The origin recognition complex (ORC) plays a central role in the initiation of DNA replication in eukaryotic cells. It interacts with origins of DNA replication in chromosomal DNA and recruits additional replication proteins to form functional initiation complexes. These processes have not been well characterized at the biochemical level except in the case of *Saccharomyces cerevisiae* ORC. We report here the expression, purification, and initial characterization of *Schizosaccharomyces pombe* ORC (SpORC) containing six recombinant subunits. Purified SpORC binds efficiently to the *ars1* origin of DNA replication via the essential N-terminal domain of the SpOrc4 subunit which contains nine AT-hook motifs. Competition binding experiments demonstrated that SpORC binds preferentially to DNA molecules rich in AT-tracts, but does not otherwise exhibit a high degree of sequence specificity. The complex is capable of binding to multiple sites within the *ars1* origin of DNA replication with similar affinities, indicating that the sequence requirements for origin recognition in *S. pombe* are significantly less stringent than in *S. cerevisiae*. We have also demonstrated that SpORC interacts directly with Cdc18p, an essential fission yeast initiation protein, and recruits it to the *ars1* origin in vitro. Recruitment of Cdc18p to chromosomal origins is a likely early step in the initiation of DNA replication in vivo. These data indicate that the purified recombinant SpORC retains at least two of its primary biological functions and that it will be useful for the eventual reconstitution of the initiation reaction with purified proteins.

In bacteria, bacteriophage, and animal viruses the initiation of DNA replication takes place at defined nucleotide sequences known as origins of replication (1). Initiator proteins bind to such origin sequences and promote the biochemical steps leading to the establishment of replication forks. The interaction of initiator proteins with origins is less well understood in the case of eukaryotic cells where initiation of DNA replication occurs at multiple sites along chromosomal DNA (2). The best characterized eukaryotic chromosomal origins of replication are those of the budding yeast *Saccharomyces cerevisiae*. Like the origins of prokaryotes and animal viruses, budding yeast origins are modular in nature and are composed of several short, well defined sequence blocks distributed over a region of ~100–150 bp (3–5). The most highly conserved sequence block of budding yeast origins is the A domain which contains an essential 11-bp ARS consensus sequence. An additional, less well conserved sequence block, referred to as the B domain, serves to enhance the efficiency of origin utilization (3–5). The six-subunit *S. cerevisiae* Origin Recognition Complex (ScORC) binds specifically to the ARS consensus sequence in a reaction requiring ATP (6). Genetic and biochemical studies have established that ORC plays a central role in the initiation of DNA replication and that it functions, at least in part, to recruit essential replication factors to origins of DNA replication to form the pre-replication complex (7–11). One such factor is Cdc6p which is required, together with ScORC, to load the MCM complex, a putative DNA helicase, onto DNA (10, 12) (for review, see Ref. 2).

Homologues of ScORC subunits have been identified in a variety of eukaryotic species including humans (13). In addition, protein complexes containing ORC-related subunits have been identified in extracts of *Xenopus laevis* eggs, *Drosophila melanogaster* embryos, *Schizosaccharomyces pombe* cells, and human HeLa cells (14–17). Thus, ORC has been highly conserved during evolution, suggesting the existence of common mechanisms for initiating DNA replication in all eukaryotes. However, it is not yet clear whether the interaction of ORC with origins of DNA replication in other species is similar to that in *S. cerevisiae*. Indeed, there is considerable evidence that the initiation of DNA replication in metazoans can occur at many sites within broad replication zones, suggesting that the sequence requirements for initiation may be more relaxed than in budding yeast (18, 19).

In previous studies we and others have shown that fission yeast origins of DNA replication differ dramatically from their budding yeast counterparts (20–22). Fission yeast origins have a minimal size of 500 to 1000 bp and are very rich in AT base pairs. However, they do not share a common consensus sequence comparable to the ARS consensus sequence of *S. cerevisiae* replication origins. In addition, *S. pombe* origins are characterized by a high degree of functional redundancy. Sequence blocks that are important for origin function appear to be composed of smaller AT-rich sequence elements that can be
deleted individually without significantly affecting origin activity (23). Several genetic properties of S. pombe origins can be rationalized by the finding that the S. pombe homologue of one of the ORC subunits (SpOrc4p) contains an N-terminal DNA-binding domain consisting of nine AT-hook motifs (24). We have suggested that binding of the N-terminal domain of SpOrc4p to appropriately spaced AT-tracts serves to tether the ORC complex to S. pombe origins of DNA replication. Consistent with this possibility, we have demonstrated that the isolated SpOrc4 subunit can bind to DNA containing a known S. pombe origin (24). However, the DNA binding properties of the SpORC holo-complex have not been reported.

To understand origin recognition and the assembly of initiation complexes in S. pombe, it will be essential to characterize the biochemical properties of SpORC. For this purpose, we have expressed all six SpORC subunits in insect cells using the baculovirus system, and we have purified the complex to near homogeneity. The purified SpORC binds with high affinity to a known origin of DNA replication in S. pombe (ars1). Our data indicate that SpORC recognizes multiple sites within this origin (24). Although the DNA binding properties of the isolated SpOrc4 subunit are less sequence-specific than those of S. cerevisiae. The binding of SpORC to ars1 DNA is mediated by the N-terminal domain of the SpOrc4 subunit. We have demonstrated that this domain is essential for the viability of S. pombe. Finally, we have shown that SpORC interacts directly with Dcd18p, a key regulator of the initiation of DNA replication. This interaction recruits Cdc18p to origin DNA, which is a likely early step in the initiation of chromosomal DNA replication. Thus, the expression and purification of SpORC should facilitate biochemical analysis of the initiation reaction.

**EXPERIMENTAL PROCEDURES**

**Cloning of S. pombe orc and cdc18 Genes into Baculovirus Vectors**—The nomenclature for S. pombe orc genes follows the suggestion in Ref. 30. To construct a baculovirus expression vector containing the orc1 gene, the full-length gene was synthesized by PCR using oligonucleotides 5'-TCCCGGATCCGCTTACCATTCATATGCTTGAGAAG-3' and 5'-CCGAAAGCTTCTATCCCGC-TTATATTGGTATACCC-3' as primers and pGEX-4T-1-orc1 as template (25). The PCR product was digested with BamHI and PstI and ligated into the corresponding sites in the plasmid pFastBac1 (Invitrogen) to yield pFastBac1-SpOrc1. Expression vectors containing the orc5 and orc6 genes (pFastBac-Sporc5 and pFastBac-Sporc6) were generated in a similar way. For pFastBac1-Sporc5, the orc5 gene was amplified using oligonucleotides 5'-CCGAGAATTCATATGGTTATACCCCATCAGCGGTA-3' and 5'-CCGAAAGCTTCCTATCCCGCTATAT-3' as primers and pMYC4-His-orc5 as template. The PCR product was cloned into the EcoRI and PstI sites of pFastBac1. The orc6 gene was amplified using oligonucleotides 5'-CCGAGAATTCATATGGTTATACCCCATCAGCGGTA-3' and 5'-CCGAAAGCTTCCTATCCCGCTATAT-3'.

**Expression and Purification of SpORC and Cdc18p in Insect Cells**—S. pombe cells were cultured at 32 °C in a medium supplemented with 10% fetal bovine serum. A nearly homogeneous preparation was obtained.

**Antibody Production**—For generation of antibodies, the SpORC subunits were expressed in bacteria as recombinant fusion proteins tagged with either the His6-epitope or the glutathione S-transferase moiety. For SpOrc1p, a truncated gene containing N-terminal residues 1–285 was amplified and cloned into the BamHI site of pGEX2T (Amersham Bioscience). For SpOrc2p, a truncated gene containing residues 239–534 was amplified and cloned into the BamHI and SalI sites of pGEX 4T-1 (Amersham Bioscience). For SpOrc3p, a truncated gene containing N-terminal residues 1–344 was amplified by using oligonucleotides 5'-CCGAAAGCTTCTATCCCGC-TTATATTGGTATACCC-3' as primers and pFastBac-Sporc3 as template. The PCR product was then inserted between the BamHI and HindIII sites of the pET21b expression vector (Novagen). For SpOrc5p, the C-terminal segment containing residues 211–454 was amplified by using oligonucleotides 5'-CCGAAAGCTTCTATCCCGC-TTATATTGGTATACCC-3' as primers and pFastBac-Sporc5 as template. The PCR product was then inserted between the BamHI and HindIII sites of the pET21b expression vector. For SpOrc6p, the pET11b-orc6 containing the full-length orc6 gene was used to express recombinant protein. Recombinant glutathione S-transferase fusion proteins were expressed in bacteria and purified on a glutathione column according to the manufacturer’s instructions (Amersham Bioscience). All His6-tagged proteins were expressed in bacteria and purified by nickel-agarose (Ni-agarose) chromatography according to instructions provided by the manufacturer (Qiagen). The purified proteins were used as antigens to immunize rabbits (Covance Research Products, Denver, PA).

**Expression and Purification of SpORC and Cdc18p in Insect Cells**—S. pombe cells were cultured at 27 °C in a medium supplemented with 10% fetal bovine serum. For expression of recombinant SpOrc3p, Sf9 cells (2 × 10^6 cells/ml) were co-infected with five recombinant baculovirus expressing all six subunits at a multiplicity of infection of 2–5. After 48 h cells were harvested and washed once with ice-cold phosphate-buffered saline (10 mM phosphate buffer, pH 7.3, 140 mM NaCl, 2.7 mM KCl) and centrifuged. Infected cell pellets from 250 ml cultures were lysed in 50 ml of lysis buffer (20 mM Tris-HCl, pH 8.6, 0.4 M sorbitol, 150 mM KCl, 5 mM MgCl2, 5 mM MgSO4, 1% Triton X-100) on ice for 5 min. The suspension was centrifuged at 14,000 × g for 5 min at 4 °C, and the resulting chromatin-enriched pellet was extracted with 12 ml of E buffer (50 mM Hepes pH 7.5, 2.5 mM MgCl2, 500 mM NaCl, 10% glycerol, 1% Triton X-100) on ice for 30 min. The suspension was then centrifuged at 100,000 × g for 30 min at 4 °C. The supernatant was collected and incubated with 0.2 ml of anti-HA antibody-conjugated agarose (F7-agarose) (Santa Cruz Biotech) at 4 °C for 2 h. The beads were washed once with 25 ml of E buffer and 1 ml of F buffer (50 mM Hepes pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 5 mM MgOAc2, 10% glycerol) twice. The F7-agarose-bound proteins were eluted by incubation with 0.8 ml of F buffer containing 2 mg/ml HA peptide overnight at 4 °C. This step yielded a nearly homogeneous

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2 R. Y. Chuang and T. Kelly, unpublished data.
S. pombe ORC

SpORC which was used for most experiments. In some cases (e.g., Fig. 1) the protein was further purified by Ni-agarose affinity chromatography. The eluate from the antibody affinity step was collected and incubated with 0.2 ml of Ni-agarose for 1 h at 4 °C. The resin was washed with 5 ml of F buffer and 1 ml of E buffer. The SpORC was eluted with E buffer containing 30 mM imidazole.

The expression and purification of FLAG-Cdc18p protein was performed by methods identical to those used for SpORC except as follows. The infected cells from 180 ml of culture were lysed in 25 ml of L buffer. After centrifugation, the chromatin pellets were extracted with 8 ml of E buffer for 30 min at 4 °C. After centrifugation at 100,000 × g for 30 min, the supernatant was incubated with 0.3 ml of FLAG antibody beads (Sigma–Aldrich) at 4 °C for 2 h. The Cdc18p was eluted from the antibody beads with 0.7 ml of F buffer containing 1 mg/ml FLAG peptide (Sigma–Aldrich). All buffers used for protein purification contained the following protease inhibitors: leupeptin (10 μg/ml), aprotenin (10 μg/ml), soybean trypsin inhibitor (2 μg/ml), bestatin A (10 μg/ml), N-tosyl-l-phenylalanine chloromethyl ketone (20 μg/ml), 4-(2-aminoethyl)-benzenesulfonylfluoride hydrochloride (AEBSF) (2 μM).

For immunoprecipitation, Western blotting analyses, and DNA extracts were prepared as described above, and SpORC was purified by Ni-agarose chromatography. After elution with imidazole, the eluate was incubated with anti-SpORC65 antibody cross-linked to protein A-Sepharose (Amersham Bioscience) as described (17). After 2–3 h incubation at 4 °C, the resin was washed three times with 1 ml of E buffer. The immunoprecipitated proteins were separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by either silver staining or immunoblotting.

DNA Binding Assays—For the DNA binding experiments of Fig. 2, a 1.15-kb DNA fragment containing the ars1 origin was synthesized by PCR using 5′-biotinylated oligonucleotide, E-5′-CAAGGTTTTGCTATA-GAATCC-3′ and 5′-GAATTCGATCTAACCTT-3′ as primers and pPR20 (21) as template. The PCR product was then coupled to streptavidin-conjugated magnetic beads (Dynabeads M-280 Streptavidin; DynaCult) according to the manufacturer’s instructions. The binding assays were performed by incubating 2 pmol of purified SpORC with beads containing 1 pmol of ars1 DNA in 20 μl of F buffer with 1 mg/ml bovine serum albumin at 4 °C for 1 h. Where indicated, 1 mM ATP was included in the reaction mixtures. Beads were washed three times with 200 μl of F buffer containing 0.1% Nonidet P-40. The bound proteins were released from the beads by incubation with 1% SDS loading buffer and separated by 10% SDS-PAGE, followed by Western blotting analysis with antibodies against SpORC subunits (1, 2, 3, 5, and 6). Anti-HA antibody, 12CA5, (Roche Molecular Biochemicals), was used for detecting recombinant HA-His6-SpOrc4p.

The immunoprecipitation (McKay) DNA binding assays of Fig. 3, B and C, were performed as described (27) with the following modifications. A 1.5-kb DNA fragment containing the 1.2-kb ars1 origin and 47 bp of DNA upstream was amplified by PCR with NotI and XhoI sites of pArg3HA, yielding a competitor DNA. This plasmid was then transfected into S. pombe N121 cells, yielding the N-terminal deletion could rescue an S. pombe

 Yeast Strains, Plasmids, and Rescue Assay—All S. pombe strains were grown on yeast extract plus supplement agar plates or Edinburgh minimal media with appropriate supplement using standard methods (29). The plasmid pRCE81X (20) and pRCE81X-orc4 were constructed as follows. The plasmid pRCE81X was derived from the plasmid pREP81X (30) by replacing the promoter-terminator DNA fragment of pSLF172 (31). The orc4-1 gene was synthesized by PCR and inserted into the XhoI site of pRCE81X, yielding plasmid pRCE81X-orc4. The plasmid pRCE81X was derived from the plasmid pREP81X (30) by replacing the promoter-terminator DNA fragment of pSLF172 (31). The orc4-1 gene was synthesized by PCR and inserted into the XhoI site of pRCE81X, yielding plasmid pRCE81X-orc4.

The plasmid pArg3HA was derived from the plasmid pSLF272 (31) by replacing the ars4 marker and 1 pmol of SpORC-Cdc18p promoter DNA fragment with the pORC11 promoter-terminator DNA fragment of pSLF172 (31). The orc4-1 gene was synthesized by PCR and inserted into the XhoI site of pRCE81X, yielding plasmid pRCE81X-orc4. The latter was synthesized by PCR with oligonucleotides 5′-TTCCCCGGGCTATTTCTACAAAGTAC-3′ and 5′-TTCCCCGGGCTATTTCTACAAAGTAC-3′ as primers and the plasmid pRCE81X was derived from the plasmid pREP81X (30) by replacing the promoter-terminator DNA fragment of pSLF172 (31). The orc4-1 gene was synthesized by PCR and inserted into the XhoI site of pRCE81X, yielding plasmid pRCE81X-orc4.

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RESULTS

Reconstitution and Purification of Recombinant SpORC—To express all six subunits of SpORC in insect cells using the baculovirus expression system. To facilitate the purification of the...
the protein complex, hexahistidine (His6) and hemagglutinin (HA) epitope tags were added to the N terminus of SpOrc4p. We have previously observed by rescue of an orc4 deletion that the SpOrc4p with a His6-epitope tag in this position is functional in vivo (data not shown). Insect cells were co-infected with recombinant baculoviruses encoding each of the SpORC subunits. Since SpOrc4p contains nine copies of the AT-hook-DNA binding motif, we expected that the recombinant SpORC complex would be tightly associated with chromosomal DNA (24). Therefore, the first step in the purification procedure was to prepare a chromatin-enriched fraction in a buffer containing 1% non-ionic detergent. We recovered greater than 90% of the expressed SpOrc1p-SpOrc5 and about half of the SpOrc6p in the chromatin fraction. To find conditions for release of SpORC in a soluble form, the chromatin fraction was extracted with buffers containing increasing concentrations of NaCl. We observed that more than 80% of SpORC was released from the chromatin-enriched fraction in 0.5 M NaCl. This observation is consistent with analysis of the stability of complexes between DNA and purified SpORC (see below) and with previous observations (16).

The solubilized SpORC was purified by sequential affinity chromatography on F7 anti-HA antibody-agarose and Ni-agarose. The eluate from the Ni-agarose affinity matrix was analyzed by SDS-PAGE and silver staining (Fig. 1A). Six major polypeptides with mobilities consistent with the calculated molecular weights of SpOrc1p-SpOrc6p were observed. The identity of the six polypeptides was confirmed by Western blotting with specific antibodies against each SpORC subunit (Fig. 1B). The SpOrc2 subunit consisted of a doublet band (Fig. 1, A and B). The upper band of the doublet is likely a phosphorylated form of the protein that accumulates in the G1 and M phases of the cell cycle (32, 33).

Additional fractionation experiments were performed to verify the association of the SpORC subunits. For example, chromatin extracts were subjected to two sequential steps of affinity purification, one specific for HA-His6-SpOrc4p (Ni-agarose) and one specific for SpOrc5p (immunoprecipitation with anti-SpOrc5 antibodies). As shown in Fig. 1, C and D, all six subunits were recovered by this procedure. In addition, the six SpORC subunits co-eluted when subjected to Mono-S column chromatography (data not shown). We conclude that the recombinant SpORC represents a holo-complex of all six subunits.

Interaction of SpORC with Origin DNA—We have previously shown that the isolated SpOrc4 subunit binds to S. pombe ars1 DNA in vitro via its N-terminal AT-hook domain (24). We have hypothesized that the AT-hook domain serves to tether the SpORC holo-complex to origins of DNA replication. To investigate this possibility we analyzed the DNA binding activity of purified SpORC using several different assays. In the first assay, the fission yeast ars1 origin of replication was synthesized by PCR using a 5′-biotin labeled primer. The labeled DNA was then immobilized on streptavidin-conjugated magnetic beads. After incubation with purified SpORC, the ars1 beads were washed, and the bound proteins were eluted and analyzed by SDS-PAGE and immunoblotting with specific antibodies against each subunit of SpORC (Fig. 2). All six subunits were recovered in the bound fraction with similar efficiencies (~50% in this experiment). Unlike S. cerevisiae ORC, SpORC bound to ori in the absence of ATP. Little, if any, SpORC bound to streptavidin beads in the absence of ars1 DNA.

To begin to explore the specificity of the interaction of SpORC with DNA, we performed binding assays using the method developed by McKay (27). In this assay, radioactively labeled DNA fragments were incubated with purified HA-His6-SpORC, and the resulting protein-DNA complexes were collected with F7 anti-HA antibody-agarose beads. We made use of three non-overlapping fragments of 0.2, 0.4, and 0.5 kb spanning the ars1 origin and a 0.3-kb non-ars1 control frag-
At relatively low ionic strengths, all four DNA fragments were bound by the purified SpORC (Fig. 3B). At 350 mM NaCl, the binding of the control fragment was completely eliminated and only the fragments derived from *ars1* showed significant binding. At 500 mM NaCl, the binding of *ars1* fragments was abolished. These data indicate that the purified SpORC has significant nonspecific DNA binding activity, but the affinity of the protein for the AT-rich fragments derived from *ars1* is significantly greater than that for control DNA with a lower AT content. Interestingly, under the conditions of our experiments the stability of DNA-SpORC complexes formed with the three different origin fragments were almost indistinguishable. This result indicates that SpORC can bind to multiple sites within *ars1* and is consistent with our previous genetic data suggesting that the origin is composed of redundant elements (21).

The DNA binding properties of the purified six subunit SpORC are similar to those that we have previously reported for the isolated SpOrc4 subunit (24). Fig. 3C shows a direct comparison of the DNA binding properties of the purified SpORC and SpOrc4p. The fractions of the various fragments bound and the salt sensitivity of binding were indistinguishable for the isolated SpOrc4p and the holo-complex. These results are consistent with the hypothesis that the N-terminal domain of SpOrc4p plays a central role in targeting SpORC to origins of DNA replication (24).

We also employed the McKay assay to study the binding of SpOrc4 to additional *S. pombe* origins of DNA replication. As shown in Fig. 3D, SpOrc4 bound to *ars3001* and *ars3002* to a significantly greater extent than to the control non-*ars* DNA fragment. As in the case of *ars1* the AT contents of *ars3001* and *ars3002* are quite high (74 and 80%, respectively), while the AT content of the non-*ars* fragment derived from *S. pombe* coding sequence is 60%.

To explore the specificity of SpORC binding in a more quantitative fashion, we carried out a series of competition filter binding experiments in which we measured the ability of various unlabeled DNA molecules to compete for SpORC binding to a 200-bp radioactive DNA fragment derived from the minimal *ars1* origin (4). In the first series of experiments the competing DNAs were synthetic polymers with different compositions. We observed that both poly(dA-dT)-(dA-dT) and poly(dG-dC)-(dG-dC) were extremely effective as competitors. In fact, the data in Fig. 4A indicate that the affinity of SpORC for these two AT-containing polymers is significantly greater than its affinity for *ars1* DNA itself. In contrast, poly(dG-dC)-(dG-dC) were extremely poor competitors, indicating that the affinity of SpORC for GC tracts is orders of magnitude less than for AT tracts.

To determine whether SpORC binds preferentially to a specific region of *ars1*, we generated a series of overlapping unlabeled 200-bp DNA fragments spanning the complete origin (Fig. 4B). An equal amount of each fragment was tested for its ability to compete with radioactive *ars1*-1 DNA for binding to a fixed quantity of SpORC. Strikingly, each of the eight *ars1* fragments competed effectively for SpORC binding to the radioactive *ars1*-1 fragment. Moreover, the extent of competition was quite similar for all of the fragments, ranging from 43% (*ars1*-7) to 68% (*ars1*-1). These data demonstrate that SpORC is capable of binding to multiple sites within *ars1* with similar affinities. Based upon a simple equilibrium model we estimate that the relative affinities of SpORC for the eight *ars1* fragments do not differ by more than a factor of 2–3. Thus, the high affinity of SpORC for *ars1* and other *S. pombe* origins is probably due to the cumulative effect of many potential AT-rich-binding sites. This situation is markedly different from *S. cerevisiae* where in most cases a single SCORC-binding site predominates.

![Figure 3: SpOrc4p Tethers SpORC to Origin DNA](http://www.jbc.org/Downloaded from http://www.jbc.org/July 23, 2018)

**Fig. 3.** SpOrc4p Tethers SpORC to Origin DNA. A, fragments of *ars1* used in DNA binding experiments. The DNA fragments, *ars1*-I, *ars1*-II, and *ars1*-III, were derived from the *S. pombe* *ars1* origin by restriction digestion and end-labeled with [32P]dATP as described under “Experimental Procedures.” A control non-*ars* fragment of 0.32 kb was prepared in a similar fashion. The shaded region indicates the minimal *ars1* origin of DNA replication defined by genetic experiments (21). B, a mixture of the four [32P]-labeled DNA fragments was incubated with purified SpORC immobilized on F7 anti-HA-agarose beads or with control beads alone. The binding reactions, containing the indicated concentrations of NaCl, were incubated at room temperature for 30 min. The bound DNA was eluted with SDS and analyzed by agarose gel electrophoresis, followed by autoradiography. C, similar DNA binding assays were performed with either the SpORC holo-complex (left) or purified SpOrc4 subunit (right) in reaction mixtures containing 250 or 350 mM NaCl. D, purified SpOrc4, tagged with the HA3 epitope, was incubated with radioactive DNA fragments containing the *S. pombe* origins *ars1*, *ars3001*, *ars3002*, or a control non-ars DNA segment. DNA-protein complexes were collected by immunoprecipitation with F7 anti-HA-agarose beads. The bound radioactive DNA was released by incubation in 1% SDS, fractionated by agarose gel electrophoresis, and quantified in a PhosphorImager.
measured by the nitrocellulose filter binding assay. The extent of binding of the radioactive ars1-1 DNA (0.4 nM) as above. The control reaction contained no competitor DNA. We introduced into this strain a plasmid carrying a 200-bp fragment spanning the B Orc4vivo. We made use of a haploid S. pombe strain in which the N-terminal domain could replace the wild-type protein. For this purpose, we asked whether a form of SpOrc4p lacking the N-terminal domain in origin selection, it was important to determine whether the domain is essential for SpORC function. Given that our DNA binding data suggested a role for the N-terminal domain of ORC from other species. The N-terminal AT-hook domain is essential for viability in S. pombe. The N-terminal AT-hook domain present in SpOrc4p has not been observed in ORCs from other species. Given that our DNA binding data suggested a role for the N-terminal domain in origin selection, it was important to determine whether the domain is essential for SpORC function. For this purpose, we asked whether a form of SpOrc4p lacking the N-terminal domain could replace the wild-type protein in vivo. We made use of a haploid S. pombe strain in which the chromosomal copy of orca- was deleted and viability was maintained by a plasmid expressing SpOrc4p under the control of the nmt1 promoter. This strain is non-viable in the presence of thiamine which strongly represses transcription from the nmt1 promoter. We introduced into this strain a plasmid carrying a mutant orca- gene (orca-ΔN), lacking the N-terminal segment which encodes all nine AT-hooks, but retaining the C-terminal segment which is homologous to Orc4 in other species. The mutant protein was expressed under control of the orca- promoter. As shown in Fig. 5, the mutant gene was unable to rescue the viability of the test strain when expression of the wild-type orca- was repressed in the presence of thiamine. Similar results were obtained after introduction of control plasmids carrying either no inserted orca- gene or the wild-type orca- gene lacking its promoter. In contrast, the wild-type orca- gene under the control of its own promoter readily rescued the viability of the test strain in the presence of thiamine. Thus, the N-terminal domain of SpOrc4p is essential, presumably because it is required to target SpORC to origins.

Interaction of SpORC with Cdc18p—There is evidence that one major function of ORC may be to recruit a regulator of DNA replication, Cdc6p/Cdc18p, to origins of DNA replication. Biochemical studies in budding yeast and X. laevis are consistent with the hypothesis that ORC interacts with Cdc6p/Cdc18p and is essential for the association of Cdc6p/Cdc18p with origin DNA (7, 11, 34). In our initial experiments to explore the possible interaction between S. pombe Cdc18p and ORC, we tagged Cdc18p at its N terminus with the FLAG epitope and expressed it in the baculovirus expression system. Previous studies have demonstrated that epitope tags placed at the N terminus of Cdc18p do not interfere with its function (35). The recombinant FLAG-Cdc18p was extracted from a chromatin-enriched fraction and purified by affinity chromatography. Analysis of the purified protein by SDS-PAGE revealed a major band (or in some cases a closely spaced doublet orca-ΔN) or with vector alone. Transformed colonies were selected and streaked onto plates without (A) or with (B) added thiamine to repress the expression of the endogenous orca- gene. The orca-ΔN plasmid contains the wild-type orca- promoter and a mutant orca- gene that lacks the N-terminal AT-hook domain. Two control plasmids containing the wild-type orca- gene were also tested. One plasmid (orca-) contains the wild type orca- promoter and gene, while the other contains the wild-type orca- gene without a functional promoter.

**Fig. 5.** The N-terminal AT-hook domain of SpOrc4p is essential for cell viability. An S. pombe strain (YRC23) expressing the S. pombe orca- gene under the repressible nmt1 promoter, was transformed with the indicated plasmids carrying derivatives of the orca- gene or with vector alone. Transformed colonies were selected and streaked onto plates without (A) or with (B) added thiamine to repress the expression of the endogenous orca- gene. The orca-ΔN plasmid contains the wild-type orca- promoter and a mutant orca- gene that lacks the N-terminal AT-hook domain. Two control plasmids containing the wild-type orca- gene were also tested. One plasmid (orca-) contains the wild type orca- promoter and gene, while the other contains the wild-type orca- gene without a functional promoter.
are independent of ATP (11), suggesting that there may be Cdc6p-ScORC interactions that motifs binds efficiently to the complete six-subunit ScORC, containing alterations in either the Walker A or Walker B ing site (36). However, it has also been shown that the Cdc6p S. cerevisiae absence of ATP as indicated. Beads alone or ars1 beads lacking SpORC served as controls. The bound proteins were analyzed by SDS-PAGE followed by immunoblotting with anti-FLAG antibody to detect complex of Cdc18p to origin DNA. Importantly, formation of the ternary DNA by itself, but that the association of Cdc18 with origin DNA differs significantly from those of S. cerevisiae, suggesting that there may be substantial differences in the mecha- nisms of origin recognition employed by the two species (20, 21, 37). S. pombe origins are large, highly AT-rich sequences composed of functionally redundant elements. Importantly, they lack a common consensus sequence analogous to the ARS consensus sequence element recognized by ScORC. These proper- ties have raised the question of whether origin recognition in fission yeast is less specific than in budding yeast. Our studies of the interaction of purified SpORC strongly suggest that this is the case. The binding of SpORC to ars1 DNA is governed by the SpORC4 subunit, as demonstrated by the fact that the binding of the isolated SpORC4 subunit is indistinguishable from that of the complete SpORC holo-complex. We have previously shown that the N-terminal domain of SpORC4p, which contains nine AT-hook motifs, is necessary and sufficient for DNA binding by the isolated subunit (24), and the present study has shown that this domain is essential for viability of fission yeast. The binding of SpORC to ars1 origin DNA appears to have a relatively low level of sequence specificity. The protein is capable of binding to multiple sites within ars1 DNA with similar affinity. Although the protein binds with the highest affinity to AT-rich DNA, we also observed detectable binding to a control DNA with significantly lower AT content than that generally found in S. pombe origins. Our data are consist- ent with a model in which SpORC is targeted preferentially to long AT-rich regions within the S. pombe chromosomal DNA without regard to the specific nucleotide sequence of such regions. We expect that such regions would contain multiple and potentially overlapping binding sites for SpORC as in the case of ars1. One consequence of this model is that SpORC would be expected to be targeted preferentially to non-coding regions of the genome which have a higher average AT content than coding sequences. Obviously, our data do not rule out the possibility that the pattern of SpORC binding and initiation of DNA replication in vivo may be modulated by other factors such as chromatin organization and local transcriptional activ- ity (38). Nevertheless, it is likely that origin recognition in S. pombe is significantly less constrained by primary nucleotide sequence than in S. cerevisiae. It is possible that the same is true for metazoans, but further work will be required to assess this possibility (18, 19).

It has been demonstrated that binding of ScORC to origin

**DISCUSSION**

To understand the initiation of DNA replication at the mo- lecular level, it will be necessary to reconstitute the initiation complex with purified proteins. Toward this end we have pu- rified recombinant S. pombe ORC and characterized two of its primary functions, origin binding and recruitment of Cdc18p. The SpORC was produced by co-expression of recombinant subunits in the baculovirus expression system. We have developed a simple affinity purification scheme to obtain SpORC from chromatin extracts of infected insect cells. This scheme relies on epitope tags placed at the N terminus of SpORC4p where they do not interfere with SpORC function in vivo. We have obtained highly purified SpORC and demonstrated that it consists of a stable complex of six subunits analogous to ORCs of budding yeast, X. laevis, D. melanogaster, and Homo sapiens (6, 14, 15, 17).

Except for the well studied case of S. cerevisiae, the nature of origins of DNA replication in eukaryotic cells remains poorly understood. Previous genetic studies have shown that S. pombe origins differ significantly from those of S. cerevisiae, suggesting that the presence of ATP (Fig. 7). It has been reported that the interaction of purified SpORC strongly suggest that this is the case. The binding of SpORC to ars1 DNA is governed by the SpORC4 subunit, as demonstrated by the fact that the binding of the isolated SpORC4 subunit is indistinguishable from that of the complete SpORC holo-complex. We have previously shown that the N-terminal domain of SpORC4p, which contains nine AT-hook motifs, is necessary and sufficient for DNA binding by the isolated subunit (24), and the present study has shown that this domain is essential for viability of fission yeast. The binding of SpORC to ars1 origin DNA appears to have a relatively low level of sequence specificity. The protein is capable of binding to multiple sites within ars1 DNA with similar affinity. Although the protein binds with the highest affinity to AT-rich DNA, we also observed detectable binding to a control DNA with significantly lower AT content than that generally found in S. pombe origins. Our data are consist- ent with a model in which SpORC is targeted preferentially to long AT-rich regions within the S. pombe chromosomal DNA without regard to the specific nucleotide sequence of such regions. We expect that such regions would contain multiple and potentially overlapping binding sites for SpORC as in the case of ars1. One consequence of this model is that SpORC would be expected to be targeted preferentially to non-coding regions of the genome which have a higher average AT content than coding sequences. Obviously, our data do not rule out the possibility that the pattern of SpORC binding and initiation of DNA replication in vivo may be modulated by other factors such as chromatin organization and local transcriptional activ- ity (38). Nevertheless, it is likely that origin recognition in S. pombe is significantly less constrained by primary nucleotide sequence than in S. cerevisiae. It is possible that the same is true for metazoans, but further work will be required to assess this possibility (18, 19).
DNA is strictly dependent upon binding of ATP to Orc1, and it seems likely that ATP binding and hydrolysis play an important role in the assembly and function of the initiation complex (6, 39). The binding of SpORC to ars1 DNA does not require ATP, but it is possible that there are additional interactions between SpORC and DNA that are dependent on ATP. Such interactions could be difficult to detect in the presence of the strong ATP-independent binding mediated by SpORC-4p, especially if they are weak or transient in nature. One function of the N-terminal domain of SpORC-4p may be to facilitate additional protein-DNA interactions by tethering the complex to origins, thereby increasing its local concentration in the vicinity of the DNA. While such interactions may not directly contribute to origin selection, they may be critical for the downstream steps in initiation of DNA replication. This possibility is currently under investigation.

Cdc6p/Cdc18p plays a key role in the initiation of S. pombe DNA replication and appears to be essential for the loading of the putative MCM helicase at origins (40, 41). It has been assumed that Cdc18p is targeted to origins via interactions with ORC, but there have been few biochemical studies to probe this interaction directly. In vitro studies with X. laevis egg extracts have shown that ORC is required for the association of Xenopus Cdc6p with chromatin (7). More recently, biochemical studies with purified S. cerevisiae proteins have demonstrated interactions between Cdc6p and ScORC that alter the conformation of ScORC and modulate its DNA binding properties (11). Our data indicate that purified S. pombe ORC interacts specifically and efficiently with Cdc18p in the presence or absence of origin DNA. This finding differs from the results of studies in S. cerevisiae demonstrating that the Cdc6p-ScORC interaction is stabilized by the presence of origin DNA (11). This difference could be due to species variation or may simply reflect differences in experimental conditions, since special washing conditions were required to observe origin dependence of the Cdc6p-ORC interaction in S. cerevisiae, and the dependence was not observed in other studies (34, 36). Neither the binary SpORC-Cdc18p complex nor the ternary SpORC-Cdc18p-ars1 complex requires ATP for formation. As noted above, the possible role of ATP in the interaction between S. cerevisiae Cdc6p and ScORC is not yet clear, although it has been demonstrated that mutations in the nucleotide-binding motifs of Cdc6p do not abolish the interaction in vitro (11). It appears that Cdc18p has some affinity for ars1 DNA in the absence of SpORC. This observation may be related to the recent finding that S. cerevisiae Cdc6p has an intrinsic nonspecific DNA binding activity (42), but further work will be required to assess this affinity, since we cannot yet completely rule out the presence of a contaminating DNA-binding protein in our purified Cdc18p. In any case, the association of Cdc18p with ars1 DNA is greatly enhanced by the presence of SpORC, consistent with the hypothesis that ORC functions to recruit Cdc18p to origins via direct interactions. The ability to form ternary complexes of SpORC, Cdc18p, and ars1 in vitro should facilitate the further biochemical analysis of the assembly of initiation complexes.

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