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ATPase-dependent, cooperative binding of ORC and Cdc6p to origin DNA

Christian Speck¹, Zhiqiang Chen², Huilin Li², and Bruce Stillman^{1,*}

¹Cold Spring Harbor Laboratory, 1 Bungtown Road, Cold Spring Harbor, NY 11724, USA

²Brookhaven National Laboratory, P.O. Box 5000; Upton, NY 11973-5000, USA

Abstract

Binding of Cdc6p to the Origin-Recognition-Complex (ORC) is a key step in the assembly of a pre-replication complex (pre-RC) at origins of DNA replication. ORC recognizes specific origin DNA sequences in an ATP-dependent manner. Here we demonstrate cooperative binding of *Saccharomyces cerevisiae* Cdc6p to ORC on DNA in an ATP-dependent manner, inducing a change in the pattern of origin binding that requires the Orc1p ATPase. The reaction is blocked by specific origin mutations that do not interfere with the interaction between ORC and DNA. Single particle reconstruction of electron microscopic images shows that the ORC-Cdc6p complex forms a ring-shaped structure with dimensions similar to the ring-shaped MCM helicase. The ORC-Cdc6p structure is predicted to contain six AAA+ subunits, analogous to other ATP-dependent protein machines. We suggest that Cdc6p and origin DNA activate a molecular switch in ORC that contributes to pre-RC assembly.

Pre-replication complex (pre-RC) assembly at origins of DNA replication is essential to license chromosomes before initiation of DNA synthesis occurs during S phase of the cell division cycle^{1–3}. The Origin-Recognition-Complex (ORC), a six-subunit, ATP-dependent DNA binding protein binds to specific DNA sequences at origins of DNA replication and is the foundation for pre-RC assembly^{4–6}. The Orc1p and Orc5p subunits are known to interact with ATP, however only the interaction between the Orc1p subunit and ATP is required for DNA binding and is essential in yeast. Once ORC is bound to origin DNA, the ORC ATPase is reduced or blocked^{7–9}. Binding of Cdc6p to ORC is a key step in the assembly of the pre-RC^{10–20}. In particular, the levels and activity of Cdc6 regulate the frequency with which any given origin is utilized during the cell cycle^{10,11,21}.

In yeast origins of DNA replication contain specific DNA elements, A, B1 and B2, not all of which are conserved at the nucleotide sequence level²². The A-element is essential and represents the principal binding site for the ORC. The B1 element is partially involved in ORC-DNA interaction and B2 mutants are impaired in loading Mini-Chromosome Maintenance (MCM) proteins on chromatin^{5,6,22–24}. The pre-RC was first described using an *in vivo* DNase I footprinting method²⁵. During the G2 phase of the cell cycle, the DNase I cleavage pattern at origins was consistent with ORC bound to DNA (i.e. protection over the A and B1 elements). In G1 phase, however, the pattern changed and a prominent hypersensitive site within B1 disappeared and the regions between B1 and B2 and the B2 element itself were protected. Since this change corresponded with chromatin binding of the MCM proteins and DNA licensing it has been assumed that MCM binding to DNA was responsible for the larger,

*Correspondence and Requests for materials should be addressed to: Bruce Stillman, 1, Bungtown Road, Cold Spring Harbor, NY 11724, USA. Stillman@cshl.edu, Telephone: (516) 367-8383, Fax: (516) 367-8879.

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extended DNase I footprint in G1. Loading of the MCM proteins depends on Cdc6p and Cdt1p, a protein first described in *S. pombe* that binds to the MCMs and Cdc6p directly and may act as a chaperone to bring the MCMs to the origin^{1–3}.

To understand the function and mechanism of the proteins involved in pre-RC formation, a reconstituted system with purified proteins is ultimately required since it enables detailed biochemical analysis. Here we focused on the first part of pre-RC reconstitution; the ATP-dependent interactions between ORC and Cdc6p in the presence or absence of origin DNA. Biochemical conditions have been found that allow efficient interaction between ORC, Cdc6p and origin DNA. With this system in hand we have investigated how origin DNA mutants, ATP binding mutants of ORC and Cdc6p and an ORC ATPase mutant influence the interaction between ORC and Cdc6p on and of DNA. Moreover, using electron-microscopic reconstructions, we have determined the structure of ORC and ORC-Cdc6p complexes in the absence of DNA, revealing a ring-like structure with dimensions consistent with the DNA binding studies. Finally, we predict that additional subunits of the ORC have a structure related to known ATP binding proteins. Our results suggest that Cdc6p activates an ATP-dependent switch in the ORC that is regulated by specific origin DNA elements to license origins of DNA replication *in vivo*.

RESULTS

ORC and Cdc6p form an pre-RC like footprint on DNA

The potential DNA binding activity of Cdc6p²⁶ was probed using deoxyribonuclease I (DNase I) footprinting with the *ARS1* origin of DNA replication²² as a DNA substrate. No specific DNA binding activity for Cdc6p could be observed on either strand (Fig. 1a). Binding of ORC to *ARS1* DNA was as reported⁴. Addition of increasing amounts of Cdc6p to ORC, however, caused extra protection of the regions between the *ARS1* B1 and B2 elements, and part of B2 (Fig. 1a, dash; note particularly the arrowed region) and the nuclease hypersensitive sites induced by ORC were reduced (Fig. 1a, *). Thus Cdc6p did not bind to DNA specifically but cooperatively bound the ORC-DNA complex, producing an extended footprint. At 10-fold lower ORC concentrations than those used in Figure 1, extended footprint formation was more sensitive to salt concentrations (supplementary Fig. 1). The extended footprint is very similar to the footprint observed *in vivo* when the pre-RC is formed in the G1 phase of the cell cycle¹² (Fig. 1b). Cdc6p induced a similar change in the ORC footprint pattern on the 2 μ origin of replication (Fig. 1c), suggesting that the Cdc6p-induced extended footprint is a general feature of replication origins.

Stable complex formation of ORC and Cdc6p on DNA

Next we determined whether the extended footprint was due to stable binding of Cdc6p to the ORC-DNA complex using a gel-shift assay. Addition of ORC, but not Cdc6p, to wild-type *ARS1* DNA resulted in a slower migrating band (Fig. 1d). Addition of stoichiometric amounts of Cdc6p to the ORC-DNA complex induced a supershift, indicating the formation of a stable ternary ORC-Cdc6p-DNA complex. Under stringent conditions ORC binds specifically to wild-type *ARS1* DNA as compared to *ARS1* A and B1 linker-scan mutants^{5,22}. We next asked if the ORC-Cdc6p complex conferred higher sequence specificity than ORC alone under less stringent conditions. ORC formed a complex with the B1 mutant DNA and addition of Cdc6p lead to a supershift that required 2-fold more Cdc6p than with wild-type DNA (Fig. 1d). This complex on the B1[–] DNA, however, did not induce a Cdc6p-dependent extended footprint (Fig. 1e). One problem could be that the B1 linker scan mutation has an effect on ORC DNA binding⁵. Therefore we used an *ARS1* point mutant 838A→G within the B1 element that had no ORC DNA binding defect (Fig 1d, ref. 5), but still had a defect in plasmid stability⁵. The 838A→G point mutant produced a normal Cdc6p-induced gel-shift (Fig. 1d) but showed a

significant reduction in the ORC-Cdc6p induced footprint (Fig. 1f; note particular regions indicated with arrows). The *ARS1* A⁻ mutant induced a Cdc6p-dependent gel-shift only at the highest Cdc6p concentration that also allowed weak, nonspecific binding of Cdc6p to DNA (supplemental Fig. 2). In the footprint assay with the *ARS1* A⁻ mutant, ORC bound to the B2 element as reported⁴, but addition of Cdc6p was unable to produce an extended footprint (Fig. 1g). These data show that Cdc6p preferentially binds to ORC when it is bound only to wild-type origin DNA and that the ORC-Cdc6p complex has increased DNA sequence specificity compared to ORC alone, consistent with previous observations¹⁸. The B1⁻ and 838A→G mutant data suggests that once an ORC-Cdc6p-DNA complex was established, formation of the extended footprint required either specific DNA sequences or a specific DNA structure. Mutations in the B1 element of *ARS1* lead to increased plasmid loss *in vivo*²², suggesting that the inability of Cdc6p to induce a pre-RC-like footprint on a B1 mutant *ARS1* DNA is one reason for the plasmid loss.

ATP requirements for ORC-Cdc6p-DNA complex formation

Although ORC requires ATP for origin DNA binding^{4,7}, ADP can substitute at about a thousand-fold higher nucleotide concentration²⁷ (supplemental Fig. 3). In contrast, high levels of ADP did not support binding of Cdc6p to the ORC-DNA complex in the gel-shift assay (supplementary Fig. 4) or the footprint assay (supplementary Fig. 5). This suggests that ORC, Cdc6p or both require ATP to form the ORC-Cdc6p-DNA complex. To investigate the ATP dependence in more detail, complexes were used that had mutations in the Walker A ATP binding sites of Orc1p (ORC-1A complex contains the Orc1p-K485T subunit), Orc5p (ORC-5A; Orc5p-K43E) or Cdc6p (Cdc6p-K114E)^{7,9,15–17,28,29}. In both the DNase I footprint assay (Fig. 2a) and the gel-shift assay (Fig. 2b) ORC-5A yielded results similar to wild-type ORC, suggesting that the Orc5p subunit of ORC had no ATP binding requirement for these reactions. This was expected, since the *orc5-K43E* mutant was viable in yeast^{9,30}. Cdc6p-K114E is lethal in yeast, fails to load MCM proteins on chromatin^{15–17} and was unable to form an extended footprint (Fig. 2a) or induce a supershift of the ORC-DNA complex (Fig. 2b). At the highest concentrations used, the Cdc6p-K114E mutant blocked ORC binding to origin DNA using the footprint assay (Fig. 2a) and at even higher concentrations blocked the ORC induced gel-shift (data not shown). In the footprint assay with the highest concentration of Cdc6p the ORC footprint was lost. This suggests that Cdc6p-K114E at high concentration can form a complex with ORC but the complex is unable to interact with DNA.

Orc1p ATP-hydrolysis promotes extended footprint formation

The ORC-1A mutant complex did bind to DNA, but only at elevated ATP concentrations⁹ (Fig. 2a and b). Using the gel-shift assay we found that Cdc6p bound to the ORC-1A-DNA complex (Fig. 2b), but an extended footprint pattern was not observed (Fig. 2a). One possibility is that ATP-hydrolysis is defective in this mutant, but since ORC-1A did not bind to DNA like wild-type ORC, we further analyzed two other ORC ATPase mutants. The first mutant, ORC-d1, has a mutation (D569Y) in the Walker B motif, resulting in reduced ATPase activity in the absence of DNA, however in the presence of DNA the ATPase was similar to wild-type ORC. ORC-d1 is viable in yeast when expressed at low copy, but lethal when over-expressed with wild-type *ORC2–6*. ORC-d1 has a significant DNA binding defect in comparison to wild-type ORC (Fig. 2c), formed a complex with Cdc6 on DNA (Fig. 2c), but was unable to produce the extended footprint (Fig. 2d). However 5-fold higher ORC-d1 concentrations, which compensated the DNA binding defect, lead to extended footprint formation (data not shown). Possibly the DNA binding defect of ORC-d1 can be tolerated *in vivo* since origins function redundantly and can compensate ORC DNA binding mutants¹¹. The second mutant ORC complex, ORC-4R, has a mutation in the arginine-finger of Orc4p (R267A)³¹. This mutant had no observable ATPase activity, but in contrast to ORC-1A, bound ATP like wild-type ORC31. In the gel-shift assay, ORC-4R behaved like the wild-type ORC (Fig. 2c) and formed

a complex with Cdc6p on DNA. However the ability of ORC-4R to form the extended footprint was reduced or absent (Fig. 2d, note particularly region indicated with the large arrow). Instead, the limited footprint over the B2 element induced by ORC-4R alone was slightly enhanced by Cdc6p, but not to the extent observed when wild type ORC was used.

orc1-K485T and *orc4-R267A* are lethal in yeast, suggesting that formation of the extended footprint by the ORC-Cdc6p-DNA complex is essential *in vivo*. The gel-shift data suggest that formation of an ORC-Cdc6p complex on DNA is not dependent on ATP hydrolysis. In contrast, formation of the extended footprint requires ATP hydrolysis by Orc1p, which is activated by Orc4p. Consistent with this hypothesis, the non-hydrolysable ATP analogue ATP γ S supported formation of the ORC-Cdc6p-DNA complex as judged by the gel-shift assay (supplementary Fig. 4), however formation of the extended footprint was hindered and instead a weak, Cdc6p-induced footprint over the B2 element and just upstream of B1 appeared, as for the ORC-4R ATPase mutant (supplementary Fig. 6). At high concentrations, Cdc6p bound nonspecifically to the entire DNA and caused a non-specific DNase 1 footprint (supplementary Fig. 6).

ORC and Cdc6p only interact in the presence of DNA

We next analyzed if ORC or its mutants (ORC-1A, ORC-5A, ORC-d1, ORC-4R) could complex with Cdc6p in the absence of DNA using a glycerol gradient sedimentation assay. The proteins were assayed in the presence of ATP, ATP γ S (Fig. 3) or ADP (ORC and Cdc6p; supplementary Fig. 7). Wild-type ORC, all the ORC mutants and Cdc6p individually fractionated as if they were monomers⁴ (Fig. 3a). ORC, ORC-5A, ORC-d1 and ORC-4R each formed a complex with Cdc6p in the presence of ATP γ S, but not in the presence of ATP (Fig. 3a) or ADP (supplemental Fig. 7), as evidenced by the disappearance of monomeric Cdc6p and its concomitant appearance in the same fraction as ORC (Fig. 3a), or by western blot of the peak fraction (Fig. 3b). In contrast, ORC-1A was unable to complex with Cdc6p. The glycerol gradient sedimentation data suggest that under physiological conditions of ATP, ORC and Cdc6p bind to each other only in the presence of origin DNA. ATP γ S (but not the ORC-4R ATPase mutant) can bypass this DNA requirement, probably by inducing structural changes in the AAA+ subunits that stabilize the interaction.

The ORC and ORC-Cdc6p structures

To better understand the interaction between ORC and Cdc6p, structures of yeast ORC and ORC-Cdc6p complexes in the presence of ATP γ S and in the absence of DNA were determined by single particle reconstruction from electron micrographs. The 3D maps of ORC and ORC-Cdc6p complexes were determined independently from ~18,000 and ~20,000 particles, respectively. The resolution of the maps was estimated to be ~2.5 nm. At this resolution, ORC is an elongated structure with roughly three domains labeled α , β , and γ , respectively, along the length of the density (Fig. 4 a–c). A less structured ω region completed a small ring the central domain. The length of ORC is about 16 nm, approximately the length of the 48 base-pair ORC-induced footprint on origin DNA. Upon binding of Cdc6p (Fig. 4 d–f), ORC maintained its basic three-domain structure, in spite of numerous changes across the map. The most dramatic change is a gain of density to the left of the ω domain of the ORC structure. Since this is the only major density gain in ORC-Cdc6p as compared to the ORC structure, we attribute it to Cdc6p, and it is so labeled in Fig. 4d. Another significant change is located in γ -domain. It appears that one side of γ -domain in the ORC-Cdc6p structure changes (Fig 4 d and e, red arrows), compared to the same region in the ORC structure (Fig. 4a and b). The atomic structure of an archaeal Cdc6p/Orc1p orthologue can be fitted into the presumed yeast Cdc6p density, as shown by the pink ribbons (Fig. 4 d–f). The binding of Cdc6p onto ORC gives rise to a pronounced ring-like feature that is illustrated by dashed blue circles (Fig. 4 d). Interestingly, the inner and outer diameters of the ring-like density, about 6 nm and 12 nm, respectively, match the collar and external diameters of an archaeal MCM structure (Fig. 4 g–

I)³². The similarity of the physical dimensions of the two structures suggests that the ring-like feature of the complex might facilitate loading or assembly of the putative MCM replicative helicase onto origin DNA.

Conserved secondary structure in Orc1–5p and Cdc6p

Replication Factor C (RFC) contains five AAA+ protein subunits and loads in an ATP dependent process the ring-like PCNA DNA polymerase clamp onto DNA³³. A similarity between RFC and Cdc6p based on secondary structure prediction was proposed¹⁵, however we suggest that it is the ORC-Cdc6p complex that is analogous to RFC and the ORC-Cdc6p complex loads or assembles the hexameric ring-like MCM2–7 complex onto DNA in an ATP-dependent process. To date, four AAA+ proteins have been identified within the ORC-Cdc6p complex (Orc1p, Orc4p, Orc5p and Cdc6p^{15,29,30,34}). Intrigued by the analogy with RFC, we asked if there were other AAA+ proteins in the ORC-Cdc6p complex. In Orc2p we could detect all the conserved alpha-helix and beta-sheet secondary structure elements typical for the AAA+ fold, and less reliably in Orc3p (Fig. 5a; data not shown). The classical Walker A and B sequences seemed to be altered in Orc2p and Orc3p, however, both domains contain amino acids that are completely conserved in all species examined, particularly for Orc2p (Fig. 5b). In addition, based on the secondary structure predictions, we predict a C-terminal winged-helix domain in Orc2p, Orc3p, and Orc5p related to those known or predicted to exist in Cdc6p, Orc1p and Orc4p^{35–38}. Our analysis predicts two potential winged-helix domains in Orc5p, one at the very C-terminus and one internally. The potential winged-helix domain in Orc3p is also internal. In an ORC-DNA complex Orc1p, Orc2p, Orc4p and Orc5p were found to crosslink with DNA⁹. Cdc6p was found in this study to promote an extended footprint in the ORC-Cdc6p-DNA complex. We suggest that multiple winged-helix domains make DNA contacts in the ORC-Cdc6p-DNA complex.

DISCUSSION

Yeast cells arrested in G2 produce a footprint pattern like ORC alone and upon exit from mitosis, they exhibit the pre-RC extended footprint that indicates that the DNA has been licensed for initiation of DNA replication in S phase^{1,2,39}. The ATPase-dependent footprint induced by the ORC-Cdc6p on origin DNA *in vitro* closely resembles the pre-RC footprint *in vivo*^{12,14}. Interestingly a Cdc6p Walker B mutant produced almost the same footprint¹⁵ and was unable to load MCM proteins on chromatin. Therefore MCM proteins cannot reflect the major part of the pre-RC footprint as has been generally believed. Once loaded, the MCM proteins may form a ring around DNA and slide away from the origin³¹, again suggesting that the stable complex at origins during G1 consists of ORC and Cdc6p.

The length of the footprint found *in vivo* during G2 and *in vitro* with ORC bound to DNA (48–49 bp) is similar to the length of the ORC structure observed by electron microscopy (assuming an rigid B-form double-helix). This suggests that ORC binds DNA along its long axis. Interestingly, the extended footprint induced by ORC-Cdc6p *in vitro* and the pre-RC footprint found during G1 *in vivo* (76–82 bp) is longer than the observed ORC-Cdc6p complex, suggesting that if ORC-Cdc6p were solely responsible for the extended DNA footprint, the DNA must be bent or wrapped around the protein. ATP-dependent DNA wrapping has been observed for DnaA, the bacterial analogue of ORC.

Our data suggest a multi-step mechanism for formation of the ORC-Cdc6p-DNA complex. First, ORC binds to origin DNA in a sequence specific manner and the ORC ATPase activity is reduced⁹. We have shown that Cdc6 cannot form a stable complex with ORC in the absence of DNA, possibly because the ATPase activity of Cdc6p or ORC destabilizes the interaction between these two proteins. In the presence of origin DNA, however, Cdc6p stably binds to the ORC-DNA complex. Interestingly, the ORC-Cdc6p complex has an increased DNA

binding specificity compared to ORC alone, as seen with the *ARS1* A- mutant, (Fig. 1g) and as shown previously¹⁸. Cdc6p binding to ORC on DNA induces ATP hydrolysis (Speck and Stillman, unpublished) and the ORC-Cdc6p-DNA complex then adopts a new conformation, yielding a footprint similar to the pre-RC footprint *in vivo*. Based on data using ORC-4R or ATP γ S, this step involves ORC-mediated ATP hydrolysis. Now, however, instead inhibiting ORC ATPase, origin DNA sequences (B1) are required to form the ATPase-dependent extended footprint.

Thus, during pre-RC formation, origin DNA serves as a dual switch; first by reducing the ATPase of Orc1p in an origin sequence-specific manner to ensure that ATP-ORC is bound to origin DNA^{9,40,41}. Second, once Cdc6p binds, origin DNA is required to activate ORC-Cdc6p-DNA complex to produce an extended footprint. The second switch greatly changes the conformation of ORC¹⁸. Based on our studies using the *S. cerevisiae* system, we suggest that Cdc6p may increase the DNA binding of ORC from metazoan species, where DNA recognition by ORC alone has little DNA sequence specificity. Indeed, Cdc6p induced stabilization of ORC binding to sperm chromatin has been observed in *Xenopus* crude egg extracts in the presence of ATP γ S⁴². We further suggest that in metazoan species, it is the ORC-Cdc6p complex that determines the location of origins of DNA replication, mostly likely in conjunction with other DNA binding proteins⁴³.

Recently it has been shown that ORC-4R mediated loading of MCM proteins on DNA in a crude yeast extract is less efficient than with wild type ORC³¹. Less MCM proteins were loaded onto each origin in the presence of ORC-4R compared to ORC-mediated loading, suggesting that efficient MCM loading requires ORC ATPase activity. We suggest that the activated ORC-Cdc6p complex we observe on origin DNA is the complex that allows multiple MCM proteins to be loaded onto chromosomes during the G1 phase of the cell cycle. In contrast, in the absence of the Orc1p-ATPase activity the ORC-Cdc6p complex on DNA is in a conformation that can bind to the MCM proteins, however the MCM proteins are either not loaded correctly or not released from the ORC-Cdc6p complex.

The ORC-Cdc6p structure we observe here is predicted to contain six AAA+ related subunits, five from ORC. This makes the ORC-Cdc6p structure analogous to other hexameric AAA+ protein machines⁴⁴. Other DNA replication initiation complexes in bacteria and archaea also have AAA+ subunits and may function like the ORC-Cdc6p complex^{35,36}, although their higher order structures are still under investigation.

Both ORC and Cdc6p are subject to post-translational modification by cyclin-dependent protein kinases^{45–48}. It will be interesting to determine whether the ATPase-induced ORC-Cdc6p complex discussed herein will be regulated by these kinases.

METHODS

Plasmids

The *CDC6* gene was amplified from genomic DNA using primers fwd-BamHI (5'-CAGGGGCCCCCTGGGATCCATGTCAGCTATACCAATAACTCC) and rev-NotI (5'-TCAGTCACGATGCGGCCGCCTAGTGAAGGAAAGGTTTCAAATTG) and BamHI – NotI cloned into pGex-6P1. *ARS1* A-, B1-, B2-, B3- (858–865, 835–842, 802–808, 756, 758) were cloned by cleaving pARS1 A- and pARS1 B123- with BglII – NdeI. The *ARS1* A- large fragment and the *ARS1* B123- small fragment were joined to produce the A-B123- mutant.

Protein preparation

ORC was purified as described⁹. Cdc6p was purified from *E. coli* BL21 codon-plus cells (Stratagene) transformed with pGex-6P1-*CDC6* (Amersham). A 1-liter culture at OD₆₀₀ 0.6

was induced with 0.5 mM IPTG for 5 h at 18°C in a shaking water bath. The cells were lysed using lysozyme and sonication in PG buffer (50 mM potassium phosphate, 150 mM potassium glutamate [KGlu], 5 mM magnesium chloride, 2 mM ATP, 1 mM DTT, 1% Triton) with 2× complete protease inhibitor + EDTA (Roche) and centrifuged at 15,000 rpm for 15 min in a SS34 rotor. The supernatant was incubated for 2 h with 2 ml 50% slurry of glutathione-agarose on a rotating wheel. The beads were washed 3 times with 10 ml of PG buffer (+ 2× complete protease inhibitor + EDTA) and 3 times with 10 ml of PG buffer. 20 µl of PreScission Protease (Amersham) in 1 ml of PG buffer was added to the beads and incubated on a rotating wheel for 2 h. The eluted protein was collected and pooled with 3× 500 µl washes using PG buffer (75 mM KGlu). 1500 µl of PG buffer (0 mM KGlu) was added along with 450 µl 50% slurry of Hydroxyapatite beads and incubated on a rotating wheel for 15 min. Beads were washed with 3× 500 µl PG buffer (75 mM KGlu, -ATP), 3× 200 µl PG buffer (150 mM KGlu, 15% glycerol, -ATP), eluted with 200 µl fractions of PG buffer (400 mM KGlu, 15% glycerol, -ATP) and snap frozen in liquid nitrogen.

DNase I footprint assay

DNase I footprinting was performed as described⁴ with minor modifications. Origin DNA fragments were amplified using primers RSP1201 (5' AACAGCTATGACCATG) and SP1211 (5'-GTAAAACGACGGCCAGT), one primer was 5' end-labeled with γ -³²P and T4 polynucleotide kinase. Binding reactions were carried out in 25 µl of binding buffer (25 mM HEPES pH 7.6, 100 mM KGlu, 5 mM magnesium acetate, 5 mM calcium chloride, 5 mM DTT, 0.1% Triton X-100, 1 mM adenine nucleotide) and the indicated concentrations of ORC and Cdc6p. Cdc6p was in general preincubated with 1 mM adenine nucleotide as indicated for at least 4 h on ice. During the course of this study we found that Cdc6p had a high but nonspecific DNA binding activity in the absence of ATP, and addition of ATP suppressed this activity (data not shown). Mixtures were incubated on ice for 10 min, moved to 30°C for 5 min, 10 fmol of labeled PCR fragments, when not otherwise indicated, were added into the mixture and the solution was incubated for 5–10 min at RT. DNase I was added and samples were incubated at RT for 2 min. After addition of an equal volume of stop buffer (1% SDS, 200 mM NaCl, 20 mM EDTA pH 8.0, 1 mg/ml glycogen), the samples were purified by phenol-chloroform extraction, DNA was precipitated with ethanol and resuspended in 5 µl of sequencing gel loading buffer (98% formamide, 0.025% bromophenol blue, 0.025% xylene cyanol). Samples were incubated at 96°C for 5 min and loaded onto 6 % sequencing gels. After electrophoresis gels were dried and autoradiographed.

Gel-shift assay

The samples were prepared in general as for the DNase I footprint assay. Binding buffer contained additional 5% glycerol and 2 mg/ml BSA (Roche). A 290 bp GC-rich PCR fragment amplified from pBluescript using the primers GC-fwd (5'-GAGCCCCCGAGGTAGAGCTTGACGGGG) and GC-rev (5'-GGGAAAACCCCTGGCGTTACC) served as competitor. 37.5 ng competitor DNA was added along with 10 fmol specific DNA and the mixture was incubated for 10 min at RT. 5 µl of the sample were loaded on a 3.5% 80:1 polyacrylamide gel containing 0.5× TB (0.045 M Tris-borate at pH 8.3), the gel was run for 4 hr at a constant voltage of 150 V at 4°C, dried and autoradiographed.

Glycerol Gradient

Cdc6p was preincubated with 1 mM adenine nucleotide for at least 4 h. 20 pmol of protein were preincubated in 200 µl binding buffer (+ 10% glycerol) for 10 min on ice, 10 min at 30 °C and then loaded onto a 5 ml 15–50% glycerol gradient in binding buffer (0.1 mM adenine nucleotide) and centrifuged at 49,000 rpm for 10 hr in an SW55Ti rotor. Fractions (200 µl)

were collected from the top of the gradient. The last fraction was not always 200 μ l in volume resulting in quite different concentrations of the aggregated proteins at the bottom of the tube. The precipitated proteins were resuspended in 200 μ l 2 $\times$ SDS loading buffer. Every other fraction was separated on a 10% SDS-PAGE and silver stained. Gradients with standard proteins were run in parallel. For Western blotting relevant fractions were separated on a 10% SDS-PAGE, transferred to nitrocellulose membrane, and probed with the monoclonal anti-Cdc6p antibody 9H8 at 1:500 dilution¹⁴.

Specimen preparation and electron microscopy

Specimens for electron microscopy were prepared on glow-discharged 300-mesh copper grids covered with a thin layer of carbon film. 5 μ l of ORC or ORC-Cdc6p complexes at a concentration of 0.025 mg/ml in 50mM Hepes, pH 7.6, 100 mM KGlu, and 1mM EDTA, was pipetted to the grid and after 1 min incubation, blotted to leave a thin layer of sample on the surface. The grid was then stained by a 5 μ l drop of 2% uranyl acetate aqueous solution. The stain solution was blotted after 1min and the grid was left for air-drying. The specimen grids were examined in a Jeol-1200EX microscope operated at 120 kV. The underfocus value used for recording images ranged from $\sim$ 1 to 2 μ m. The micrographs were recorded on Kodak SO-163 negative films at a magnification of 50,000 with an exposure dose of $\sim$ 10 e/ $\text{\AA}^2$. To obtain an initial model of ORC with random conical tilt method⁴⁹, tilt pairs at 0° and 45° of the same area were recorded. Films were developed in full-strength D19 for 12 min. Images were examined on an optical diffractometer and only those without drift or astigmatism were digitized in a Nikon supercool ED 8000 scanner at 12.7 μ m step, corresponding to 2.54 $\text{\AA}$ at specimen level.

Image analysis and model reconstruction

Computer processing of the ORC and ORC-Cdc6p particle images was carried out with standard single particle image processing techniques. The defocus value for each electron micrograph was determined by software SPIDER. The particles were picked up interactively in WEB, an image display and manipulation interface of SPIDER. We manually selected about 20,000 particles from 50 selected micrographs of ORC-Cdc6p sample and $\sim$ 18,000 particles from another 50 selected micrographs of ORC sample. 2D particle images were corrected for the effect of contrast transfer function and then normalized before reference free alignment. The initial 3D model was reconstructed with the random conical tilt techniques⁴⁹. The refinement was performed with the software package EMAN⁵⁰. The iterative refinement procedure includes an extra step during each cycle for reference-based 2D image classification that effectively avoids the initial model bias problem. In addition, the final ORC structure was used to refine the ORC-Cdc6p data set, and the resultant map converged to the ORC-Cdc6p structure; conversely, the ORC-Cdc6p structure was used as a starting model to refine the ORC data set, and the resultant map converged to the ORC structure. The cross refinement test further confirms that the 3D reconstructions are not biased by the starting model. The resolution of the final map was estimated by applying the 0.5 threshold to the Fourier shell correlation between two maps calculated from two halves of the data. The maps were contoured at levels to include volumes corresponding to $\sim$ 413 kDa for the ORC and $\sim$ 470 kDa for ORC-Cdc6p complex. Docking of the crystal structures of the Cdc6p (PDB code 1FNN) and the MCM (PDB code 1LTL) was performed manually using the program O. There are three domains in the archaeal Cdc6 structure³⁸. The nucleotide-binding site is located at the interface between the N-terminal two domains, and the third flexible C-terminal domain is a winged helix domain typically found in DNA-binding structures. Domain III was separated from the first two domains in order to fit the atomic structure into the observed density.

Supplementary Material

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Figure 1. ORC and Cdc6p form a pre-RC like complex on DNA. Important elements of the *ARS1* origin of replication are indicated as A, B1, B2 and B3. **a**, DNase I footprints were done with 2 ng (0.4 nM) of a 290 bp DNA *ARS1* fragment labeled with γ -³²P at the 5'-end of either strand (lanes 1–10, T-rich strand of the *ARS* consensus sequence; lanes 11–20, A-rich strand). Lanes 1 and 11 contain no protein. Lanes 2–5, 7–10, 12–15 and 17–20 contain 3.4, 8.5, 17 and 42.5 nM Cdc6p. Lanes 6–10 and 16–20 contain 40 nM ORC. Regions protected by the addition of Cdc6p are indicated by a vertical bar. Hypersensitive sites are indicated by a star. The arrow points to one region discussed in the text. **b**, *ARS1* sequence including the A, B1 and B2 element. Comparison of *in vivo* footprint data of G2 and G1 arrested yeast cells¹² and *in vitro* footprint data with ORC or ORC and Cdc6p. **c**, DNase I footprint with ORC and Cdc6p at the 2μ origin of replication. Lane 1 contains no protein. Lanes 5–8 contain 40 nM of ORC. Lane 2–4 and 6–8 contain 1.4, 7 and 17 nM Cdc6p. **d**, Gel-shift assay of p³² labeled *ARS1* DNA with ORC and Cdc6p. Protein concentrations are indicated in the figure. Wt and mutant *ARS1* sequences are indicated in the figure, A-, B1-, B2-, B3- (858–865, 835–842, 802–808, 756, 758), B1 point-mutant 838A→G, B1- (835–842). **e**, DNase I footprint with ORC and Cdc6p at the wt and B1 mutant (835–842) *ARS1* origin of replication as indicated. Lane 1 and 6 contain no protein. Lanes 2–5 and 7–10 contain 40 nM ORC. Lanes 3–5 and 8–10 contain 1.4, 7 and 17 nM Cdc6p. **f**, DNase I footprint with ORC and Cdc6p at the wt and B1 point mutant 838A→G *ARS1* origin of replication. Protein concentrations as in **a**. The arrow points to regions discussed in the text. **g**, DNase I footprint with ORC and Cdc6p at the wt and A-mutant (858–865) *ARS1* origin of replication. Protein concentrations as in **e**.

Figure 2. Analysis of ATP binding and hydrolysis mutants in ORC and Cdc6p. **a**, DNase I footprint with ORC and Cdc6p ATP binding mutants at the T-rich strand of *ARS1* origin of replication. Protein concentrations of ORC and Cdc6p as in Figure 1a. For ORC-1A (lanes 36–38) is shown a titration with 1 μ M, 10 μ M, 100 μ M and 1 mM ATP. **b**, Gel-shift assay of γ -³²P labeled *ARS1* DNA with ORC and Cdc6p ATP binding mutants. Proteins used and concentrations of ORC and Cdc6p are indicated in the figure. **c**, Gel-shift assay of γ -³²P labeled *ARS1* DNA with wt ORC, ORC1 ATP hydrolysis mutants and Cdc6p. Proteins used and concentrations of ORC and Cdc6p are indicated in the figure. **d**, DNase I footprint with wt ORC, ORC1 ATP hydrolysis mutants and Cdc6p at the *ARS1* origin of replication. Protein concentrations as in figure 1a.

Figure 3. ORC - Cdc6p interaction analyzed by glycerol gradient sedimentation. **a**, The load onto the gradient (lane L) and fractions as well as any material in the pellet (lane B) were analyzed by gel electrophoresis and silver staining. Arrows indicate the sedimentation position of protein standards from a gradient prepared in parallel. Proteins analyzed and adenine nucleotide are indicated. **b**, Western blot analysis of ORC - Cdc6p peak fractions with an monoclonal α Cdc6p antibody.

Figure 4. Three-dimensional structures of the yeast ORC. **a, b, c**, ORC alone. **d, e, f**, ORC in complex with the yeast Cdc6p. **g, h, i**, Density maps of a hexameric archaeal MCM N-termini complex³². The MCM complex was generated by filtering the atomic structure to a resolution of 2.5nm. An archaeal Cdc6 ortholog³⁸ is displayed by pink ribbons. See supplementary movies 1 and 2.

Figure 5.

Analysis of AAA+ signatures within the proteins Orc1p, Orc2p, Orc3p, Orc4p and Orc5p and Cdc6p. **a**, secondary structure prediction of Orc1p, Orc2p, Orc3p, Orc4p, Orc5p and Cdc6p. Amino acids shown are indicated. Red corresponds to predicted α -helical and green to a β -sheet prediction using the SAM-T02 method (Karplus, K. et al. Combining local-structure, fold-recognition, and new fold methods for protein structure prediction. Proteins 53 Suppl 6, 491-6 (2003); <http://www.soe.ucsc.edu/research/compbio/HMM-apps/>. Deletions of sequence corresponding to no structure alignment are indicated by // and insertions by v. The amino acid sequence of the Walker A and B boxes and their position within the sequence is indicated in the bottom. **b**, Sequence conversation within the Walker A and B boxes from different species (*S. cerevisiae*, *S. pombe*, *H. sapiens*, *M. musculus*, *D. melanogaster*, *X. laevis*) for Orc1p, Orc2p, Orc3p, Orc4p, Orc5p and Cdc6p are shown in the sequence logo format (Crooks, G. E., Hon, G., Chandonia, J. M. & Brenner, S. E. WebLogo: a sequence logo generator. Genome Res 14, 1188-90 (2004) <http://weblogo.berkeley.edu/logo.cgi>.