Multiple Mechanisms Contribute to *Schizosaccharomyces pombe* Origin Recognition Complex-DNA Interactions*

Christopher R. Houchens‡, Wenyan Lu‡, Ray-Yuan Chuang‡, Mark G. Frattini§, Alex Fuller‡, Pam Simancek‡, and Thomas J. Kelly‡

From the ‡Program in Molecular Biology and §Department of Medicine, Memorial Sloan-Kettering Cancer Center, New York, New York 10021

Eukaryotic DNA replication requires the assembly of multiprotein pre-replication complexes (pre-RCs) at chromosomal origins of DNA replication. Here we describe the interactions of highly purified *Schizosaccharomyces pombe* pre-RC components, SpORC, SpCdc18, and SpCdt1, with each other and with *ars1* origin DNA. We show that SpORC binds DNA in at least two steps. The first step likely involves electrostatic interactions between the AT-hook motifs of SpOrc4 and AT tracts in *ars1* DNA and results in the formation of a salt-sensitive complex. In the second step, the salt-sensitive complex is slowly converted to a salt-stable complex that involves additional interactions between SpORC and DNA. Binding of SpORC to *ars1* DNA is facilitated by negative supercoiling and is accompanied by changes in DNA topology, suggesting that SpORC-DNA complexes contain underwound or negatively writhe DNA. Puriﬁed human origin recognition complex (ORC) induces similar topological changes in origin DNA, indicating that this property of ORC is conserved in eukaryotic evolution and plays an important role in ORC function. We also show that SpCdc18 and SpCdt1 form a binary complex that has greater afﬁnity for DNA than either protein alone. In addition, both proteins contribute signiﬁcantly to the stability of the initial SpORC-DNA complex and enhance the SpORC-dependent topology changes in origin DNA. Thus, the formation of stable protein-DNA complexes at *S. pombe* origins of replication involves binary interactions among all three proteins, as well as interactions of both SpORC and SpCdt1-SpCdc18 with origin DNA. These ﬁndings demonstrate that SpORC is not the sole determinant of origin recognition.

The timely and faithful duplication of eukaryotic genomes involves the coordinated initiation of DNA replication from hundreds or thousands of chromosomal replication origins during S phase. The initiation reaction requires the assembly and activation of pre-replication complexes (pre-RCs) at replication origins, leading to the establishment of two replication forks that move in opposite directions (reviewed in Refs. 1–4). Pre-RC formation involves the selection of replication origins by the origin recognition complex (ORC), followed by ORC-dependent recruitment of Cdc6 and Cdt1. The resulting complex catalyzes the ATP-dependent loading of the heterohexameric minichromosome maintenance helicase complex onto DNA to establish a functional pre-RC.

Eukaryotic chromosomal origins were ﬁrst identiﬁed in the budding yeast *Saccharomyces cerevisiae* as isolated chromosomal DNA fragments capable of supporting the autonomous replication of extrachromosomal plasmids (5, 6). Budding yeast origins, most of which are utilized efﬁciently in their chromosomal context, are relatively small DNA segments comprised of a highly conserved and essential 11-bp ARS consensus sequence and auxiliary B elements that enhance origin efﬁciency (7–12). *S. cerevisiae* ORC binds in a sequence-speciﬁc and ATP-dependent manner to the ARS consensus sequence and B1 elements. Notably, mutations in these elements that reduce binding of *S. cerevisiae* ORC in vitro eliminate origin function in vivo (13, 14). The properties of *S. cerevisiae* origins suggested that the initiation of eukaryotic DNA replication might conform to the replicon hypothesis posited by Jacob et al. over 40 years ago (15).

However, work in the fission yeast *Schizosaccharomyces pombe* and metazoan cells suggests that the budding yeast paradigm may not be as generally applicable to all eukaryotes as ﬁrst imagined. Most important, conserved and essential ARS consensus sequence-like sequences have not been identiﬁed in eukaryotic replication origins outside of budding yeast. In fission yeast, both the size and AT content of origins, but not speciﬁc ARS consensus sequence-like sequences, contribute to origin activity (16–20). Similarly, in *Drosophila* and *Xenopus* embryos, virtually any DNA fragment is capable of supporting DNA replication (21–25), and in mammalian cells, DNA replication appears to initiate randomly from numerous chromosomal sites within large zones of initiation (26–30).

Consistent with these observations, both *S. pombe* and metazoan ORCs, although lacking sequence-speciﬁc DNA-binding activities, preferentially bind AT-rich DNA sequences, a common feature of both fission yeast and metazoan replication origins (24, 31). The *S. pombe* ORC subunit SpOrc4 possesses an N-terminal extension, also present in other Ascomycota Orc4 homologs, that is not found in budding yeast and metazoan Orc4 homologs. The N-terminal domain of fission yeast SpOrc4 is composed of nine AT-hook DNA-binding motifs.

---

*This work was supported, in whole or part, by National Institutes of Health Grant GM 50806 from the NIGMS (to T. K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 To whom correspondence should be addressed: 609 Rockefeller Research Laboratories, 430 East 67th St., New York, NY 10021. Tel.: 212-639-3264; Fax: 646-422-2189; E-mail: chouchens@sainc.com.

2 Present address: J. Craig Venter Inst., 9704 Medical Center Dr., Rockville, MD 20850.

3 The abbreviations used are: pre-RCs, pre-replication complexes; ORC, origin recognition complex; SpORC, *S. pombe* ORC; HsORC, *Homo sapiens* ORC.
that are necessary and sufficient for the DNA-binding activities of SpORC in vitro (31–37). Biochemical and structural studies have shown that AT-hook motifs bind the minor groove of short AT tracts. Proteins with repeated AT-hook motifs can bind in a multivalent manner to sequential AT tracts along DNA with nanomolar binding affinities (38–40). It is likely that some or all of the SpOrc4 AT-hook motifs target the SpORC holocomplex to AT-rich fission yeast chromosomal origins and that the relative DNA-binding affinity of SpOrc4 is a function of the total number and spatial arrangement of simple AT-core sequences distributed throughout an origin.

Because *S. pombe* and metazoan origins, unlike those of *S. cerevisiae*, do not appear to contain highly conserved sequence elements, it becomes important to define the factors that contribute to origin recognition in these species. We have examined the interactions of highly purified *S. pombe* ORC, Cdc18, and Cdt1 proteins with one another and with the fission yeast ars1 origin of replication. We have found that SpORC binds to ars1 DNA in at least two distinct steps. The first step results in formation of a salt-sensitive SpORC-DNA complex that is likely mediated by the AT-hook domain of SpOrc4. The second step involves the slow conversion of the salt-sensitive complex to a salt-resistant complex that involves additional non-electrostatic interactions between SpORC and DNA. We also found that negative supercoiling facilitates SpORC-DNA interactions, and as previously observed, that binding of SpORC induces topological changes in DNA consistent with untwisting or negative writhing. Finally, we show that SpCdt1 and SpCdc18 form a binary DNA-binding complex and that each protein facilitates SpORC-DNA interactions and SpORC-dependent changes in DNA topology. Our data demonstrate that SpORC is not the sole determinant of origin recognition, but that DNA structure and interactions with other pre-RC proteins also make major contributions.

**EXPERIMENTAL PROCEDURES**

**Expression and Purification of SpORC, SpCdc18, SpCdt1, and HsORC in Insect Cells**—Recombinant SpORC was purified essentially as described previously (31) with the following modifications. S9 insect cells were co-infected with five recombinant baculoviruses expressing SpORC subunits 1, 2, 3, 4, and 6 and a recombinant baculovirus expressing FLAG epitope-tagged SpORC5. After clarification of the salt-extracted chromatin-enriched pellet, the collected supernatant (12 ml) was incubated with 1 ml of anti-FLAG antibody-conjugated agarose (Sigma) at 4 °C for 2 h. The beads were washed twice with 10 ml of E buffer and once with 10 ml of F buffer (31). The M2-agarose-bound proteins were eluted by incubation with 1.5 ml of F buffer containing 0.4 mg/ml FLAG peptide (M2-agarose) (Sigma) at 4 °C for 2 h. The beads were washed twice with 10 ml of E buffer and once with 10 ml of F buffer (31). The M2-agarose-bound proteins were eluted by incubation with 1.5 ml of F buffer containing 0.4 mg/ml FLAG peptide at 4 °C for 2 h.

**DNA Binding Experiments**—For DNA-bead binding experiments, a biotinylated 1153-bp DNA fragment containing the ars1 origin was synthesized by PCR and coupled to streptavidin-conjugated magnetic beads as described previously (31). The binding assays were performed by incubating the indicated amounts of purified SpORC, SpCdc18, and SpCdt1 with beads containing 0.5 pmol of ars1 DNA in 25 μl of B buffer (50 mM Hepes-NaOH, pH 7.5, 60 mM or 500 mM NaCl, 1 mM EDTA, 1 mM EGTA, 5 mM magnesium acetate, 10% glycerol, 1 mM dithiothreitol) with 0.1 mg/ml bovine serum albumin, 1 mM ATP, and 1 × protease inhibitor mixture (Roche Applied Bioscience) for 30 min at 30 °C. Where indicated, 5 pmol of ars1 competitor DNA was included in the reaction mixtures. Beads were washed twice for 1 min each with 200 μl of B buffer containing 0.1% Nonidet P-40 (low salt wash) or B buffer containing 500 mM NaCl and 0.1% Nonidet P-40 (high salt wash). The bound proteins were released by the addition of 1 × SDS loading dye and separated by 10% SDS-PAGE, followed by Western blot analysis using rabbit polyclonal antibodies against SpOrc3, SpORC5, or SpCdt1. Anti-FLAG antibody (M2, Sigma) was used for detecting recombinant FLAG–SpCdc18.

For competition nitrocellulose filter binding experiments, a 1153-bp DNA fragment containing the ars1 origin was synthesized and radiolabeled with [α-32P]dATP by PCR. The binding assays were performed by incubating 50 fmol (2 nM) of purified SpORC with 12.5 fmol (0.5 nM) of radiolabeled ars1 DNA in 25 μl of B buffer with 0.1 mg/ml bovine serum albumin, 1 mM ATP, and 1 × protease inhibitor mixture for 30 min at 30 °C in the absence or presence of supercoiled and relaxed pARS1 plasmid competitor DNA. The reaction mixtures were passed through nitrocellulose filters (HA, Millipore Corp.), washed with 2 ml of low salt wash buffer, dried, and counted in a scintillation spectrometer.

**Topoisomerase I Assay**—The 3737-bp pPAR1 plasmid containing the 1153-bp ars1 origin DNA sequence was generated by removing a 2967-bp PstI-BamHI DNA fragment containing the LEU2 gene from pRC20 (41) and ligation of the product. The supercoiled and relaxed pARS1 plasmids used in Figs. 2B and 3B were generated by incubating 5 μg of pPAR1 for 2 h at 37 °C in the presence or absence of 60 units of topoisomerase I (Invitrogen). The plasmids were ethanol-precipitated and dissolved in Tris/EDTA buffer. The topoisomerase I assays were performed by incubating purified SpORC, SpCdc18, and SpCdt1 with 40 ng of pARS1 in 25 μl of B buffer with 0.1 mg/ml bovine serum albumin, 1 mM ATP, and 1 × protease inhibitor mixture for 10 min at 30 °C, followed by addition of 30 units topoisomerase I for 30 min at 37 °C. The reactions were stopped and deproteinized by addition of 5.5 μl of stop buffer (6% SDS, 30% glycerol, 10 mM EDTA, bromphenol blue, and
and incubation at 42 °C for 10 min. The samples were electrophoresed in 0.8% agarose in 0.5 M Tris/EDTA buffer lacking or containing 1 μg/ml chloroquine for 16 h at 45 V. Gels were washed three times for 20 min in 0.5 M Tris borate/EDTA and then stained with SYBR gold diluted 1:10,000 in 0.5 M Tris borate/EDTA for 2 h. The DNA was detected using a UV transilluminator.

RESULTS

SpORC Binds DNA in a Biphasic Manner

S. pombe ORC, Cdc18, and Cdt1 were purified from Sf9 insect cells infected with baculoviruses expressing FLAG epitope-tagged recombinant proteins (Fig. 1A). The purity of each protein preparation, as analyzed by silver staining of SDS-polyacrylamide gels, was determined to be >90%. In initial experiments, we made use of an ars1 DNA-bead binding assay to examine the formation and stability of SpORC-DNA complexes at different salt concentrations. In this assay, SpORC was incubated with an ars1 DNA fragment coupled to magnetic beads (ars1-beads) for 30 min in buffer containing either 60 mM NaCl (low salt) or 500 mM NaCl (high salt). The beads were collected and washed with either low salt buffer (lanes 2 and 4) or high salt buffer (lanes 3 and 5; ars1-beads were collected, washed with low salt buffer (lanes 2 and 3) or high salt buffer (lanes 4 and 5), and analyzed by immunoblotting for SpORC3. C. SpORC and ars1-beads were incubated for 1–30 min in low salt buffer (ars1-beads were collected, washed with low salt wash (LSW) buffer containing 60 mM NaCl (lanes 2–6) or high salt wash (HSW) buffer containing 500 mM NaCl (lanes 7–11), and analyzed by immunoblotting for SpORC5. D. SpORC and ars1-beads were incubated for 1 min in low salt buffer in the absence (lanes 3, 4, 6, and 7) or presence (lanes 2 and 5) of ars1 competitor DNA (comp), followed by the addition of ars1 competitor DNA (lanes 3 and 6) for an additional 30 min; ars1-beads were collected, washed with low salt wash buffer (lanes 2–4) or high salt wash buffer (lanes 5–7), and analyzed by immunoblotting for SpORC3. (Note that lane 1 in B–D is a loading control and represents a fraction equal to the input for all subsequent lanes).
These results suggested that SpORC can form two distinct complexes with origin DNA. To investigate this possibility further, we examined the kinetics of formation of salt-sensitive and salt-resistant complexes (Fig. 1C). For this purpose, SpORC was incubated with ars1-beads for 1–30 min in low salt buffer, followed by washing with buffer containing either a low or a high salt concentration. A significant fraction of SpORC bound to ars1 DNA within 1 min, but this complex was almost completely sensitive to dissociation by high salt (compare lanes 2 and 7). However, a second SpORC-DNA complex, resistant to washing with high salt, formed gradually and by 30 min accounted for most of the bound SpORC (compare lanes 6 and 11). Similar results were obtained when the reactions were carried out in the absence of ATP (data not shown). Thus, two SpORC-DNA complexes form with different kinetics: a salt-sensitive complex (complex I) that forms rapidly and presumably involves significant electrostatic interactions and a second, salt-resistant complex (complex II) that forms slowly and presumably involves additional non-electrostatic SpORC-DNA interactions. Complex II is quite stable, as no appreciable dissociation was observed in the high salt wash buffer during a 30-min incubation.

**SpORC-DNA Complex I Is a Precursor to Complex II**—There are two possible interpretations of the binding data described above: either the two SpORC-DNA complexes form independently of one another or complex I is an obligatory precursor to complex II. To distinguish between these two possibilities, we incubated SpORC with ars1 DNA-beads for 1 min in low salt buffer to allow complex I to form and then added competitor ars1 DNA to prevent further binding of SpORC to ars1 DNA-beads. If complex I is a precursor to complex II, then the presence of ars1 competitor DNA should not affect formation of the salt-stable complex II on the ars1 DNA-beads. Alternatively, if the two SpORC-DNA complexes form independently of one another, then addition of the competitor DNA should completely prevent formation complex II on the ars1 DNA-beads. We confirmed that the concentration of added ars1 competitor DNA was sufficient to prevent SpORC binding to ars1-beads when added to the binding reaction prior to SpORC (Fig. 1D, lanes 2 and 5). However, when this concentration of competitor DNA was added after formation of complex I, the amount of complex II formed in 30 min was completely unaffected (compare lanes 6 and 7). These results demonstrate that SpORC binds ars1 DNA to form an initial SpORC-DNA complex I that does not readily dissociate at low salt concentrations (compare lanes 3 and 4) and is slowly converted to the salt-stable complex II.

**SpORC Preferentially Binds Negatively Supercoiled DNA**—It has been reported that Drosophila melanogaster ORC has an affinity for negatively supercoiled plasmid DNA that is ~30-fold greater than its affinity for relaxed plasmid DNA (42). To determine whether this is the case for *S. pombe* ORC, we used a competition nitrocellulose filter DNA-binding assay to compare the relative binding affinities of SpORC for a negatively supercoiled or relaxed plasmid DNA containing the ars1 origin (pARS1) (Fig. 2A). In this assay, SpORC was incubated for 30 min with a radiolabeled ars1 DNA probe in the presence of increasing concentrations of unlabeled negatively supercoiled or relaxed pARS1, followed by filtration through a nitrocellulose filter. Under these conditions, the apparent binding affinity of SpORC for ars1 DNA was determined to be ~2 nM (data not shown), consistent with previously reported measurements (35). Negatively supercoiled pARS1 was a significantly more effective competitor for SpORC binding to the ars1 probe (Fig. 2B, blue line) than relaxed pARS1 DNA (red line). Binding of SpORC to the ars1 probe was reduced by ~50% in the presence of a 4-fold excess of negatively supercoiled pARS1 competitor DNA, whereas the addition of an ~50-fold excess of relaxed pARS1 competitor DNA was required for a similar reduction in SpORC binding to the ars1 probe. These results demonstrate that SpORC, like *D. melanogaster* ORC, has a significantly higher apparent affinity for negatively supercoiled plasmid DNA than for relaxed plasmid DNA. These results are highly reproducible and are consistent with the existence of a SpORC-DNA complex in which the bound DNA is structurally distorted, so as to induce local untwisting or negative writhe or both. In covalently closed circular DNA molecules, such a local change in DNA structure upon SpORC binding would induce compensatory positive writhe in the plasmid DNA, increasing the free energy change of the binding reaction relative to that of the binding reaction with topologically unconstrained (e.g. linear) DNA. In a negatively supercoiled DNA, the positive writhe cancels negative writhe, decreasing the relative free energy change for the reaction.

**SpORC and HsORC Alter the Structure of Bound DNA**—To verify that binding of SpORC can induce changes in the twist or writhe of bound DNA, we carried out topoisomerase relaxation

---

**FIGURE 2. SpORC preferentially binds negatively supercoiled DNA.** A, negatively supercoiled pARS1 (lane 1) and pARS1 relaxed with topoisomerase I (lane 2) were purified and analyzed on an agarose gel. B, SpORC (2 nM) was incubated with radiolabeled ars1 probe (0.5 nM) and the indicated fold excess amount of unlabeled negatively supercoiled (blue line) or relaxed (red line) pARS1 plasmid DNA for 30 min in low salt buffer containing 60 mM NaCl and analyzed by nitrocellulose filter binding.
assays. In these assays, pARS1 was incubated with increasing concentrations of SpORC to allow complex formation, and then topoisomerase I was added to relax unconstrained regions of the plasmid. Following deproteinization, the plasmid DNA was analyzed by native agarose gel electrophoresis in the absence (Fig. 3A, left) or presence (Fig. 3A, right) of chloroquine. We first confirmed previous observations (33) that binding of S. pombe ORC can alter the topology of negatively supercoiled plasmid DNA. Incubation with SpORC resulted in a shift in the topoisomer distribution toward lower mobilities when gel electrophoresis of the topoisomerase-treated DNA was carried out in the absence of chloroquine (Fig. 3A, left, compare lanes 2 and 6). Conversely, the topoisomer distribution shifted toward higher mobilities when gel electrophoresis was carried out in the presence of chloroquine (Fig. 3A, right). Similar results were obtained when the reaction mixtures lacked ATP (data not shown). Increasing the topoisomerase I concentration in the assay or the duration of the incubation with topoisomerase I did not alter the topoisomer distributions (data not shown), indicating that SpORC was not simply inhibiting the activity of topoisomerase I. The results shown in Fig. 3A, together with analysis of the distribution of pARS1 topoisomers by agarose gel electrophoresis in different concentrations of chloroquine (data not shown), demonstrated that the linking number of pARS1 DNA bound to SpORC was lower than that of free pARS1 DNA following relaxation with topoisomerase I. This observation is consistent with the hypothesis that the binding of SpORC can untwist DNA or stabilize left-handed negative writhe, perhaps via wrapping.

We next used the topoisomerase relaxation assay to examine the effect of SpORC binding on the topology of relaxed pARS1 plasmid DNA (Fig. 3B). Unlike supercoiled plasmid DNA (compare lanes 2 and 3), the relaxed plasmid DNA did not exhibit a change in topoisomer distribution as a result of incubation with increasing concentrations of SpORC (compare lanes 5 and 6). Thus, under the conditions of these experiments, negative supercoiling facilitates the change in DNA structure that accompanies SpORC binding.

Our data indicate that the properties of S. pombe ORC are similar to those of D. melanogaster ORC, which also binds preferentially to supercoiled DNA and alters the topology of supercoiled, but not relaxed, DNA. Thus, the ability to untwist or induce negative writhe may be a general property of ORC. To examine this possibility further, we carried out topoisomerase relaxation assays with highly purified recombinant human ORC (HsORC) (Fig. 3C). We used supercoiled pARS1 plasmid DNA in the topoisomerase assay and analyzed the plasmid DNA by agarose gel electrophoresis in the absence (Fig. 3C, left) or presence (Fig. 3C, right) of chloroquine. The results shown in Fig. 3C, together with analysis of the distribution of pARS1 topoisomers by agarose gel electrophoresis in different concentrations of chloroquine (data not shown), demonstrated that the linking number of pARS1 DNA bound to HsORC was lower than that of free pARS1 DNA following relaxation with topoisomerase I. This observation is consistent with the hypothesis that the binding of HsORC can untwist DNA or stabilize left-handed negative writhe, perhaps via wrapping.
DNA in these experiments because we have shown previously that HsORC has relatively little sequence specificity. The binding of HsORC to pARS1 DNA resulted in a shift in the topoisomer distribution (compare lanes 2 and 6), similar in magnitude to that observed with SpORC at similar concentrations (lane 7). Thus, the ability of ORC to alter DNA structure and topology is an evolutionarily conserved property of ORC that can be found in yeast, flies, and humans.

**SpCdc18 and SpCdt1 Facilitate SpORC-DNA Interactions**—We examined the interactions of purified SpORC, SpCdc18, and SpCdt1 with each other and with *ars1* DNA during the formation of salt-stable nucleoprotein complexes. For this purpose, we incubated various combinations of SpORC, SpCdc18, and SpCdt1 with *ars1*-beads for 10 min and then washed the beads with high salt (500 mM NaCl) buffer (Fig. 4A). In the absence of the other proteins, a small amount of SpCdt1 bound to the *ars1* DNA (lane 6). Under the same conditions, no DNA binding by SpCdc18 was observed (lane 5). When the two proteins were incubated together, we observed a significant enhancement of the binding of both proteins to *ars1* DNA (compare lanes 5 and 6 with lane 7). This observation strongly suggests that SpCdt1 and SpCdc18 form a complex that has an intrinsic DNA-binding activity greater than that of the individual proteins and is independent of SpORC.

The presence of SpORC increased the association of SpCdt1 or SpCdc18 with *ars1*-beads, indicating that both proteins interact independently with SpORC (Fig. 4A, compare lanes 5 and 6 to lanes 8 and 9). Conversely, we observed that the presence of either SpCdt1 (lane 8) or SpCdc18 (lane 9) enhanced SpORC binding to *ars1* DNA to form the salt-stable complex. SpORC, SpCdc18, and SpCdt1 all bound DNA most efficiently when all three proteins were present (lane 10). Similar results were observed when the reactions were carried out in the absence of ATP (data not shown). We also investigated the effects of SpCdt1 and SpCdc18 on the formation of the salt-sensitive SpORC-DNA complex. For this purpose, the incubation was shortened to 1 min, and the beads were washed with either low salt buffer (Fig. 4B, left) or high salt buffer (Fig. 4B, right). Under low salt wash conditions, we observed a similar enhancement of SpORC binding by SpCdt1 or SpCdc18 (Fig. 4B, left). However, these complexes were not resistant to washing with high salt buffer (Fig. 4B, right). These results suggest that SpCdc18 and SpCdt1 facilitate the initial interaction between SpORC and *ars1* DNA that leads the formation of a salt-stable complex.

We also carried out a topoisomerase I relaxation assay to examine whether SpCdc18 and/or SpCdt1 enhances SpORC-dependent structural changes in negatively supercoiled pARS1 plasmid DNA. In this assay, we used a concentration of SpORC that we previously determined had a minimal effect on altering pARS1 topology (Fig. 4C, lane 3). Neither SpCdc18 (lane 4) nor SpCdt1 (lane 5) had a significant effect on pARS1 topology either alone or in combination (lane 6). However, incubation of SpORC with either SpCdc18 (lane 8) or SpCdt1 (lane 7) resulted in a significant shift in the distribution of topoisomers, with the greatest effect observed when all three proteins were present (lane 9). These changes in pARS1 topology are similar to those we observed using higher SpORC concentrations.
SpORC-DNA Interactions

FIGURE 5. Multiple factors contribute to SpORC-DNA interactions. SpORC binds DNA in a biphasic manner: formation of complex I involves the AT-hooks of SpOrc4 and serves as a precursor for the formation of a second complex II that involves additional non-electrostatic SpORC-DNA interactions. SpCdc18 and SpCdt1 form a complex that binds DNA and also facilitates initial SpORC-DNA interactions that promote formation of complex II. Negative supercoiling also has a positive affect on SpORC-DNA interactions that promote SpORC-dependent changes in DNA topology such as unwinding or wrapping that lead to more stable SpORC-DNA interactions.

DISCUSSION

We have used highly purified recombinant S. pombe ORC, Cdc18, and Cdt1 proteins to examine the molecular interactions that occur during pre-replication complex assembly at the fission yeast ars1 origin of DNA replication. SpORC binds ars1 DNA in a biphasic manner in which the initial salt-sensitive complex I is slowly converted to the highly salt-resistant complex II (Fig. 5). The formation of complex I likely involves interactions between the nine AT-hook motifs in the SpOrc4 subunit and AT tracts in ars1 DNA. Previous studies have shown that the AT-hook motifs are necessary and sufficient to target SpORC to DNA and that the binding affinity of SpORC for a segment of DNA is determined largely by its length and AT content (31–37). Moreover, thermodynamic studies have demonstrated that salt-sensitive electrostatic interactions are the dominant forces in the binding of AT-hooks to DNA (43). The nature of complex II is not yet clear, but its salt resistance indicates that additional non-electrostatic interactions between SpORC subunits and DNA make a substantial contribution to its stability. The formation of complex II is a slow step that only occurs at an appreciable rate when SpORC is tethered to origin DNA via complex I. Thus, the AT-hooks of SpOrc4 likely function to properly localize SpORC to AT-rich fission yeast chromosomal origins of DNA replication, which are preferentially located in intergenic DNA (18, 20, 44–46). Because AT tracts are highly abundant in the genome, particularly in intergenic DNA, SpORC does not bind to a highly specific site like S. cerevisiae ORC, but rather is distributed at multiple sites even within a single ars element like ars1. Once SpORC is tethered to the origin DNA via the AT-hooks, additional SpORC-DNA interactions occur that are presumably required for the subsequent steps in the assembly of the pre-RC. In addition to simply recruiting SpORC to the vicinity of origin DNA, it is possible that the AT-hook motifs more directly facilitate the formation of complex II. For example, a number of proteins containing AT-hooks (e.g. high mobility group) appear to facilitate the formation of regulatory protein-DNA complexes at promoters (e.g. the interferon-β promoter) (47). Depending on the case, high mobility group can facilitate the binding of other transcriptional regulatory proteins either by direct protein-protein interactions or by modulating the structure of the DNA. Further work will be required to determine whether the AT-hook motifs of SpOrc4 act in a similar way. Interestingly, it has been reported that the stability of Xenopus ORC binding to chromatin changes during the cell cycle (48). In metaphase, the Xenopus laevis ORC-DNA complexes are salt-sensitive, but they become salt-resistant in early interphase. The complexes become salt-sensitive again after XLMcm2–7 loading completes the formation of active pre-RCs.

Several lines of evidence indicate that complex II does not simply represent an aggregated or insoluble form of SpORC. First, no precipitation of SpORC was observed in control experiments in which SpORC was incubated with beads lacking DNA. Second, excess competitor DNA was sufficient to prevent formation of both complexes I and II, indicating that both species represent DNA-bound forms. Finally, the SpORC-DNA complexes formed under conditions that generate the salt-stable complex are associated with a distinct and stable topological state of the bound DNA (see below).

We observed that SpORC in complex II induces a significant change in the structure of the bound DNA. Consistent with previous studies of D. melanogaster and S. pombe ORCs, topology assays with closed circular DNA demonstrated that the DNA is underwound or has negative writhe or both (33, 42). We observed that highly purified human ORC induced a similar topological change, indicating that this property of ORC has been conserved during eukaryotic evolution and probably plays an important role in ORC function. The loading of the Mcm2–7 helicase likely requires local unwinding of the origin DNA, so it is possible that the ORC-induced structural alteration in the DNA contributes to this function. We observed that SpORC, like D. melanogaster ORC, has little effect on the topology of covalently closed, but relaxed, plasmid DNA in the topoisomerase assay. This observation is consistent with the expectation that the SpORC-induced changes in topology would be disfavored in such a DNA molecule because of the energetic penalty resulting from the accumulation of compensatory positive writhe. On the other hand, it would be expected that binding and the concomitant changes in DNA structure would be facilitated by negative supercoiling. These effects explain our obser-
vation that the affinity of SpORC for relaxed plasmid DNA is much less than its affinity for supercoiled plasmid DNA.

The higher affinity of SpORC for supercoiled DNA may be relevant to SpORC function in vivo. It is interesting that the *Escherichia coli* replication initiator DnaA protein, which has structural similarity to ORC1, -4, and -5 (49) in the AAA+ domains, preferentially binds to negatively supercoiled oriC replication origin DNA sequences and, upon binding, wraps the DNA and promotes localized DNA unwinding (50–53). In *E. coli*, negative supercoiling is maintained by the activity of gyrase, but there is no comparable mechanism in eukaryotes. However, in eukaryotic cells, chromosomal origins are packaged into nucleosomes that must be cleared during the assembly of pre-replication complexes (54–58). The removal of nucleosomes by chromatin remodeling factors might result in the transient formation of negatively supercoiled DNA that could potentially facilitate ORC binding and the accompanying perturbation of DNA structure. Of course such a mechanism presumes that the rate of relaxation of the supercoiled region by topoisomerases is not more rapid than that of ORC binding.

We have observed a number of interactions among *S. pombe* ORC, Cdc18, Cdt1, and *ars1* DNA. As expected, SpORC greatly enhanced the binding of SpCdt1 and SpCdc18 to *ars1* DNA, consistent with the view that ORC recruits the other pre-RC proteins to DNA (1–3). However, we observed that in the absence of SpORC, SpCdt1 has detectable affinity for *ars1* DNA. This interaction has been described previously in the *Xenopus* system, and the DNA-binding activity has been attributed to the N-terminal region of Cdt1 (59). Interestingly, we also observed that SpCdt1 can form a binary complex with SpCdc18 that has significantly greater affinity for DNA than SpCdt1 or SpCdc18 alone. We suggest that this SpCdt1-Cdc18-DNA complex may play a role in pre-RC formation, so SpORC may not be the only determinant of the chromosomal sites of pre-RC assembly. Consistent with this possibility, it has been reported that ScCdc6, the orthologue of SpCdc18, can alter origin site selection by *S. cerevisiae* ORC (21, 60, 61). Finally, we observed that SpCdt1 and SpCdc18 individually enhance the binding of SpORC to *ars1* DNA and that maximal SpORC binding is observed when all three proteins are present. The effect of SpCdc18 and SpCdt1 is to facilitate the initial salt-sensitive interaction between SpORC and DNA that leads to the formation of the salt-stable complex II. Consistent with these results, we found that SpCdc18 and SpCdt1 also facilitate SpORC-dependent changes in DNA topology in complex II. Thus, our data indicate that the formation of the pre-RC involves binary interactions among all three proteins (SpCdt1-Cdc18, SpCdt1-ORC, and SpCdc18-ORC) as well as interactions of both SpORC and SpCdt1-Cdc18 with origin DNA. Whereas SpORC is sufficient to induce topological changes in origin DNA, the stability of the SpORC-DNA complex may likely depend upon all of these interactions.

REFERENCES

1. Bell, S. P., and Dutta, A. (2002) *Annu. Rev. Biochem.* 71, 333–374
2. Kelly, T. J., and Brown, G. W. (2000) *Annu. Rev. Biochem.* 69, 829–880
3. Teer, J. K., and Dutta, A. (2006) *Results Probl. Cell Differ.* 42, 31–63
4. Nishitani, H., and Lygerou, Z. (2002) *Genes Cells* 7, 523–534
5. Fangman, W. L., Hice, R. H., and Chlebowicz-Sledziewska, E. (1983) *Cell* 32, 831–838
6. Kearsey, S. (1983) *EMBO J.* 2, 1571–1575
7. Huang, R. Y., and Kowalski, D. (1993) *EMBO J.* 12, 4521–4531
8. Marahrens, Y., and Stillman, B. (1992) *Science* 255, 817–823
9. Rao, H., Marahrens, Y., and Stillman, B. (1994) *Mol. Cell. Biol.* 14, 7643–7651
10. Theis, J. F., and Newlon, C. S. (1994) *Mol. Cell. Biol.* 14, 7652–7659
11. Breier, A. M., Chatterji, S., and Cozzarelli, N. R. (2004) *Genome Biol.* 5, R22
12. Wyrick, J. J., Aparicio, J. G., Chen, T., Barnett, J. D., Jennings, E. G., Young, R. A., Bell, S. P., and Aparicio, O. M. (2001) *Science* 294, 2357–2360
13. Bell, S. P., and Stillman, B. (1992) *Nature* 357, 128–134
14. Biswas, S. B., Khopde, S. M., and Biswas-Fiss, E. E. (2005) *Cell Cycle* 4, 494–500
15. Jacob, F., Brenner, S., and Cuzin, F. (1964) *Cold Spring Harbor Symp. Quant. Biol.* 28, 329–348
16. Dubey, D. D., Kim, S. M., Todorov, I. T., and Huberman, J. A. (1996) *Curr. Biol.* 6, 6348–6357
17. Clyne, R. K., and Kelly, T. J. (1997) *Methods (Orlando)* 13, 221–233
18. Dai, J., Chuang, R.-Y., and Kelly, T. J. (2005) *Proc. Natl. Acad. Sci. U. S. A.* 102, 337–342
19. Okuno, Y., Satoh, H., Sekiguchi, M., and Masukata, H. (1999) *Mol. Cell. Biol.* 19, 6699–6709
20. Segurado, M., de Luis, A., and Antequera, F. (2003) *EMBO Rep.* 4, 1048–1053
21. Harvey, K. J., and Newport, J. (2003) *J. Biol. Chem.* 278, 48524–48528
22. Mechali, M., and Kearsey, S. (1984) *Cell* 35, 55–64
23. Arias, E. E., and Walter, J. C. (2004) *Front. Biosci.* 9, 3029–3045
24. Vashee, S., Cvetic, C., Lu, W., Simancek, P., Kelly, T. J., and Walter, J. C. (2003) *Genes Dev.* 17, 1894–1908
25. Chesnovik, I., Gossen, M., Remus, D., and Botchan, M. (1999) *Genes Dev.* 13, 1289–1296
26. Mesner, L. D., Li, X., Dijkwel, P. A., and Hamlin, J. L. (2003) *Mol. Cell. Biol.* 23, 804–814
27. Dijkwel, P. A., Wang, S., and Hamlin, J. L. (2002) *Mol. Cell. Biol.* 22, 3053–3065
28. Dijkwel, P. A., Mesner, L. D., Levenson, V. V., d’Anna, J., and Hamlin, J. L. (2000) *Exp. Cell Res.* 256, 150–157
29. Dijkwel, P. A., Vaughn, J. P., and Hamlin, J. L. (1994) *Nucleic Acids Res.* 22, 4989–4996
30. Norio, P., Kosiyatrakul, S., Yang, Q., Guan, Z., Brown, N. M., Thomas, S., Riblet, R., and Schildkraut, C. L. (2005) *Mol. Cell* 20, 575–587
31. Chuang, R.-Y., Chretien, I., Dai, J., and Kelly, T. J. (2002) *J. Biol. Chem.* 277, 16920–16927
32. Chuang, R.-Y., and Kelly, T. J. (1999) *Proc. Natl. Acad. Sci. U. S. A.* 96, 2656–2661
33. Gaczynska, M., Osmulski, P. A., Jiang, Y., Lee, J. K., Bermudez, V., and Hurwitz, J. (2004) *Proc. Natl. Acad. Sci. U. S. A.* 101, 17952–17957
34. Kong, D., and DePamphilis, M. L. (2001) *Mol. Cell. Biol.* 21, 8095–8103
35. Lee, J. K., Moon, K. Y., Jiang, Y., and Hurwitz, J. (2001) *Proc. Natl. Acad. Sci. U. S. A.* 98, 13589–13594
36. Takahashi, T., and Masukata, H. (2001) *Genes Cells* 6, 837–849
37. Moon, K. Y., Kong, D., Lee, J. K., Raychaudhuri, S., and Hurwitz, J. (1999) *Proc. Natl. Acad. Sci. U. S. A.* 96, 12367–12372
38. Bustin, M., and Reeves, R. (1996) *Proc. Natl. Acad. Sci. U. S. A.* 93, 6577–6580
39. Mehta, J. J., Peltier, L., and Schildkraut, C. L. (1997) *Cold Spring Harbor Symp. Quant. Biol.* 62, 467–473
40. Huth, J. R., Bewley, C. A., Nissen, M. S., Evans, J. N., Reeves, R., Gronenborn, A. M., and Clore, G. M. (1997) *Nat. Struct. Biol.* 4, 657–665
41. Heichinger, C., Penkett, C. J., Bahler, J., and Nurse, P. (2006) *EMBO J.* 25, 13589–13594
SpORC-DNA Interactions

5171–5179
47. Reeves, R., and Beckerbauer, L. (2001) Biochim. Biophys. Acta 1519, 13–29
48. Rowles, A., Tada, S., and Blow, J. J. (1999) J. Cell Sci. 112, 2011–2018
49. Clarey, M. G., Erzberger, J. P., Grob, P., Leschziner, A. E., Berger, J. M., Nogales, E., and Botchan, M. (2006) Nat. Struct. Mol. Biol. 13, 684–690
50. Bramhill, D., and Kornberg, A. (1988) Cell 54, 915–918
51. Bramhill, D., and Kornberg, A. (1988) Cell 52, 743–755
52. Messer, W. (2002) FEMS Microbiol. Rev. 26, 355–374
53. Sekimizu, K., Bramhill, D., and Kornberg, A. (1988) J. Biol. Chem. 263, 7124–7130
54. Gerbi, S. A., and Bielinsky, A. K. (2002) Curr. Opin. Genet. Dev. 12, 243–248
55. Aggarwal, B. D., and Calvi, B. R. (2004) Nature 430, 372–376
56. Melendy, T., and Li, R. (2001) Front. Biosci. 6, D1048–D1053
57. Biamonti, G., Paixao, S., Montecucco, A., Peverali, F. A., Riva, S., and Falaschi, A. (2003) Chromosome Res. 11, 403–412
58. Iizuka, M., Matsui, T., Takisawa, H., and Smith, M. M. (2006) Mol. Cell. Biol. 26, 1098–1108
59. Lee, C., Hong, B., Choi, J. M., Kim, Y., Watanabe, S., Ishimi, Y., Enomoto, T., Tada, S., Kim, Y., and Cho, Y. (2004) Nature 430, 913–917
60. Speck, C., Chen, Z., Li, H., and Stillman, B. (2005) Nat. Struct. Mol. Biol. 12, 965–971
61. Speck, C., and Stillman, B. (2007) J. Biol. Chem. 282, 11705–11714