The pre-replicative complex (pre-RC) is formed at all potential origins of replication through the action of the origin recognition complex (ORC), Cdc6, Cdt1, and the Mcm2-7 complex. The end result of pre-RC formation is the loading of the Mcm2-7 replicative helicase onto origin DNA. We examined pre-RC formation in vitro and found that it proceeds through separable binding events. Origin-bound ORC recruits Cdc6, and this ternary complex then promotes helicase loading in the presence of a pre-formed Mcm2-7-Cdt1 complex. Using a stepwise pre-RC assembly assay, we investigated the fate of pre-RC components during later stages of the reaction. We determined that helicase loading is accompanied by dissociation of ORC, Cdc6, and Cdt1 from origin DNA. This dissociation requires ATP hydrolysis at a late stage of pre-RC assembly. Our results indicate that pre-RC formation is a dynamic process.

Eukaryotic DNA replication initiates at origins of replication throughout the genome. The first event in this tightly regulated process is origin selection. In the budding yeast *Saccharomyces cerevisiae*, specific nucleotide sequences mark sites where replication can initiate. These sites are bound by the origin recognition complex (ORC) (1, 2). As cells enter G1, ORC recruits Cdc6, and the Mcm2-7 replicative helicase to the origin DNA resulting in pre-replicative complex (pre-RC) formation (3). Activation of S phase kinases leads to the recruitment of numerous additional factors to the Mcm2-7 complex resulting in the formation of a pair of bi-directional replisomes (4). Multiple mechanisms regulate pre-RC formation to ensure that each origin can only initiate once per cell cycle (5). Thus, the pre-RC marks all potential sites of replication initiation in G1 and forms the foundation for the assembly of the replisome, which is tightly regulated to ensure complete genome replication.

Studies of *S. cerevisiae* DNA replication initiation have been greatly facilitated by the short (80–120 bp), well defined origins of replication of this organism (6). These sequences include a conserved 11-bp autonomous replicating sequence consensus sequence and multiple 10–15-bp B-elements. Much of our understanding of the pre-RC has come from *in vitro* origin binding assays. It was shown that the ORC-origin interaction is ATP-dependent (7) and that Cdc6 cooperatively binds the ORC-DNA complex (8). This ternary complex is also regulated by Cdc6 ATPase activity (9). *In vitro* pre-RC assembly in budding yeast extracts indicates sequential association of ORC, Cdc6, Cdt1, and Mcm2-7 with the origin (10, 11), and ATP hydrolysis by Cdc6 is required for Mcm2-7 loading (12).

In this study, we examine pre-RC formation *in vitro* and find that the process can be separated into three distinct sequential binding events. The final step of Mcm2-7 complex loading correlates with reduced origin association of ORC, Cdc6, and Cdt1. The dissociation of these proteins from the origin requires ATP hydrolysis at a late step in pre-RC assembly. Our findings indicate that the pre-RC proteins associate with the origin in a dynamic fashion.

**EXPERIMENTAL PROCEDURES**

**Strains, Extracts, and Protein Purification—Yeast strain ySC15 (Gal1,10-ORC1-6 (13)) was used to prepare ORC-loading whole cell extract. Purified ORC was prepared from F1ORC1, an equivalent strain with the addition of a FLAG tag at the amino terminus of Orc1 (kind gift of S. Kang). Cdc6-HA and Cdt1 were purified from *Escherichia coli* as described previously (12). Purified Mcm2-7 complex was prepared from strains VTY167 (Gal1,10-Mcm2-7) and VTY169 (Gal1,10-Mcm2-7 and Gal-Cdt1). Whole cell extracts were prepared in lysis buffer (100 mM HEPES (pH 7.6); 0.8 mM sorbitol; 10 mM magnesium acetate; 2 mM EDTA; 300 mM potassium glutamate) using the Sample Prep 6870 Freezer/Mill (Spex Certi Prep Group; 8 cycles of 2 min grinding at setting 10). Lysates were dialyzed against H buffer, 300 mM potassium glutamate (H buffer; 50 mM HEPES (pH 7.6); 5 mM magnesium acetate; 1 mM EDTA; 1 mM EGTA; 10% glycerol; 0.01% Nonidet P-40). FLAG purification of ORC and Mcm2-7-Cdt1 was performed using anti-FLAG M2 affinity gel (Sigma) per the manufacturer’s instructions. Briefly, 4 liters of culture were grown, induced with galactose, and arrested in G1 phase with α-factor. Whole cell extract was prepared as above except the lysate was not dialyzed. FLAG resin was equilibrated in FLAG buffer (50 mM HEPES (pH 7.6); 300 mM potassium glutamate; 2 mM dithiothreitol; 10% glycerol; 1 mM EDTA; 0.02% Nonidet P-40). 2 ml of FLAG resin was incubated with extract for 1 h at 4 °C, washed with 15-column volumes of FLAG buffer, and eluted with 0.15 mg/ml triple FLAG peptide (Sigma) in FLAG buffer. FLAG-ORC was further purified on a 1-ml SPHP column (GE Healthcare) and eluted with H buffer, 500 mM KCl buffer.

**Pre-replicative Complex Assembly—** A 1,116-bp DNA fragment containing the wild type or A-B2− mutant *ARS1* origin sequence was amplified using an oligonucleotide primer with a photocleavable biotin attachment (Integrated DNA Technologies) at one end. The biotinylated origin DNA was coupled to...
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streptavidin M-280 Dynabeads (Invitrogen) as described previously (13). For each assembly reaction, 250 fmol of bead-bound origin DNA was incubated with either extract or purified proteins in a buffer containing 25 mM HEPES (pH 7.6), 20 mM phosphocreatine, 2 mM dithiothreitol, 40 μg/ml creatine kinase, 12 mM magnesium acetate, 3 mM ATP, and 300 mM potassium glutamate. Reaction times were 10 min for ORC and Cdc6 binding and 20–30 min for Mcm2-7-Cdt1 binding. All reactions were performed at 25 °C. For two- or three-step reactions, beads were washed three times with wash buffer (H buffer containing 300 mM potassium glutamate, 1 mM dithiothreitol, and 0.05% Nonidet P-40) before the next step. At the completion of the assembly assay, beads were washed three times with wash buffer. Salt extractions involved substitution of the middle wash with H buffer, 400 mM KCl. Washed beads were resuspended in 8 μl of 10 mM Tris (pH 7.6), 1 mM EDTA. A 254-nm UV light was used to cleave the DNA-protein complexes off the beads on ice for 15–30 min. The photocleavage supernatant was transferred to a new tube; 2 μl of × SDS-PAGE sample buffer was added, the sample was boiled for 5 min and analyzed by SDS-PAGE and immunoblotting. For Cdc6 and Mcm2-7-Cdt1 titration experiments, reactions were supplemented with the appropriate purification buffer to maintain equivalent reaction conditions across the titration. Assays comparing ATP and ATPγS were as above except nucleotide concentration was increased to 5 mM to overcome any effect of residual nucleotide in the extracts used.

RESULTS

Mcm2-7 Loading Leads to Reduced Origin-associated ORC—In our studies of in vitro pre-RC assembly, we frequently observed reduced ORC levels at origins upon Mcm2-7 loading (12). These observations were difficult to interpret due to extract-to-extract variations in the efficiency of ORC and Mcm2-7 loading. Background binding of proteins to the beads used to immobilize and precipitate origin DNA further obscured these apparent effects on origin-associated ORC. To better study the fate of ORC in pre-RC assembly, we used a photocleavable biotin linkage on the origin DNA to cleave the DNA from streptavidin magnetic beads with minimal disturbance of DNA-protein complexes.

We analyzed the association of ORC and Mcm2-7 with the UV-released DNA in the presence or absence of pre-RC formation. We performed pre-RC assembly using a G1-arrested whole cell extract from a strain overexpressing all six ORC subunits. Because the levels of Cdc6 were very low in the G1-arrested cells from which the extract was prepared, no pre-RC formation was observed in the absence of added Cdc6. Adding recombinant Cdc6 protein allowed pre-RC formation to occur (12). We observed substantially reduced levels of ORC on the origin DNA when pre-RC formation occurred relative to the same reaction in the absence of Cdc6 (Fig. 1).

Pre-RC Assembly Can Be Separated into Distinct Binding Steps—A one-step extract-based pre-RC assembly assay was ill-suited to study ORC dissociation because of the continued presence of high concentrations of ORC in the extract through-out the reaction. To eliminate the possibility that ORC dissociates and rebinds the origin during the pre-RC assembly assay, we separated the ORC binding step from the rest of the assembly reaction. Our source for ORC was either a whole cell extract from an ORC-overexpressing strain (“extract ORC”) or FLAG-ORC purified from a similar strain (Fig. 2D, lane 2). In the absence of added Cdc6, neither one of these sources of ORC supports pre-RC formation (13) (data not shown). Recombinant Cdc6 was incubated with DNA-bound ORC in a second binding step. Next, we sought to eliminate unknown factors that might influence ORC-origin association during helicase loading by purifying the helicase and its associated loading factor Cdt1 rather than using a cell extract to provide these components. Mcm2-7-Cdt1 was purified from a strain overexpressing all seven proteins (Fig. 2D, lane 3). We used purified Mcm2-7-Cdt1 to complete pre-RC formation in a third binding step. Washes after each step eliminated unbound proteins.

We found that we could still observe Mcm2-7 association when pre-RC formation was separated into three sequential steps (Fig. 2A). This result indicates that Cdc6 binds origin-associated ORC stably in the absence of Mcm2-7-Cdt1 and that the ternary origin-ORC-Cdc6 complex can support helicase loading. Furthermore, purified ORC, Cdc6, and Mcm2-7-Cdt1 were sufficient to bring Mcm2-7 to the origin in our reconstituted in vitro assembly assay indicating that no other factors are required in this system. As observed with the extract reaction (Fig. 1), origin association of ORC was significantly reduced in the presence of pre-RC formation (Fig. 2A, compare lanes 2 and 3 to lanes 1 and lanes 5 and 6 to lane 4). ORC released from origin DNA was detectable in the supernatant of Mcm2-7 loading reactions suggesting that proteolysis is not involved in ORC dissociation (data not shown).
Both extract-derived ORC and purified ORC were able to load purified Mcm2-7 proteins that were resistant to salt extraction (Fig. 2B, lanes 5 and 7). Although purified ORC bound origins efficiently and was active in Cdc6 and Mcm2-7-Cdt1 recruitment (Fig. 2B, lane 3) we found that it exhibited more origin-independent DNA binding (Fig. 2B, lane 4). Extract-derived ORC did not bind DNA in the absence of the A and B2 autonomous replicating sequence elements (A-B2 mutant, Fig. 2B, lane 2). This is likely due to other proteins in the extract blocking weak, non-origin-specific ORC-binding sites present on the DNA. As we were interested in studying origin association of ORC during pre-RC assembly, all subsequent experiments were carried out using the extract-derived ORC.

We also purified Mcm2-7 complex from a strain overexpressing Mcm2-7 but not Cdt1 (Fig. 2D, lane 4). We note that Mcm2-7-Cdt1, while keeping Cdc6 constant (Fig. 3B). We found that Mcm2-7 loading increased as a function of Cdc6 or Mcm2-7-Cdt1 input and ORC loss correlated with the level of Mcm2-7 loading. ORC was stabilized in the absence of Cdc6 and Mcm2-7-Cdt1, while keeping Cdc6 constant (Fig. 3A, lane 1) or by titrating Cdc6, while keeping Mcm2-7-Cdt1 constant (Fig. 3A), or by titrating helicase purification in the presence of Cdt1 co-overexpression stabilizes Mcm3 and Mcm5 in the complex. The yield of these subunits is much reduced in the absence of Cdt1, presumably because of reduced association with Mcm4 (purification is based on an Mcm4 FLAG tag and therefore enriches for Mcm4 and Mcm4-containing complexes). We found that Mcm2-7 proteins derived from this strain were unable to load onto origin DNA even when recombinant Cdt1 was supplied in the reaction (Fig. 2C). The same recombinant Cdt1 complements the ability of an extract depleted of Cdt1 to load Mcm2-7 (Ref. 12; data not shown). We speculate that the Mcm2-7 complex is inactive when not associated with Cdt1 and that this association is facilitated by factors in the yeast extract prior to pre-RC assembly.

Increased Mcm2-7 Loading Correlates with Reduced ORC-Origin Association—To determine whether ORC loss from the origin is an effect of Cdc6 addition or the extent of Mcm2-7 loading, we performed two titration experiments. In each case, ORC was loaded in a first step; unbound ORC was washed away, and the DNA-bound ORC was divided into 5 equal aliquots. Pre-RC formation was performed in a second reaction with added Cdc6 and Mcm2-7-Cdt1. We varied the amount of pre-RC formation in these reactions by titrating either Cdc6, while keeping Mcm2-7-Cdt1 constant (Fig. 3A), or by titrating Mcm3 and Mcm5 in the complex. The yield of these subunits is much reduced in the absence of Cdt1, presumably because of reduced association with Mcm4 (purification is based on an Mcm4 FLAG tag and therefore enriches for Mcm4 and Mcm4-containing complexes). We found that Mcm2-7 proteins derived from this strain were unable to load onto origin DNA even when recombinant Cdt1 was supplied in the reaction (Fig. 2C). The same recombinant Cdt1 complements the ability of an extract depleted of Cdt1 to load Mcm2-7 (Ref. 12; data not shown). We speculate that the Mcm2-7 complex is inactive when not associated with Cdt1 and that this association is facilitated by factors in the yeast extract prior to pre-RC assembly.

**FIGURE 2. Pre-RC assembly can be reconstituted in separate steps.** A, pre-RC assembly can be separated into three sequential steps. For each reaction, 250 fmol of ARS1 origin DNA coupled to magnetic beads was incubated with either whole cell extract overexpressing all ORC subunits (extract ORC) or purified ORC (FLAG-ORC) (step 1), followed by recombinant Cdc6 (step 2), followed by reaction buffer (buf), purified Mcm2-7-Cdt1 (M/C) or Mcm2-7-Cdt1, and recombinant Cdc6 (W/C/B) (step 3). The DNA beads were washed between each step to eliminate unbound protein. Following the three incubations, all reactions were washed, and DNA-protein complexes were released from the beads by photolysis and analyzed by immunoblotting with polyclonal sera for the Mcm2-7 (MCM) and ORC complexes. B, purified ORC exhibits higher nonorigin DNA binding than extract ORC. 500 fmol of bead-bound wild type or A-B2 mutant origin 5′ARS1 DNA was incubated with either whole cell extract from a strain overexpressing all ORC subunits or purified ORC. Unbound ORC and extract were washed off and Mcm2-7-Cdt1 was loaded in the presence of Cdc6 in a subsequent reaction. Each reaction was then divided into two parts. One set was washed in H buffer, 300 mM potassium glutamate (lanes 1–4) and the other in H buffer, 400 mM potassium chloride (salt-extracted, lanes 5–8). C, co-overexpression of Cdt1 is required for loading of purified Mcm2-7. Mcm2-7 was purified from a strain overexpressing (o/e) Mcm2-7 (lanes 1 and 2) or Mcm2-7 and Cdt1 (lanes 3 and 4). ORC was loaded onto bead-bound 5′ARS1 DNA from whole cell extract; unbound proteins were washed away, and the reaction was divided into four parts. Recombinant Cdc6 was added to all reactions, and Mcm2-7, Mcm2-7-Cdt1, and recombinant Cdt1 were added as indicated. D, Coomassie Brilliant Blue staining of purified proteins. Purified proteins in the amounts used per 250-fmol origin DNA assay were separated on 7% SDS-PAGE (lane 1, Bio-Rad broad range molecular weight standards; lane 2, 0.9 μg of FLAG-ORC; lane 3, 2.5 μg of Mcm2-7-Cdt1; lane 4, 2.5 μg of Mcm2-7; lane 5, 0.8 μg of Mcm2-7; lane 6, 0.1 μg of Cdt1).
difficulty by using a wild type yeast whole cell extract as a source of Mcm2-7-Cdt1, expressed at endogenous levels. Wild type extracts lack sufficient ORC or Cdc6 to participate in the \textit{in vitro} pre-RC assembly reaction. We performed a three-step pre-RC assembly assay during which ORC was first loaded from an ORC-overexpressing whole cell extract. Recombinant Cdc6 was added in a second reaction. Finally, Mcm2-7-Cdt1 was added from a wild type extract. Each step was performed in either 5 mM ATP or 5 mM ATP-S, and washes between steps eliminated unbound nucleotide and protein.

We found that ATP hydrolysis was required for loss of origin-associated ORC at a late step during \textit{in vitro} pre-RC assembly. The nucleotide used in the ORC binding step did not affect the ability of ORC to associate with DNA or the subsequent steps of pre-RC formation (Fig. 4A, \textit{lanes} 7–10 to \textit{lanes} 11–14). In contrast, addition of ATPγS in the Cdc6 binding step stabilized Cdc6 on the origin DNA (Fig. 4A, \textit{lanes} 4 and 6) but had no effect on the levels of ORC-DNA association. Robust Mcm2-7 protein loading was only observed when ATP was present in the final step (Mcm2-7-Cdt1 addition). When ATPγS was present in the final step, independent of which nucleotides were present in the ORC and Cdc6 binding steps, little or no Mcm2-7 loading was observed (Fig. 4A, \textit{lanes} 8, 10, 12, and 14). The increase in Mcm2-7 loading observed in the reactions that included ATPγS in the second step (Cdc6) and ATP in the third step (Fig. 4A, \textit{lanes} 9 and 13) was likely due to the ATPγS-induced increased level of Cdc6 on DNA prior to the third reaction step (Fig. 4A, \textit{lanes} 4 and 6), consistent with Mcm2-7 loading increasing as a function of Cdc6 input (Fig. 3A). Importantly, ORC loss was only dependent on the presence of ATP in the final step of the reaction (Fig. 4, \textit{lanes} 7, 9, 11, and 13). ATPγS in the Mcm2-7-Cdt1 binding step led to stabilization of ORC in all cases (Fig. 4A, \textit{lanes} 8, 10, 12, and 14).

As observed previously (12), Cdt1 was stabilized on DNA in the presence of ATPγS. Cdt1 signal was at background levels when ATP was used in both the second (Cdc6 binding) and third (Mcm2-7-Cdt1 binding) steps of the pre-RC assembly assay (Fig. 4A, \textit{lanes} 7 and 11). When the third step was carried out in ATPγS, some Cdt1 protein was stabilized on origin DNA (Fig. 4A, \textit{lanes} 8 and 12). The absence of appreciable Cdt1 stabilization in reactions where ATPγS was present in the second step and ATP in the third step could be due to greater importance of ATP hydrolysis by Mcm2-7 or nucleotide exchange by Cdc6 during the course of the 20-min incubation time of this third...
block the conversion of DNA-"associated" but salt-labile Mcm2-7 to a "loaded" salt-resistant complex (12). We performed two-step pre-RC assembly reactions in which ORC binding was followed by Mcm2-7-Cdt1 binding in the presence of increasing amounts of Cdc6. Wild type whole cell extract was used as the source of Mcm2-7-Cdt1 in this experiment. First, titration of Cdc6 was carried out in the presence of ATP (which allows Mcm2-7 loading to proceed) or ATPγS (which allows Mcm2-7 association but prevents subsequent loading). Under these conditions, the levels of DNA-associated Mcm2-7 were much lower in ATPγS (Fig. 4B, left panel). To reduce DNA-associated Mcm2-7 levels in ATP and approach the low levels of the remaining pre-RC components. Previous studies of in vitro pre-RC assembly failed to detect the dissociation of origin-associated ORC and Cdc6 due to technical limitations. Our use of photocleavable origin DNA, purified components, and a stepwise assembly reaction improved the signal-to-noise ratio in our experiments, minimized rebinding of ORC and other components during the course of the reaction, and revealed the destabilization of ORC, Cdc6, as well as Cdt1 upon Mcm2-7 loading. Loss of origin-associated ORC, Cdc6, and Cdt1 was dependent on ATP hydrolysis event in the late steps of pre-RC assembly (most likely linked to the loading of Mcm2-7 onto origin DNA) stimulated the origin release of the remaining pre-RC components.

**DISCUSSION**

In this study, we examined the assembly of pre-RCs in vitro and found that loading of Mcm2-7 onto origin DNA stimulated the release of ORC, Cdc6, and Cdt1 at the origin required ATP hydrolysis even under conditions where equal levels of Mcm2-7 were associated with origin DNA. In ATP reactions, no Cdc6 or Cdt1 protein could be detected on origin DNA, and ORC levels dropped significantly in the presence of ATPγS (Fig. 4B, lanes 1–5 and 11–15). In contrast, ATPγS led to stabilization of Cdc6, Cdt1, and ORC on origin DNA (Fig. 4B, lanes 6–10). Based on these findings, we conclude that the initial association of Mcm2-7 does not lead to ORC and Cdc6 release. Instead, an ATP hydrolysis event in the late steps of pre-RC assembly (most likely linked to the loading of Mcm2-7 onto origin DNA) stimulated the origin release of the remaining pre-RC components.
ORC and Cdc6 Release during Pre-RC Formation

Pre-RC Assembly as a Series of Sequential Binding Events—Using our modified pre-RC assembly assay, we found that it is possible to completely separate the ORC, Cdc6, and Mcm2-7-Cdt1 binding steps into three sequential reactions. It was shown previously that Cdc6 can bind the ORC-DNA complex stably (8). We now show that this ternary complex is both stable and capable of loading Mcm2-7 onto DNA in the absence of free ORC or Cdc6 in the final loading step.

As had been observed previously (11), Mcm2-7 and Cdt1 co-purified as a complex. Omission of Cdt1 co-overexpression resulted in reduced Mcm3 and Mcm5 levels in our protein purification yield, suggesting that Cdt1 may stabilize these subunits in the complex. Interestingly, we found that when purified and added to the assembly reaction separately, Mcm2-7 and Cdt1 did not reconstitute pre-RC formation. In contrast, recombiant Cdt1 complements Mcm2-7 loading activity in a Cdt1-immunodepleted extract (12). We suggest that the whole cell extract contains cofactors that help form the active Mcm2-7-Cdt1 complex prior to pre-RC assembly. This seven-protein complex is then recruited to origin-bound ORC-Cdc6 complex to form the pre-RC. The requirement for a pre-formed Mcm2-7-Cdt1 complex could reflect a role for Cdt1 in maintaining the helicase in a state that is competent for loading. Cdt1 also plays a direct role in helicase recruitment to the origin through interaction with ORC (15).

ATPase-dependent Dissociation of Pre-RC Components—In our experiments, it was the nucleotide used in the Mcm2-7-Cdt1 binding step that most affected ORC, Cdc6, and Cdt1 association with origin DNA. This finding could suggest a role for one or more of the Mcm2-7 subunits, all of which are AAA+ proteins, in the release of the remaining pre-RC subunits. Alternatively, it is possible that during the lengthy incubation (20 min) required for Mcm2-7 loading, there is nucleotide exchange in ORC and/or Cdc6 and that hydrolysis by these ATPases stabilizes the release of the other proteins. Consistent with the latter possibility, it was previously observed that inhibition of Cdc6 ATP hydrolysis prevents release of Cdt1 from the origin DNA (12).

ATP hydrolysis regulates the binding of AAA+ pre-RC components (7–9). ATP yS allows Mcm2-7 association with the origin while inhibiting salt-stable Mcm2-7 loading (12). Release of ORC, Cdc6, and Cdt1 could occur concurrently with and as a consequence of salt-stable helicase loading, so that the ATPase activity required for ORC, Cdc6, and Cdt1 dissociation is the same activity shown to mediate Mcm2-7 loading. It is also possible that the pre-RC complex is dismantled following helicase loading in one or more separate ATP hydrolysis-dependent events stimulated by helicase loading but directly involved in the release of ORC, Cdc6, and Cdt1 from the origin. Mutants that target the ATPase activity of individual pre-RC subunits may help to distinguish among these possibilities. In either case, the ATP hydrolysis event(s) required for ORC, Cdc6, and Cdt1 dissociation occur late in pre-RC assembly, after the stable association of ORC and Cdc6 with the origin and in the presence of Mcm2-7-Cdt1.

The persistence of a fraction of ORC on origin DNA after Mcm2-7 loading may be indicative of inefficiency in the in vitro pre-RC assembly assay. Cdc6 and Mcm2-7-Cdt1 input was set at saturating levels for helicase loading, and the Cdc6 and Cdt1 proteins that participated in the loading reaction appeared to completely dissociate from the origin in ATP. This suggests the possibility that only a subset of DNA-bound ORC in our assay successfully participated in the reaction (i.e., recruited Cdc6, loaded Mcm2-7, and dissociated from the origin). We speculate that the ORC remaining on DNA at the end of the reaction was not able to bind the origin in a conformation competent for Cdc6 recruitment and helicase loading.

As a population-based experiment, the in vitro pre-RC assembly assay does not distinguish between different species of origin-bound ORC. Because we cannot show complete removal of ORC and complete helicase occupancy of origin DNA in vitro, we cannot confirm that ORC is lost at Mcm2-7-loaded origins. It remains formally possible that ORC is lost at origins that have failed to load Mcm2-7, whereas ORC is retained at origins that assemble a “full” pre-RC. Single molecule experiments would be needed to distinguish between the two possibilities. We note that we failed to detect protein complexes significantly larger than predicted for ORC or Mcm2-7 alone on DNA in electron micrographs of our pre-RC assembly products. Given the dose response and ATP hydrolysis dependence of ORC, Cdc6, and Cdt1 loss with increasing Mcm2-7 loading (Figs. 3 and 4), our experiments do show that Mcm2-7 loading activity causes dissociation of these proteins from the origin in vitro.

Pre-RC as a Dynamic Complex—Our results suggest that the pre-RC is a dynamic complex. ORC and Cdc6 are stably bound to the origin until Mcm2-7-Cdt1 loading stimulates their ATPase-dependent dissociation. As demonstrated previously, Cdt1 is a transient part of the pre-RC (12). Pre-RC formation in Xenopus egg extracts also involves changes in the DNA binding of ORC and Cdc6. Upon licensing, these proteins become salt-sensitive (16). Whereas multiple Mcm2-7 complexes appear to bind Xenopus chromatin per ORC (17), the Cdt1 inhibitor geminin stabilizes ORC on DNA to levels equimolar to Mcm2-7 with no reduction of Mcm2-7 levels (18). Thus, as in our yeast in vitro system, ORC is normally lost from Xenopus origins when Mcm2-7 is loaded, and this loss requires Cdt1 activity.

Cdc6 and the Mcm2-7 complex specifically associate with origins only in G1 (19, 20). On the contrary, chromatin spin down (21) and chromatin immunoprecipitation experiments (20) have found that S. cerevisiae ORC associates with the chromatin fraction throughout the cell cycle. Inefficiency and asynchrony of pre-RC formation, as well as rapid rebinding of ORC, may obscure ORC dissociation from the origin in these in vivo experiments. Furthermore, as our experiments are performed on a chromatin-free linear DNA template, our data only suggest that ORC is released from the origin DNA as a consequence of Mcm2-7 loading. Pre-RC assembly (or disassembly) in vivo may involve additional protein contacts that retain factors on chromatin. Therefore, ORC may still be maintained on chromatin through interactions with histones (22) after its release from the DNA. Such retention or rapid rebinding of ORC would be required for the reiterative helicase loading described previously (13).

5 V. Tsakraklides, S. P. Bell, and J. D. Griffith, unpublished data.
Yeast origins exhibit a genomic footprint that is altered in a Cdc6-dependent manner in G1 (23, 24). In vitro evidence suggests that the pre-replicative pattern is equivalent to that proposed for origins in G1 with no evidence for ORC dissociation, Mcm2-7 loading, or a more extended region of protection that might correspond to a stable pre-RC (ORC-Cdc6-Mcm2-7-Cdt1 complex) at the origin. As Mcm2-7 origin association has been well established by other methods, we suggest that genomic footprinting is not suited to detect the short-lived single molecule events surrounding Mcm2-7 loading.

Previous studies have shown that immunoprecipitation of ORC-origin complexes was reduced as Mcm2-7-origin complexes formed in G1 (20). Preventing pre-RC formation by inactivating Cdc6 resulted in increased ORC-origin immunoprecipitation (20). This is consistent with the possibility that ORC is removed from the origin once it successfully completes helicase loading in vivo as it is in our in vitro assay.

After pre-RC formation, ORC and Cdc6 can be removed from the chromatin fraction selectively by salt extraction without removing the Mcm2-7 complex (26), as is true for pre-RCs assembled in vitro (13). This suggests that ORC, Cdc6, and Cdt1 are required for the loading but not the maintenance of Mcm2-7 at origins of replication. In agreement with such a model, downstream Mcm2-7 phosphorylation by CDC-Dbf4 requires loaded Mcm2-7 but is independent of ORC, Cdc6, and Cdt1 (27). The clearing of DNA-bound ORC, Cdc6, and Cdt1 could play a role in facilitating formation of the pre-initiation complex and the passage of the replication fork through the origin. Rebinding of ORC to origin DNA for the next cycle of pre-RC formation would not be a novel challenge for the cell as the newly replicated origin requires at least one new ORC complex to be recruited to the second copy of the origin.

Another line of evidence suggests that continued ORC function is required for pre-RC maintenance. Execution point analysis showed that ORC is required for viability in late G1 phase after pre-RC formation has already taken place (28, 29). Targeted degradation of Orc6 in G1-arrested cells led to loss of Mcm2-7 association with all sites of pre-RC formation across the genome (15, 29). A continued requirement for ORC function is not incompatible with a pre-RC assembly pathway that leads to ORC release from origin DNA. We suggest the possibility that loaded Mcm2-7 is unstable in the prolonged G1 phase examined in these experiments, and ORC is required to restart pre-RC assembly when the helicase is lost from the origin.

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