with a certain sequence or structure in the replication origins. Fission yeast ORC is composed of six subunits, as has been shown for the budding yeast ORC as well as its homologues in *Drosophila* and human (Tugal *et al.* 1998; Chesnokov *et al.* 1999; Moon *et al.* 1999; ...
The results of immunoprecipitation from the *orp1-FLAG* or *orp2-FLAG* cell extracts with anti-FLAG antibody show that the Orc1-IPs or Orc2-IPs contain at least four subunits, SpOrc1, SpOrc2, SpOrc4 and SpOrc5 (Fig. 1). It is not clear, at present, whether these IPs contain the remaining SpOrc3 and SpOrc6 subunits. We have demonstrated that both Orc1-IPs and Orc2-IPs bind to the *ars2004* fragment. FLAG-IPs prepared from an untagged strain do not contain the binding activity. Furthermore, deletions of C-terminal regions of SpOrc1 abolish both ORC complex formation and ARS binding. These results show that the ARS DNA binding activity is associated with the SpORC complex.

The results of competition experiments show that SpORC-IPs bind to regions I and III but not to region II (Fig. 5). Thus, the essential role of regions I and III for initiation of DNA replication appears to be the interaction with SpORC. Previously, we have shown that regions I and III are more important than region II in *in vivo* functional replacement studies (Okuno et al. 1999). This is consistent with the idea that the essential function of the replicator is to interact with the initiator protein. Interestingly, the sequences of regions I and III significantly differ from each other, although both are highly AT-rich (Fig. 5A). The results of competition with synthetic oligonucleotides suggest that the motif required for the interaction is represented by $A_n(A/T)$, where $n \geq 3$. We have previously shown that only some artificial sequences can substitute for region I of the *ars2004* in an *in vivo* ARS assay (Okuno et al. 1999). $A_{40}/T_{40}$ and $(AAAT/TTTA)_{10}$ can substitute for region II.
I without decreasing ARS activity. Replacement with (AAT/TTA)$_{13}$ reduced the ARS activity to about one-tenth, while substitution with (AT/TA)$_{20}$ completely abolished the ARS activity (Table 1). The sequences required for \textit{in vivo} ARS activity are in good agreement with those required for the \textit{in vitro} interaction with SpORC-IPs (Table 1). Thus, the results presented here strongly suggest that the \textit{in vitro} interaction of ORC-IPs with adenine/thymine stretches in \textit{ars2004} represents the essential function of ORC in the initiation of DNA replication from the origin. Other ARS fragments, such as \textit{ars1}, \textit{ars3002}, \textit{ars3001}, \textit{ars2002} and \textit{ars2003}, contain clustered adenine/thymine stretches in the regions required for ARS activity (Dubey \textit{et al}. 1994; Zhu \textit{et al}. 1994; Clyne \\& Kelly 1995) (Y. Yamada and H. Masukata, unpublished results). These adenine/thymine stretches might be binding sites for SpORC. In fact, SpORC-IPs interact with replication origins such as \textit{ars2002} and \textit{ars1} (Fig. 3). This is consistent with the above conclusion that the interaction of SpORC with adenine/thymine stretches in the replication origins is

\begin{table}[!h]
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\begin{tabular}{|l|c|c|c|c|}
\hline
Sequence & A$_{40}$/T$_{40}$ & (AAAT/TTTA)$_{10}$ & (AAT/TTA)$_{13}$ & (AT/TA)$_{20}$ \\
\hline
\textit{ARS activity}\textsuperscript{a} & ++ & ++ & + & – \\
\textit{In vitro binding}\textsuperscript{b} & ++ & ++ & + & – \\
\hline
\end{tabular}
\caption{Sequence specificity for \textit{in vitro} ORC binding and \textit{in vivo} ARS activity}
\end{table}

\textsuperscript{a} The ++ and + signs show ARS activity as, and high as 1/10 of that of pARS2004M, respectively (Okuno \textit{et al}. 1999). The – sign shows no ARS activity.

\textsuperscript{b} The ++ and + signs show the extents of interaction with ORC-IPs as efficient as that with the region I fragment and the intermediate level, respectively, obtained from results of the competition experiments shown in Fig. 6.
essential for replication in fission yeast. It should be noted that SpORC-IPs bind to *ars2004* more efficiently than *ars2002* and *ars1* (Fig. 3) and this is consistent with the *in vivo* ARS activity (Okuno *et al*. 1997, 1999). Thus, the efficiency of utilization of the origins appears to depend on the affinity with ORC. On the other hand, the efficiency of initiation of replication is also affected by certain other regions that do not interact with SpORC-IPs, such as region II of *ars2004*. Fission yeast replication origins are thus composed of ORC-binding sites and additional functional elements.

The *in vitro* results show that SpORC-IPs can bind to both regions I and III, which are several hundred bp distant in the *ars2004*. These results raise the possibility that multiple ORC molecules bind to the *ars2004* origin *in vivo*. Our previous results from a ChIP analysis are consistent with this possibility, although the exact ORC-binding sites cannot be determined by this method (Ogawa *et al*. 1999). Some other replication origins also contain multiple adenine/thymine stretches. In *ars1*, two segments—segments 1 and 9—which are important for ARS function, contain adenine/thymine stretches (Clyne & Kelly 1995). In *ars3001*, three essential regions named domains α, β and γ are composed of highly clustered adenine/thymine tracts (Kim & Huberman 1998). These results are consistent with the idea that the multiple ORC binding is required for function of some fission yeast replication origins. The ORC, Cdc6/Cdc18 and Cdt1 proteins are required for loading of MCM on to the chromatin (Ogawa *et al*. 1999; Nishitani *et al*. 2000) (T. Asahara & H. Masukata, unpublished results). A possible association of multiple SpORC molecules within a replication origin might stimulate the loading of MCM proteins on to the origin.

What molecular mechanism underlies the interaction of SpORC with adenine/thymine stretches? Single-stranded dA40 or dT40 oligonucleotides do not compete with the binding of SpORC-IPs with *ars2004* (data not shown), suggesting that SpORC interacts with adenine/thymine tracts in the double stranded DNA. Considering that a highly specific sequence is not required for interaction with SpORC-IPs, the minor groove of the DNA or DNA structure specific to adenine/thymine tracts might be recognized by SpORC. It has been shown that the N-terminal half of SpOrc4, which contains AT-hook motifs, binds to the *ars1* fragment (Chuang & Kelly 1999). The AT-hook motifs have been found in a variety of DNA binding proteins, including high mobility group protein (HMG-I), and are thought to be responsible for binding to the minor groove of AT-rich DNA (Maher & Nathans 1996). However, it is not known whether an AT-hook motif can interact preferentially with adenine/thymine tracts rather than simple AT-rich sequence. In contrast to binding of AT-hook motif to AT-rich DNA, which does not require ATP, the interaction of ORC-IPs with ARSs is stimulated by ATP (Fig. 4). The other ORC subunits may be required for stimulation by ATP or for a preferential interaction with adenine/thymine stretches rather than AT-altarates.

In higher eukaryotes, several well-characterized replication origins, such as the human β-globin locus, the DHFR locus in Chinese hamster and the chorion gene locus in *Drosophila* do not contain any short consensus sequence, although these origins contain AT-rich sequences (Aladjem *et al*. 1998). It has been shown by *in vivo* croslinking experiment that the *Drosophila* ORC complex (DmORC) is located at the replication elements, the ACE3 and AER-d regions, which are required for amplification of chorion gene loci in the follicle cells (Austin *et al*. 1999). These findings suggest that ORC, or a complex containing ORC subunits, preferentially interacts with at least some replication origins in metazoan cells. Although the DNA elements required for this association have not been identified, it might be possible that ORC preferentially interacts with AT-rich sequences in the replication origins. Alternatively, ORC alone does not specifically bind to the replication origins in higher eukaryotes, but in association with an additional factor containing AT-hooks, ORC may interact with AT-rich sequences in the replication origins. Such a factor might confer sequence dependency on ORC-DNA binding. Fission yeast Orc4 is thought to be a fusion protein with such a factor.

The sequence specificity of fission yeast ORC binding determined in this study has given an answer to the question why a clear consensus sequence is not found in fission yeast replication origins. Requirement of longer ARS fragments in fission yeast than those in budding yeast might be due to a requirement of multiple ORC-binding sites for efficient replication. It should be noted that this is the first case in which ORC binding sites other than those in budding yeast have been determined. Recently, the 'replication initiation point (RIP)' mapping method, which is used to determine the 5′-end of the leading strand, was developed (Gerbi & Bielinsky 1997). Using this method, the initiation points in the budding yeast ARS1 have been mapped proximally to the ORC binding site (Bielinsky & Gerbi 1999), suggesting that all the processes for initiation of DNA replication takes place around the ORC binding site. On the other
hand, using the same technique, the initiation point in the fission yeast *ars1* has been located at positions approximately 130 bp distant from a long adenine/thymine region (Gomez & Antequera 1999), which could be the binding site for SpORC. Using two-dimensional gel electrophoresis method, we have previously mapped the replication initiation region in the *ars2004* (Okuno et al. 1997). The initiation region is about 200 bp distant from both the regions I and III, to which the ORC-IPs binds in *in vitro*. These results suggest that certain reactions in the initiation process, such as pre-RC formation or loading of DNA polymerases, would occur at a place distant from the ORC binding sites. Thus, fission yeast replication origins, such as the *ars2004* ORC binding sites, would occur at a place distant from the regions I and III, which would involve a structural change in the DNA. Analyses of locations of initiation factors in the replication origins are important for understanding the precise mechanism of initiation of DNA replication in many eukaryotes.

**Experimental procedures**

**Strains and media**

*Schizosaccharomyces pombe* haploid strains used were 972 *h−*, 975 *h−* and HM123 *h−* *leu1-32* (Miyake et al. 1993) and JY741 *h−* *ade6-M216* *ura4-D18* *leu1-32* and JY746 *h−* *ade6-M210* *ura4-D18* *leu1-32*. They were cultured in a complete medium, YE (0.5% yeast extract, 3% glucose), and an Edinburgh minimal medium, EMM (Moreno et al. 1991). Media containing 2% agar were used for plating. Transformation of *S. pombe* was performed by electroporation (Hood & Stachow 1990).

**Construction of FLAG-tagged orp1 strain**

Construction of TTY15 *h−* *orp1::5xFLAG-orp1* was as described below. The N-terminal region of *orp1* on pKG9 (Gavin et al. 1995) was amplified by the polymerase chain reaction (PCR) using primers 5′-AACATATGGACTACAAGGACGACGATGACGAAAGGATACGCTGTCATCGTCGTCATCGTCGTCC-3′ containing a *NdeI* site and single FLAG epitope sequence, and 5′-CAATTTGCTTTTTCTGAC-3′, and the products were digested with *NdeI* and *HindIII*. The C-terminus of the gene was PCR amplified using primers 5′-TATGGACTCAAGGACGACGATGACGAAAGGATACGCTGTCATCGTCGTCATCGTCGTCC-3′ and 5′-TAAGCTTACGCTGTCATCGTCGTCATCGTCGTCC-3′, containing two copies of *FLAG*-epitope sequences, were inserted into the *NdeI* site of pTO5.7FLAG, resulting in pTO5.7FLAGS carrying a 5xFLAG epitope sequence fused with the coding region of the *orp1*. The region upstream of the initiation codon of the *orp1* gene of pKG9 was amplified by PCR using an M13 reverse primer and 5′-AACAATATGGACGATAAAGTCTAACCAC-3′. The 0.2 kb *NdeI-* *NdeI* fragment of the PCR products and the 1.9 kb *PstI*- *NdeI* fragment upstream of the *orp1* ORF prepared from pKG9, and the 2.7 kb *NdeI*- *NdeI* fragment from pTO5.7FLAGS were cloned into the *PstI*- *BamHI* sites of pBluescriptII SK +, resulting in an integration plasmid pTO7.8FLAGS. Integration of the *orp1*-*FLAG* gene into the chromosomal *orp1* locus was carried out as follows. In the first step, the chromosomal *orp1* gene was replaced with the *orp1* ORF fused with the thiamin-repressible *nmt** promoter. The *PstI* site of pREP81 (Basi et al. 1993) was changed into *NdeI* site by insertion of a *NdeI* linker. The 1.2 kb *NdeI*- *NdeI* fragment containing the *nmt** promoter and the *PstI*- *NdeI* fragment upstream of the *orp1* gene from pKG9 were cloned into the *PstI*- *NdeI* site of pBluescriptII SK +, to make pTO6.3. The 1.7 kb *nmt**-fragment, whose *HindIII* ends were changed into *NdeI* sites by linker ligation, was inserted at the *NdeI* site of pTO6.3, and then the 2.7 kb *NdeI*- *NdeI* fragment of pTO5.7FLAGS was integrated at the *NdeI*- *NdeI* sites of pTO6.3. The resulting pTO11.81 was digested with *PstI* and *ApaI*, and used for transformation of JY741. Ura + and thiamin sensitive transformants were selected and integration of the *nmt**-*orp1-*FLAG* gene at the *orp1* locus was confirmed by Southern blotting, resulting in TTY13. In the second step, TTY13 was transformed with pTO7.8FLAGS digested with *PstI* and *ApaI* and 5′-Fluoroorotic acid (5FOA) resistant transformants were selected. Integration of the region upstream of the *orp1* start codon was confirmed by Southern blotting. TTY15 carrying the *orp1*- *FLAG* gene without auxotrophy was obtained by crossing with 975 *h−*. The TTY15 grew as the wild-type strain, showing that the tagged protein is functional.

**Construction of FLAG-tagged orp2 strain**

The 0.5 kb fragment upstream of the initiation codon of *orp2* gene was amplified by PCR using a T7 primer and 5′-AAGGATCCATATGAGGATACGCTGTCATCGTCGTCATCGTCGTCC-3′ from the template pHS1-002 (H. Shima & H. Masukata, manuscript in preparation) which contained 2.7 kb *HindIII* fragment of pER27 (Leatherwood et al. 1993) at the *HindIII* site of pBluescriptII SK +. The PCR products digested with *HindIII* and *BamHI* were inserted at the *HindIII*- *BamHI* sites of pBluescriptII SK +, creating pBS-orp2up. The N-terminal region of the *orp2* gene was amplified from pHS1-002 using the M13 reverse primer and 5′-AAGGATCCATATGAGGATACGCTGTCATCGTCGTCATCGTCGTCC-3′, containing two copies of *FLAG*-epitope sequences, were inserted into the *NdeI* site of pTO5.7FLAG, resulting in pTO5.7FLAGS carrying a 5xFLAG epitope sequence fused with the coding region of the *orp1*. The region upstream of the initiation codon of the *orp1* gene was amplified by PCR using an M13 reverse primer and 5′-AACAATATGGACGATAAAGTCTAACCAC-3′. The 0.2 kb *NdeI-* *NdeI* fragment of the PCR products and the 1.9 kb *PstI*- *NdeI* fragment upstream of the *orp1* ORF prepared from pKG9, and the 2.7 kb *NdeI*- *NdeI* fragment from pTO5.7FLAGS were cloned into the *PstI*- *BamHI* sites of pBluescriptII SK +, resulting in an integration plasmid pTO7.8FLAGS. Integration of the *orp1*-*FLAG* gene into the chromosomal *orp1* locus was carried out as follows. In the first step, the chromosomal *orp1* gene was replaced with the *orp1* ORF fused with the thiamin-repressible *nmt** promoter. The *PstI* site of pREP81 (Basi et al. 1993) was changed into *NdeI* site by insertion of a *NdeI* linker. The 1.2 kb *NdeI*- *NdeI* fragment containing the *nmt** promoter and the *PstI*- *NdeI* fragment upstream of the *orp1* gene from pKG9 were cloned into the *PstI*- *NdeI* site of pBluescriptII SK +, to make pTO6.3. The 1.7 kb *nmt**-fragment, whose *HindIII* ends were changed into *NdeI* sites by linker ligation, was inserted at the *NdeI* site of pTO6.3, and then the 2.7 kb *NdeI*- *NdeI* fragment of pTO5.7FLAGS was integrated at the *NdeI*- *NdeI* sites of pTO6.3. The remaining pTO11.81 was digested with *PstI* and *ApaI*, and used for transformation of JY741. Ura + and thiamin sensitive transformants were selected and integration of the *nmt**-*orp1-*FLAG* gene at the *orp1* locus was confirmed by Southern blotting, resulting in TTY13. In the second step, TTY13 was transformed with pTO7.8FLAGS digested with *PstI* and *ApaI* and 5′-Fluoroorotic acid (5FOA) resistant transformants were selected. Integration of the region upstream of the *orp1* start codon was confirmed by Southern blotting. TTY15 carrying the *orp1*- *FLAG* gene without auxotrophy was obtained by crossing with 975 *h−*. The TTY15 grew as the wild-type strain, showing that the tagged protein is functional.
CAAGGACGACGATGACAAAGCTAATAAAACGAGCAGC-3’ containing a Ndel site and a FLAG epitope sequence. PCR products digested with BamHI and EcoRI were inserted at the BamHI-EcoRI sites of pBluescriptII SK+, resulting in pBS-orp2FL-NdEco. Four copies of FLAG epitope sequences were inserted at the Ndel site of the pBS-orp2FL-NdEco as described above, to make pBS-orp2FL-NdEco. The 0.3 kb Ndel-EcoRI fragment of pBS-orp2FL-NdEco and the 1.9 kb EcoRI-PstI fragment of pHS1-002 were inserted at the Ndel-PstI sites of pBS-orp2up, resulting in an Integration plasmid pBS-orp2FL5. For construction of the orp2Δ gene disrupted with ura4Δ, pBS-orp2FL5 was digested with Ndel and partially with EcoRI, followed by end-filling with T4 DNA polymerase. The resulting 4.1 kb fragment lacking orp2 ORF was ligated with the blunt-ended 1.7 kb HindIII fragment containing the ura4Δ gene. The resulting pBS-Δorp2 was digested with SpeI and SphI, and used for transformation of the JY741/JY746 diploid strain. Ura+ transformants were selected and the replacement of orp2 was confirmed by Southern hybridization. The Δorp2/orp2Δ heterodisruptant, TTY22, was transformed with pREP41-GST-orp2 (H. Shim & H. Masukata, manuscript in preparation) and the leu+ transformant was selected. Then, TTY23 h- ade6-M210 ura4-D18 leu1-32 orp2:: ura4Δ pREP41-GST-orp2 haploid strains were selected by tetrad analysis. After transformation of TTY22 with the fragment from pBS-orp2FL5. Among 5FOA resistant transformants, a strain carrying the orp2-FLAG gene without auxotrophy was obtained. TTY31 grew as the wild-type strain, indicating the tagged protein to be functional.

**Construction of orp1 deletion mutants**

The BamHI site within the orp1 deletion fragment of pARS2004M (Okuno et al. 2003) was disrupted by filling-in with the DNA polymerase I Klenow fragment and a linker fragment 5’-TCGAAGGATCCT-3’ was inserted at the XhoI site in the multicloning sites to create p940B, carrying a blunt BamHI site. The 4 kb BamHI fragment of pTO7.8FLAG5 containing orp1 ORF was cloned into the BamHI site of p940B, resulting in p940B-FLorp1. For construction of deletion mutants of orp1, the region downstream of the termination codon (inclusive) was amplified with PCR using primers 5'-AATCTAGAGCTCGAG AATAAAAGCTAATTATTTGG-3’ and 5’-AAGGATCCTT CGATTCGCTTCAATAAAT-3’, carrying the XhoI and Xhol sites. PCR products were digested with either XhoI and MdiI or XhoI and MluI, and fragments were inserted in place of the XhoI-MdiI or XhoI-MluI fragment of p940B-FLorp1, resulting in p940B-orp1-N226 lacking 482 amino acids and p940B-orp1-N390 lacking 318 amino acids from the C-terminus.

**Antisera**

To raise polyclonal antibodies against SpOrc1, SpOrc2 and SpOrc4, DNA fragments coding the 318–514 amino acid regions of SpOrc1, 1–175 amino acids of SpOrc2 and 1–329 amino acids of SpOrc4, with 6×his-tag at the N-termini, respectively, were made by PCR and cloned into the Ndel-BamHI sites of pET21a (Novagen). Peptides expressed in E. coli BL21(DE3) (Studier & Moffatt 1986) were used for immunization of rabbits essentially as previously described (Ogawa et al. 1999). Antiserum against SpOrc5 was donated by K. Tanaka. All antisera were affinity-purified with NHS-activated columns (Pharmacia) conjugated with the immunogen peptides as recommended by the manufacturers.

**Immunoprecipitation and immunoblotting**

Preparation of cell extracts and immunoblotting were carried out essentially as previously described (Ogawa et al. 1999). Briefly, 5 × 10⁸ cells suspended in 500 μL HB buffer (HB buffer (Moreno et al. 1991) supplemented with 150 mM NaCl) were disrupted with glass beads and 400 μL of cleared extract was obtained by centrifugation at 15000 g at 4 °C. Anti-FLAG antibody-associated magnetic beads were prepared by incubation of 0.5 mg anti-mouse IgG conjugated magnetic beads (Dynal) with 10 μg anti-FLAG M2 antibody (Sigma) for 10 h at 4 °C. The beads were incubated with the cleared extracts for 1 h at 4 °C and washed five times with HB buffer. For immunoblotting, the bound proteins were eluted with 50 μL of SDS-sample buffer (Laemmli 1970) and separated by SDS-PAGE. Proteins transferred on to PVDF membranes were probed with anti-FLAG M2, anti-SpOrc1, anti-SpOrc2, anti-SpOrc4 and anti-SpOrc5 antibodies at 1 : 1500, 1 : 200, 1 : 100, 1 : 1000 and 1 : 200 dilutions, respectively.

**In vitro ARS binding assay**

For preparation of probe DNA fragments, pARS2004M was digested with ApoLI, NotI and XhoI, dephosphorylated with calf-intestinal alkaline phosphatase, followed by phenol/chloroform extraction and ethanol precipitation. The DNA fragments were phosphorylated with T4 polynucleotide kinase (New England Biolabs) in the presence of [γ-³²P]ATP (0.925 MBq, > 4000 Ci/mmol, ICN) and purified by MicroBioSpin 6 column (Bio-Rad). For the ARS binding reactions, 10 ng of ³²P-labelled probe DNA was incubated for 30 min at 25 °C with proteins immunoprecipitated with anti-FLAG antibody-associated magnetic beads (equivalent to 0.15 mg magnetic beads) in 50 μL of reaction buffer containing 20 mM Tris-HCl (pH 8.0), 50 mM NaCl, 5 mM MgCl₂, 1 mM DTT, 0.1% NP-40, 1 mM PMSE, 100 ng/μL poly(dI-dC)-poly(dI-dC) (Pharmacia), 2 mM ATP, 20 mM creatine phosphate, 20 μg/mL creatine kinase. Magnetic beads were washed five times with a wash buffer containing 20 mM Tris-HCl (pH 8.0), 50 mM NaCl, 5 mM MgCl₂, 1 mM DTT, 0.1% NP-40, 1 mM PMSF and 0.5 mM ATP. The bound DNA fragments were eluted from magnetic beads by incubation for 10 min in 100 μL elution buffer containing 10 mM Tris-HCl (pH 8.0) and 500 mM NaCl and purified with phenol/chloroform extraction and ethanol precipitation. The DNA fragments were separated by 0.8%
agarose gel electrophoresis and analysed with the BAS2000 image analysing system (Fuji-film). In competition experiments, indicated amounts of unlabelled DNA fragments, together with $^{32}$P labelled probe DNA were added to the reaction mixture. DNA fragments used as competitors were 1 kb ars2004 Not-fXhol fragment prepared from pARS2004M (Okuno et al. 1999), 1 kb ars2002 SacI-HindIII fragment from pARS2002M (Y. Yamada and H. Masukata, unpublished results), 1.2 kb ars1 EcoRI fragment from pREPI (Maundrell 1990) and 1 kb non-ARS NdeI-BamHI fragment from pET21a-orp4N which contains the orp4 coding region corresponding to 1–329 amino acids. These fragments were prepared by restriction enzyme digestion followed by purification by agarose gel electrophoresis. Region I fragment were made by annealing an oligonucleotide

$$5'$$-GGTCCTACGTAaaaaaaaaatataataacaa aaaaaaaaaaaaaacgcacagtca-3'

with its complementary oligonucleotide. Region II and III fragments were PCR amplified using primers, 5'-AAAGGATCCCTAATTTTAA TTGGTTTTAAAAAG-3' and 5'-AAAGGATCCGTAAAAAA AAAATTAAAGTTAG-3' for Region II, and 5'-AAATCCTAG TTTATTTTTTTTTTTTTTTAGTAC-3' and 5'-AAAT CTCAGCTCAGAAAATAAATTTAA-3' for Region III. Oligonucleotides 5'-GGTCCTACGAGGCGCACAATCG-3', 5'-GGTCCTACG(AAT)10CGCACAGTCG-3', 5'-GGTCCTACG(AAT)10CGCACAGTCG-3', 5'-GGTCCTACG (AT)20CGCACAGTCG-3' and their complementary oligonucleotides were annealed and used as competitors.

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**References**


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