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Cdc6 ATPase Activity Regulates ORC·Cdc6 Stability and the Selection of Specific DNA Sequences as Origins of DNA Replication*

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DNA replication, as with all macromolecular synthesis steps, is controlled in part at the level of initiation. Although the origin recognition complex (ORC) binds to origins of DNA replication, it does not solely determine their location. To initiate DNA replication ORC requires Cdc6 to target initiation to specific DNA sequences in chromosomes and with Cdt1 loads the ring-shaped mini-chromosome maintenance (MCM) 2–7 DNA helicase component onto DNA. ORC and Cdc6 combine to form a ring-shaped complex that contains six AAA+ subunits. ORC and Cdc6 ATPase mutants are defective in MCM loading, and ORC ATPase mutants have reduced activity in ORC·Cdc6·DNA complex formation. Here we analyzed the role of the Cdc6 ATPase on ORC·Cdc6 complex stability in the presence or absence of specific DNA sequences. Cdc6 ATPase is activated by ORC, regulates ORC·Cdc6 complex stability, and is suppressed by origin DNA. Mutations in the conserved origin A element, and to a lesser extent mutations in the B1 and B2 elements, induce Cdc6 ATPase activity and prevent stable ORC·Cdc6 formation. By analyzing ORC·Cdc6 complex stability on various DNAs, we demonstrated that specific DNA sequences control the rate of Cdc6 ATPase, which in turn controls the rate of Cdc6 dissociation from the ORC·Cdc6·DNA complex. We propose a mechanism explaining how Cdc6 ATPase activity promotes origin DNA sequence specificity; on DNA that lacks origin activity, Cdc6 ATPase promotes dissociation of Cdc6, whereas origin DNA down-regulates Cdc6 ATPase resulting in a stable ORC·Cdc6·DNA complex, which can then promote MCM loading. This model has relevance for origin specificity in higher eukaryotes.

Jacob and Brenner (1) described the replicon model in 1963 that outlined in general terms how DNA replication might start at a specific location within chromosomes. This model formed the basis for discovery of initiator proteins that recognize origins of DNA replication. The bacterial initiator, DnaA (2), recognizes the bacterial origin of DNA replication, *oriC*, by binding to multiple 9-mer DnaA boxes within the origin (3). However, DnaA also binds frequently to DnaA boxes outside of DNA replication origins, and these sites do not promote initiation of DNA replication (4); therefore, the origin is defined by a combination of specific protein-DNA interactions, including DnaA, histone-like protein HU, and integration host factor proteins and an arrangement of DNA sequences that form multiple binding sites in a specific conformation (5). It is only this particular conformation that allows the initiator to promote origin unwinding, to recruit other DNA replication proteins such as the DnaB helicase and helicase loading protein DnaC to the DNA, and consequently to initiate DNA synthesis (5).

The mechanism that specifies the location of origins of DNA replication in eukaryotic chromosomes is less clear particularly in mammalian cells, but it involves a combination of sequence specific protein-DNA interactions, epigenetic factors, and cell cycle timing (6, 7). In *Saccharomyces cerevisiae*, specific DNA sequences are known to contribute the location of origins of DNA replication in chromosomes (8–10). Knowledge of such sequences led to the discovery of the origin recognition complex (ORC)[3] (11), a six-subunit complex. Homologues of ORC exist in all eukaryotic species. Yeast origins contain several conserved genetic elements (12); the A element, which serves as a binding site for ORC (11), the B1 element, which is partially involved in ORC binding (13, 14), and the B2 element as a potential binding site for loading the mini-chromosome maintenance (MCM) proteins (15, 16). ORC in higher eukaryotes does not bind specifically to DNA but has a preference for AT-rich (17, 18) or supercoiled DNA (19).

Our understanding of how DNA sequences or locations within the chromosome are selected as sites of initiation of DNA replication is still poor since it is unclear what defines a start site for initiation of DNA replication. *S. cerevisiae* ORC binds to origins in a DNA sequence-specific fashion; however, this feature is not conserved in higher eukaryotes. But because the protein components required for initiation of DNA replication are conserved, it is expected that studies of how this process occurs in yeast will shed light on the process in all eukaryotes. In budding yeast it has been shown that the Orc1 subunit in ORC has to be in the ATP-bound form for ORC to bind DNA (11, 20). Orc1-dependent ATPase, stimulated by an

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[3] The abbreviations used are: ORC, origin recognition complex; MCM, mini-chromosome maintenance protein; pre-RC, pre-replicative complexes; ARS, autonomously replicating sequence; ATPγS, adenosine 5′-*O*-(thiotriphosphate).

arginine residue in an adjacent Orc4 subunit, is also regulated in a DNA sequence-specific manner (20), but this ATPase activity has no influence on the selection of DNA sequences as origins of DNA replication (21). Although ATP-bound ORC can bind to origins of DNA replication specifically, its affinity cannot explain why some DNA sequences are selected as origins and others are not, *e.g.* ORC binding sites have been identified by chromatin immunoprecipitation analysis that do not act as replication sites (10).

Another protein involved in the sequential assembly of prereplicative complexes (pre-RCs) at origins of DNA replication is the Cdc6 protein (cdc18 in *Schizosaccharomyces pombe*) (22–27). Cdc6 is a member of the AAA+ family of proteins and has striking amino acid sequence similarity with Orc1, the largest subunit of ORC (28, 29). Together with ORC and Cdt1, Cdc6 is required for loading the MCM proteins onto origin DNA (30–36). Cdc6 binds to and changes the structure of ORC, and together they bind cooperatively to origin DNA with higher DNA sequence specificity than ORC binding alone (37, 38). Specific nucleotides within the origin DNA are required for ORC·Cdc6·DNA complex formation, and Orc1 ATPase mutants are less efficient in forming the ORC·Cdc6·DNA complex (38). We were interested in understanding how Cdc6 contributes to the selection of certain DNA sequences in chromosomes of yeast to function as origins of DNA replication, a process with potential relevance to origin selection in higher eukaryotes. Because Cdc6 is also an AAA+ protein, like Orc1–5 (38, 39), and many initiation proteins use their ATPase activity in a regulatory fashion (40), we analyzed the contribution of Cdc6 ATPase activity to ORC·Cdc6 and ORC·Cdc6·DNA complex assembly and its impact on the selection of DNA sequences as origins of DNA replication.

EXPERIMENTAL PROCEDURES

Proteins—ORC expressed in insect cells from recombinant baculoviruses and Cdc6 expressed in *Escherichia coli* were purified to apparent homogeneity as described (11, 20, 38).

ATPase Assays—ORC and Cdc6 ATP hydrolysis was carried out as described (20), with minor modifications. 2.5 pmol of ORC and 2.5 pmol of Cdc6 were incubated for 30 min on ice in 12 μl of ATPase buffer (25 mM HEPES, pH 7.6, 100 mM potassium glutamate, 5 mM magnesium acetate, 1 mM dithiothreitol, 1 mM EDTA, 1 mM EGTA, 0.1% (v/v) Triton X-100, 10% glycerol) containing 2.5 pmol of DNA (when indicated) and 100 μM ATP (unless otherwise indicated). After the incubation 5 μCi of [α-^{32}P]ATP (3000 Ci/mmol) was added and the reaction was started by moving the tubes from ice into a room temperature water bath. At 15, 30, 45, and 60 min 2-μl aliquots were removed and stopped with 0.5 μl of 2% SDS stop solution. 1 μl of the samples were consequently spotted on TLC plates and developed.

DNA Probes—DNA probes and competitor DNA (290 bp of GC-rich DNA) used in the gel shift and/or footprinting experiments were prepared as described (12, 38). For the ATPase experiments a 91-bp fragment (residue 790–880) was amplified from plasmid pARS1WT or from the various mutant pARS1 plasmids (in the case of the A$^-$, B1$^-$, B2$^-$ fragment, an oligo (B1$^-$) primer, and a A$^-$, B2- template DNA was used). For ARS607 and ARS607A$^-$, a 91-bp fragment was amplified from pARS607 with primer ARS 607 forward 91 bp (5′-CTTACGCTGGGTATTTTTTTTTTGG-3′) and primer ARS607 reverse 91 bp (5′-GAGCTTTGTCTTGTTTATATTTAGTTACG-3′). For ARS607A$^-$ a modified primer was used to create a linker scanning mutation as in pARS1 A$^-$ using primer ARS 607 A-REV 91 bp (5′-GAGCTTTGTCTTGTCCTCGAGGAGTTACGTTGGG-3′).

Glycerol Gradients, Footprints, and Gel Shifts—Experiments were performed as described (38).

Determination of Dissociation Constants—Dissociation constants were determined using a gel shift assay. Gel shift reactions were performed as described previously (38) in the absence of competitor DNA. After challenging the complex with 100-fold cold competitor DNA (*ARS1* 291 bp) aliquots were loaded onto a continuously running gel in the cold room at the indicated time points. k_{off}, R values, and $t_{½}$ rates were determined using KaleidaGraph 4.0 (Synergy software). All experiments were repeated at least twice with similar results.

RESULTS

Cdc6 ATPase Is Induced by ORC·Cdc6 Complex Formation—ORC and Cdc6 have been shown to form a stable complex in the presence of a slowly hydrolysable ATP analogue, ATPγS, but not in the presence of ATP (38). This finding suggests that ATP hydrolysis regulates complex stability. To address this question in more detail we analyzed ATPase activities and complex formation of ORC and Cdc6 (Fig. 1). Cdc6 on its own had no ATPase activity as reported (31, 41) (Fig. 1*A*). The ORC ATPase activity was similar to what has been reported (20). In contrast to a recent study (31) that used different salt conditions, we found very robust ATPase activity when Cdc6 was added to ORC, a 2.5-fold induction over the ATPase activity of ORC alone. This result suggests that ORC and Cdc6 form at least a transient complex in the absence of DNA that induces either ORC or Cdc6 ATPase activity. To identify the active ATPases in the ORC·Cdc6 complex we analyzed two Cdc6 ATPase mutants (Cdc6 E224G and Cdc6 N263A) and an ORC ATP hydrolysis mutant (ORC4R/5A) in ATPase assays. Cdc6 E224G has a mutation in the highly conserved Walker B motif (31, 35) and is dominant lethal when overexpressed in yeast. Cdc6 N263A (42, 43) has a mutation in the sensor 1 motif and is temperature-sensitive and lethal when overexpressed. Cdc6 belongs to the class of AAA+ proteins, and mutations in Walker B or sensor 1 motifs frequently interfere with the ability of AAA+ proteins to hydrolyze ATP (40). The two Cdc6 mutants individually displayed no ATPase activity, similar to wild type Cdc6 (Fig. 1*A*). The addition of Cdc6 N263A to ORC did not change the ATPase activity, in contrast to wild type Cdc6. Cdc6 E224G addition to ORC led to a reduction of overall ATPase activity. These results suggest that Cdc6 is a major ATPase within the ORC·Cdc6 complex. To analyze the contribution of ORC toward ATPase activity of the ORC·Cdc6 complex, we analyzed an ORC4R/5A double mutant (Fig. 1*B*). Mutations in the arginine finger of ORC4 (ORC4R) have been shown to abolish Orc1-dependent ATPase (21). ORC5A carries a mutation in the Walker A motif of Orc5 that blocks ATP binding to ORC5; however,

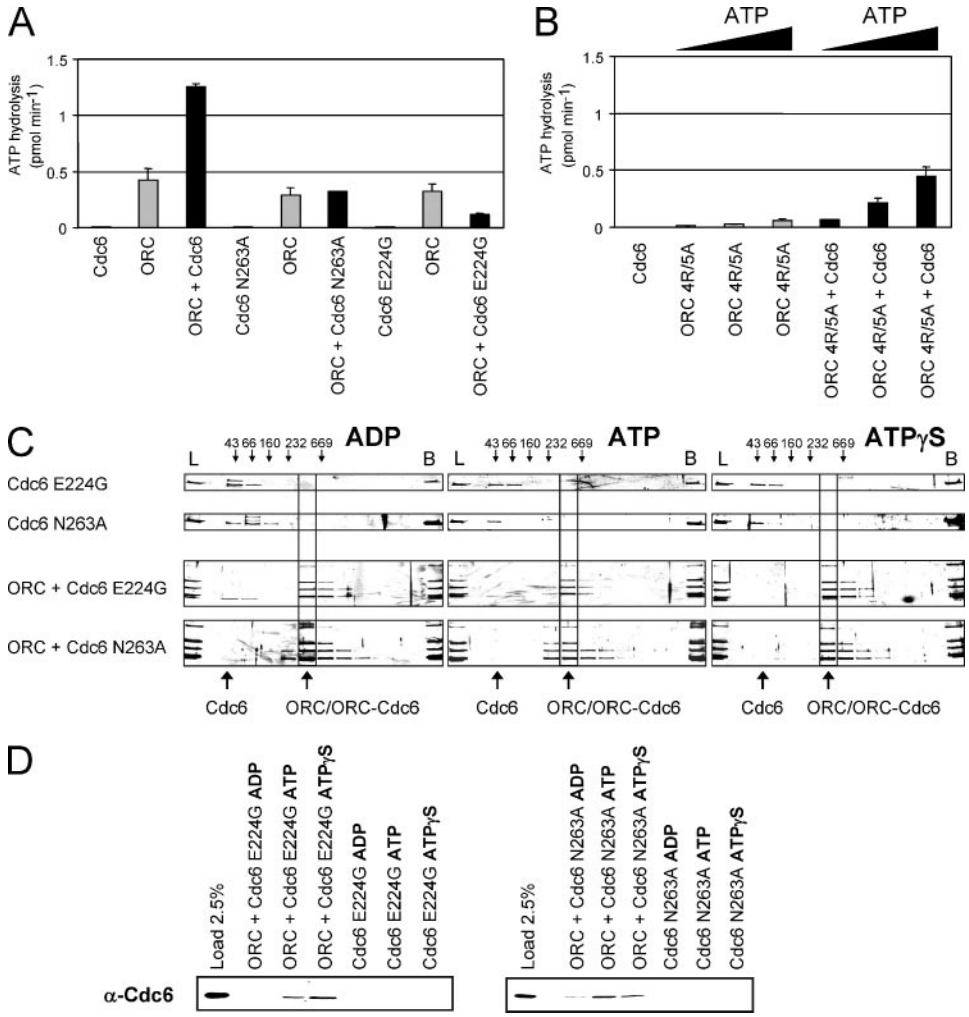

FIGURE 1. **Cdc6 and ORC interaction is regulated by ATPase activity.** *A*, ATP hydrolysis by ORC, Cdc6, Cdc6 E224G, Cdc6 N263A, ORC·Cdc6, ORC·Cdc6 E224G, and ORC·Cdc6 N263A. ATP hydrolysis rates were determined in the absence of DNA. *B*, ATPase activity in the presence of the ORC4R/5A ATPase mutant. Increasing ATP concentrations were 10, 100, and 1 mM. The *lane* containing Cdc6 alone contained 100 mM ATP. *C*, interaction between ORC and Cdc6, Cdc6 E224G, Cdc6 N263A, ORC·Cdc6 E224G, and ORC·Cdc6 N263A were analyzed by glycerol-gradient sedimentation. The load onto the gradient (*lane L*) and fractions as well as any material in the pellet (*lane B*) were analyzed by SDS-PAGE and silver staining. *Arrows* indicate the peak sedimentation position of protein standards, Cdc6, ORC, and the ORC·Cdc6 complexes. ORC and ORC·Cdc6 sedimentation positions overlap. Proteins were analyzed in the presence of the indicated nucleotide. *D*, immunoblot analysis of Cdc6 E224G, Cdc6 N263A, ORC·Cdc6 E224G, and ORC·Cdc6 N263A. Fractions corresponding to the ORC·Cdc6 peak from the glycerol gradients were subjected to SDS-PAGE analysis and analyzed by immunoblot with a monoclonal anti-Cdc6 antibody.

this mutation has no significant phenotype *in vitro* and *in vivo* (20, 44, 45). We measured ATPase of the combined ORC4R/5A mutant in the absence and presence of Cdc6 (Fig. 1*B*). ORC4R/5A showed a very weak ATPase that was dependent on high but physiological relevant ATP concentrations (up to 1 mM). The addition of Cdc6 to the ORC4R/5A mutant lead to a significant increase in ATPase activity, and this was dependent on high ATP concentrations as well. High ATP concentrations, however, had no effect on the ORC·Cdc6-induced ATPase (data not shown). These results show that ORC4R/5A can stimulate Cdc6 ATPase. Taken together, the ORC and Cdc6 ATPase data suggest that both ORC and Cdc6 are active ATPases in the ORC·Cdc6 complex since ATPase mutants in both ORC and Cdc6 did not abolish ATPase activity completely.

Cdc6 ATPase Regulates ORC·Cdc6 Stability—Using glycerol gradient sedimentation, we have shown that in the presence of ATP, ORC and Cdc6 did not form a stable complex, whereas in the presence of the non-hydrolyzable ATP analogue, ATPγS, a stable ORC·Cdc6 complex was observed (38). This suggests that ATPase activity controls the stability of the ORC·Cdc6 complex in the absence of DNA. The ORC mutants ORC-4R, an Orc1-dependent ATP hydrolysis mutant (21), and ORC-1A, an Orc1 ATP binding mutant (20), did not form a stable ORC·Cdc6 complex in the presence of ATP (38). These results indicate that ORC ATPase in the ORC·Cdc6 complex does not regulate ORC·Cdc6 complex stability, suggesting that it is the Cdc6 ATPase that is responsible. This possibility was examined by analyzing Cdc6 ATPase mutants for their ability to form a stable complex with ORC (Fig. 1, *C* and *D*). Cdc6 E224G and Cdc6 N263A were fractionated either individually or together with ORC in the presence of ADP, ATP, or ATPγS by glycerol gradient sedimentation (Fig. 1*C*). Cdc6 E224G and Cdc6 N263A individually fractionated as monomers. The Cdc6 ATPase mutants co-fractionated with ORC in the presence of ATP and ATPγS, as seen by the disappearance of monomeric Cdc6 and concomitant appearance of Cdc6 in the peak fraction containing ORC, which was verified by Western blot with anti-Cdc6 antibodies (Fig. 1*D*). In the absence of ORC, Cdc6 E224G and N263A were not found in the fraction corresponding to the ORC·Cdc6 (Fig. 1*D*). Previously we have shown that in the presence of ADP, ORC and Cdc6 do not form a complex (38). This is also true for Cdc6 E224G; however, Cdc6 N263A formed a weak complex with ORC in the presence of ADP, probably due to inefficient exchange of ATP for ADP.

The results so far show that Cdc6E224G and Cdc6N263A were capable of forming a stable complex with ORC in the presence of ATP, which was not true for wild type Cdc6 protein. The results suggest that, in the absence of DNA, the Cdc6 ATPase negatively regulates ORC·Cdc6 complex stability. Because Orc1 and Orc5 ATP binding or ATPase activity have no influence on ORC·Cdc6 complex stability (38), it is the Cdc6 ATPase within the ORC·Cdc6 complex that regulates complex stability.

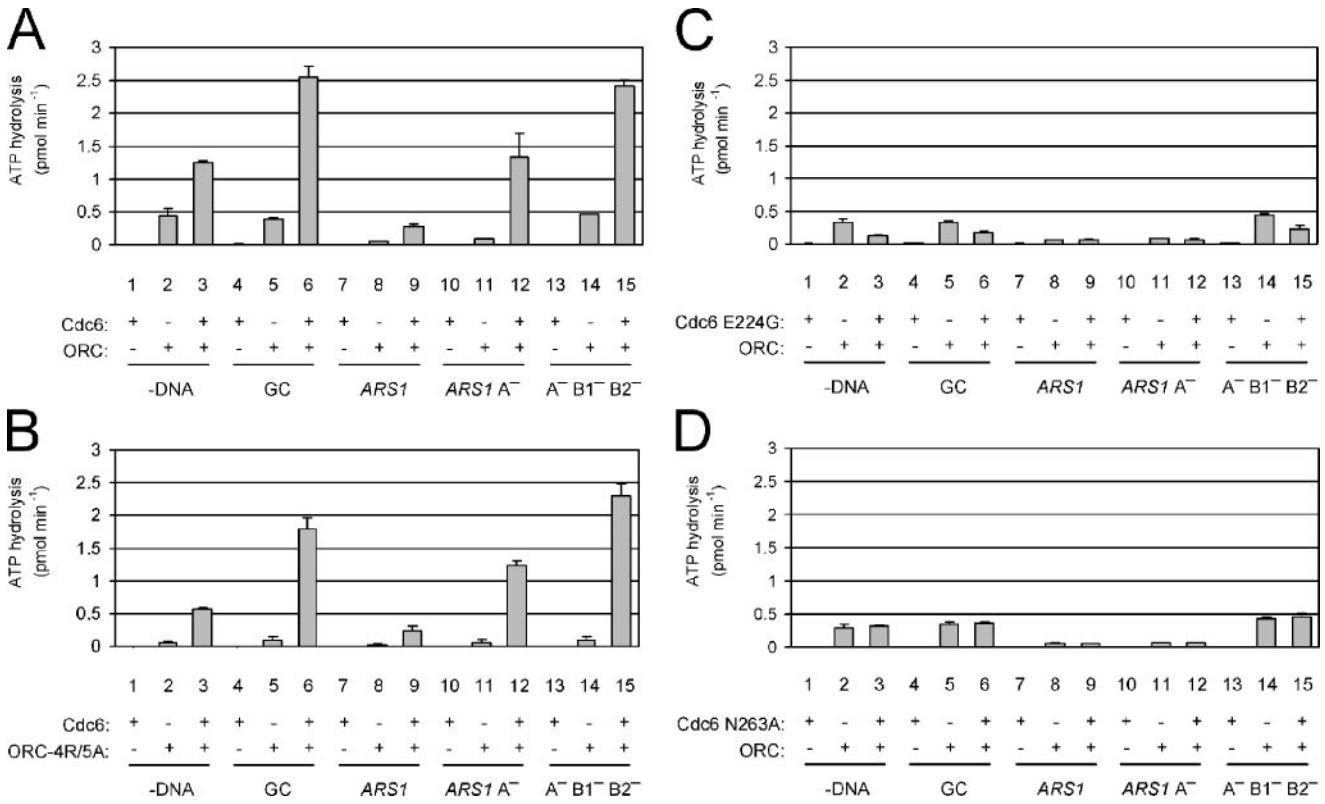

FIGURE 2. **ATPase of the ORC·Cdc6 complex is regulated by DNA sequence and is dependent on Cdc6 ATPase.** ATP hydrolysis rates were determined in the absence of DNA (−*DNA*) or in the presence of GC-rich DNA (*GC*), *ARS1*, *ARS1* with a mutant A element (*ARS1* A$^-$), and *ARS1* with mutant A, B1, and B2 elements (A$^-$B1$^-$B2$^-$). *A*, ATP hydrolysis by ORC, Cdc6, and ORC·Cdc6. *B*, ATP hydrolysis by ORC4R/5A, Cdc6, and ORC4R/5A·Cdc6. *C*, ATP hydrolysis by ORC, Cdc6 E224G, and ORC·Cdc6 E224G. *D*, ATP hydrolysis by ORC, Cdc6 N263A, and ORC·Cdc6 N263A.

DNA Sequence-dependent ATPase Induction in the ORC·Cdc6·DNA Complex—Because the ORC·Cdc6 complex is assembled on DNA, we were interested in determining how DNA modulates the ATPase activity in the complex. In performing these experiments, wild type and mutant versions of origin DNAs were used as well as nonspecific natural DNAs that do not function as an origin in yeast. Thus, the relationship between origin binding and ATPase activity could be assessed. The ATPase of ORC, ORC4R/5A, Cdc6, and the two Cdc6 ATP hydrolysis mutants (Cdc6 E224G and Cdc6 N263A) were analyzed in the absence and presence of GC-rich non-origin DNA, *ARS1* origin DNA (containing the A, B1, and B2 elements), and mutant *ARS1* A$^-$ or A$^-$B1$^-$B2$^-$ DNA (Fig. 2). Note that both the A and B2 elements are ORC binding sites; B2 is only used as an ORC binding site in the absence of a functional A element (11, 38).

Cdc6 had no ATPase in the absence or presence of DNA (Fig. 2*A*, *lanes 1*, *4*, *7*, *10*, and *13*). In the absence of DNA, ORC is an ATPase (*lane 2*), and the addition of Cdc6 to ORC led to a 2.5-fold increase in ATPase compared with ORC alone (Fig. 2*A*, *lane 3*). GC-rich DNA and *ARS1* A$^-$B1$^-$B2$^-$, which do not support initiation of DNA replication, ORC·DNA, and ORC·Cdc6·DNA complex formation (38), did not suppress ORC ATPase activity (Fig. 2*A*, compare *lane 2* with *lanes 5* and *14*). Rather, they induced ORC·Cdc6 ATPase activity 5-fold stronger than the ATPase in the presence of ORC alone (Fig. 2*A*, compare *lane 2* with *lanes 6* and *15*). This result suggests that transient binding of ORC and Cdc6 to GC-rich DNA activates the ATPase in either ORC or Cdc6. *ARS1* origin DNA suppressed ORC ATPase by 8–10-fold (Fig. 2*A*, *lanes 2* and *8*) as reported (20). *ARS1* DNA also promoted stable complex formation of the ORC·Cdc6·*ARS1* complex (38). The addition of *ARS1* DNA to reactions containing ORC and Cdc6 suppressed the ATPase activity of ORC·Cdc6 about 5-fold compared with the ATPase activity in the absence of DNA (Fig. 2*A*, compare *lanes 3* and *9*). An *ARS1* A$^-$ mutant, which supports weak binding of ORC but does not support ORC·Cdc6 complex formation on DNA (38), suppressed ORC ATPase activity likely due to the weaker, non-perfect ORC binding site within the B2 element (Fig. 2*A*, compare *lane 2* with *lane 11*). Interestingly, the *ARS1* A$^-$ mutant did not suppress ORC·Cdc6 ATPase but resulted in a similar ATPase as the ORC·Cdc6 complex in the absence of DNA (Fig. 2*A*, compare *lane 3* with *lane 12*). This result suggests that ORC can bind to the *ARS1* A$^-$ via the weak ORC binding site within the B2 element but that addition of Cdc6 triggers ATPase activity that disrupts the ORC·Cdc6 binding to the *ARS1* A$^-$ DNA. These data indicate that ORC·Cdc6 complex formation on DNA is regulated by ATPase activity and that the ORC and the ORC·Cdc6 complexes differ in the way they respond to non-origin DNA sequences, as exemplified by the striking differences between *ARS1* and *ARS1* A$^-$ DNA.

Cdc6 Is the Active ATPase in the ORC·Cdc6·DNA Complex—Next we were interested in identifying the DNA-regulated ATPase within the ORC·Cdc6·DNA complex. We, therefore, analyzed mutant versions of these proteins, including ORC4R/5A and Cdc6 E224G and N263A for their ability to

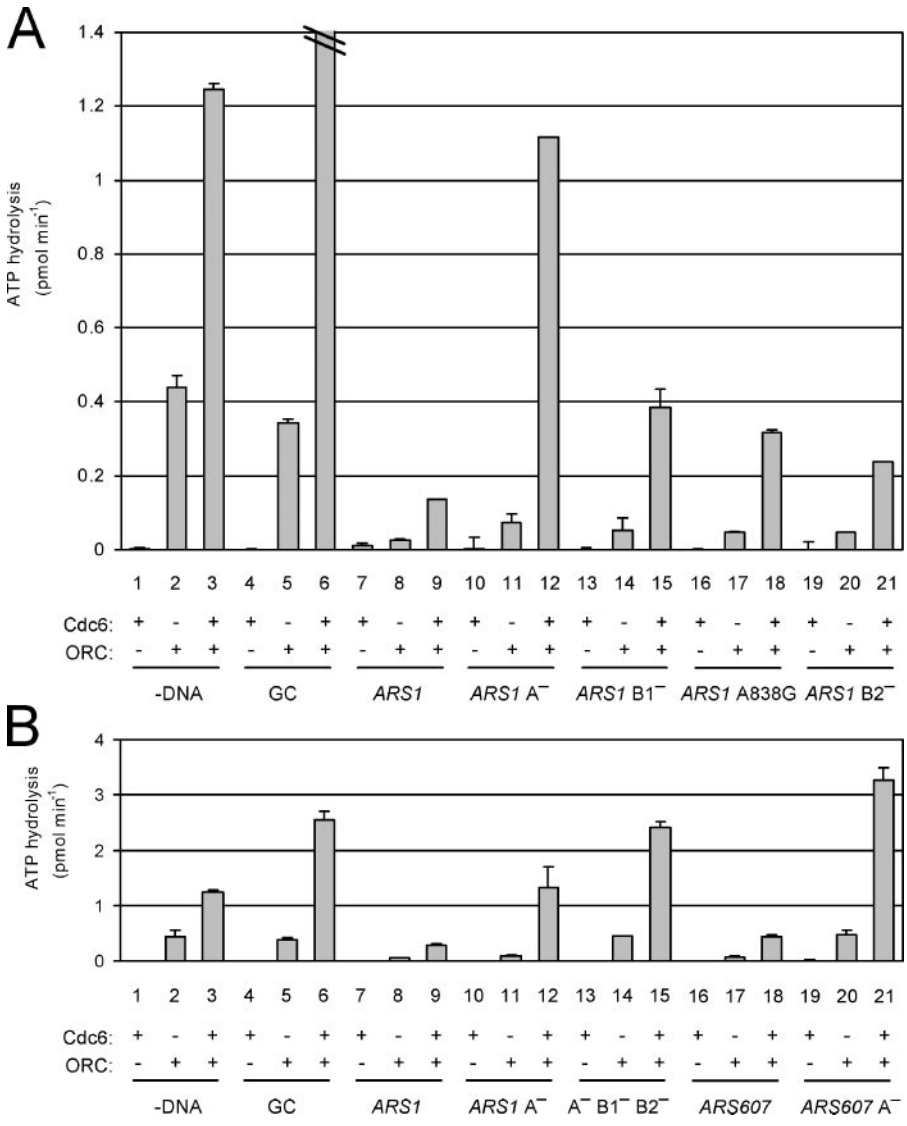

FIGURE 3. **ATPase of the ORC·Cdc6 complex is regulated by origin DNA.** *A*, ATP hydrolysis rates were determined in the absence of DNA (−*DNA*) or in the presence of GC-rich DNA (*GC*), *ARS1*, *ARS1* with a mutant A element (*ARS1* A$^-$), *ARS1* with mutant B1 element (*ARS1* B1$^-$), and *ARS1* with an point mutation in B1 (*ARS1* A838G) and *ARS1* with a mutated B2 element (*ARS1* B2$^-$). *B*, ATP hydrolysis rates were determined in the absence of DNA (−*DNA*) or in the presence of GC-rich DNA (*GC*), *ARS1*, *ARS1* with a mutant A element (*ARS1* A$^-$), *ARS1* with mutant A, B1, and B2 elements (A$^-$B1$^-$B2$^-$), and ARS607 and ARS607 with mutant A element (ARS607 A$^-$). Note that *A* and *B* have different scales.

regulate ORC·Cdc6 ATPase activity. ORC4R/5A, a very weak ATPase, was reproducibly suppressed by *ARS1* DNA but not by GC-rich DNA or *ARS1* A$^-$B1$^-$B2$^-$ (Fig. 2*B*, *lanes 2*, *5*, *8*, *11*, and *14*). The addition of Cdc6 to ORC4A/5A resulted in stronger ATPase in the presence and absence of DNA, similar to the case of ORC and Cdc6, indicating that ORC ATPase is not the major ATPase within the ORC·Cdc6·DNA complex (Fig. 2*B*, *lanes 3*, *6*, *9*, *12*, and *15*). Interestingly, based on the Cdc6·activated ATPase, this mutant complex was still able to distinguish between DNAs that acted as an origin and DNAs that did not.

The two Cdc6 mutants, Cdc6 E224G (31) and N263A, did not increase the ATPase when compared with ORC alone in the absence or presence of any DNA (Fig. 2, *C* and *D*). Interestingly, the dominant negative Cdc6 E224G mutant resulted in reducing the ATPase of ORC in solution or in complex with non-origin GC-rich or *ARS1* A$^-$ and A$^-$B1$^-$B2$^-$ DNA (Fig. 2*D*). Combined, these results suggest that Cdc6 is the major ATPase within the ORC·Cdc6 complex, independent of whether DNA is present or not. Furthermore, origin DNA, but not non-origin DNAs, suppressed the ORC·Cdc6 ATPase activity, suggesting that the ORC·Cdc6 complex and the Cdc6 ATPase within it could discriminate between functional and non-functional origin sequences. For example, even though the non-origin *ARS1* A$^-$ DNA has an ORC binding site within it and this sequence is capable of suppressing ORC ATPase activity (Fig. 2*A*, *lanes 2* and *10*), this DNA was incapable of suppressing the ORC·Cdc6 ATPase activity.

B1 and B2 Origin Mutations Induce ATPase within the ORC·Cdc6·DNA Complex—The results presented in Fig. 2, combined with those in a previous report (38), suggest that multiple DNA sequences within an origin regulate the ATPase activity of the ORC·Cdc6 complex. We were, therefore, interested in quantifying the contribution of the genetically conserved B1 and B2 origin elements to the ATPase activity (Fig. 3*A*). A linker scanning mutant in B1, a point mutant in B1 (A838G), and a B2 linker scanning mutant were analyzed. The linker mutation of the B1 element has been shown to reduce the affinity of ORC; however, a point mutant in the B1 element, *ARS1* A838G, and a linker scanning mutation of the B2 element have no influence on the ORC affinity for DNA (13). The B1 linker scanning mutation abolished and the point mutant reduced ORC·Cdc6 complex formation on DNA (38). The mutation of the B2 element had only a very weak influence on ORC·Cdc6 complex formation on DNA.[4] In ATPase assays with *ARS1* B1$^-$, B1 A838G, and B2$^-$ mutants, the addition of Cdc6 to ORC resulted in a 3-, 2.3-, and 1.8-fold activation of ATPase respectively, compared with the activity of ORC alone (Fig. 3*A*). ORC ATPase activity on its own was at the same time also slightly increased in these mutants compared with the activity on *ARS1* DNA (Fig. 3*A*). In contrast, the A$^-$ mutant resulted in 8.5-fold activation of ORC·Cdc6 ATPase activity, indicating that mutations in the B elements have a

[4] C. Speck and B. Stillman, unpublished data.

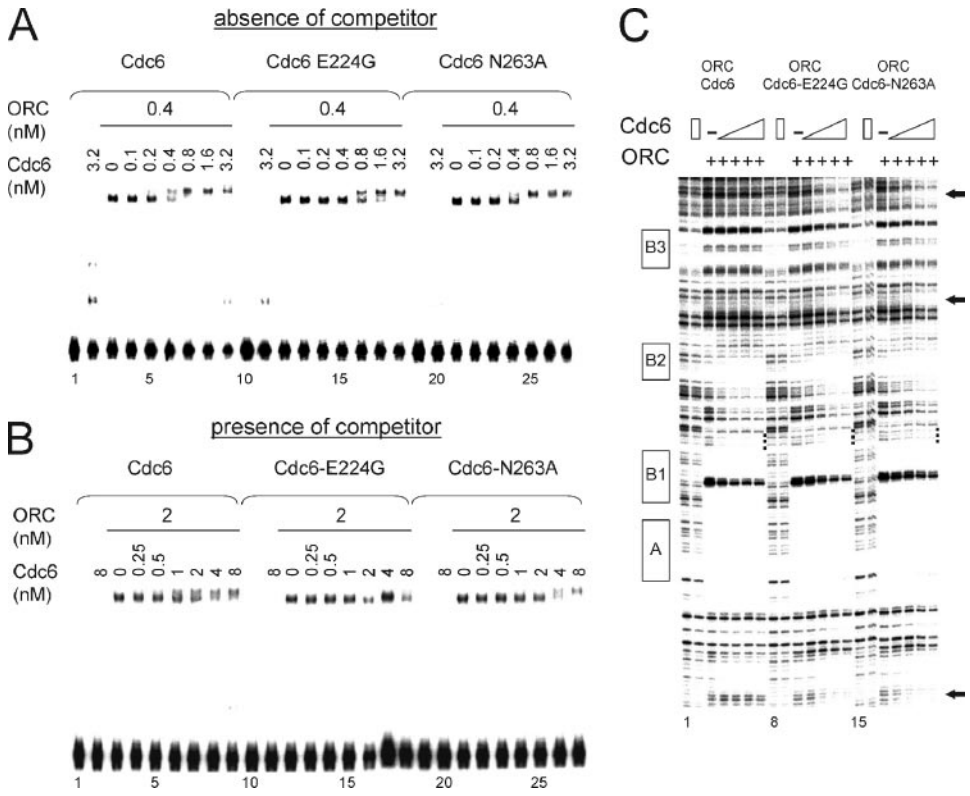

FIGURE 4. **ORC·Cdc6 binds nonspecifically to DNA in the absence of Cdc6 ATPase.** *A*, gel shift assay of ^{32}P-labeled *ARS1* DNA with ORC, Cdc6, Cdc6 E224G, and Cdc6 N263A in the absence of competitor DNA. Protein concentrations are indicated. *B*, gel shift assay of ^{32}P-labeled *ARS1* DNA with ORC, Cdc6, Cdc6 E224G, and Cdc6 N263A in the presence of 37.5 ng of GC-rich 290 bp of competitor DNA. *C*, DNase I footprints were performed with 2 ng (0.4 nM) of a 290-bp DNA *ARS1* fragment ^{32}P-labeled at the 5′ end (T-rich strand). *Lanes 4–7*, *11–14*, and *18–21* contain 3.4, 8.5, 17 and 42.5 nM Cdc6, Cdc6 E224G, and Cdc6 N263A, respectively. *Lanes 3–7*, *10–14*, and *17–21* contain 40 nM ORC. Regions with extra protection due to Cdc6 ATPase inhibition (Cdc6 E224G and N263A) are *highlighted* with *arrows*. A region of reduced protection in the absence of Cdc6 ATPase is indicated by the *dotted line*.

weaker but significant impact on activation of ATPase. Thus, Cdc6 ATPase activity in the ORC·Cdc6 complex was most influenced by ORC binding sites that contribute most to origin activity, but additional sequences that do not affect ORC binding also alter the regulation of Cdc6ATPase activity.

Origins of Replication Suppress ORC·Cdc6 ATPase—We tested whether mutations in ARS A elements at other origins have a similar impact. The ARS607 and an ARS607 A$^-$ mutant DNAs were analyzed. ARS607 suppressed ORC ATPase efficiently, and the addition of Cdc6 resulted in increased ATPase activity, similar to *ARS1* (Fig. 3*B*). Interestingly a mutation in the A element resulted in significant ORC ATPase, comparable with ORC ATPase in the absence of DNA, and the addition of Cdc6 induced very strong ATPase. This situation is similar to an *ARS1* A$^-$B1$^-$B2$^-$ mutant, suggesting that ARS607 has no secondary ORC binding site within its B2 element. The ARS607 data suggest that Cdc6-induced ATPase is down-regulated at origins of replication due to ARS A elements.

Cdc6 ATPase Mutants Promote Binding of the ORC·Cdc6 Complex to Nonspecific DNA—The above-described experiments established that Cdc6 ATPase in the ORC·Cdc6 complex was regulated in an ORC and DNA sequence-specific manner. Furthermore, we have shown that Cdc6 influences the DNA binding specificity of the ORC·Cdc6 complex (37, 38). We were, therefore, interested in determining if Cdc6 ATPase regulates the DNA sequence specificity of ORC·Cdc6 interactions with DNA.

Either wild type Cdc6 or the two Cdc6 ATPase mutants were tested for DNA binding in the absence or presence of ORC using a gel shift assay (Fig. 4, *A* and *B*) and a DNase I footprint assay (Fig. 4*C*). Cdc6 and its mutants had very weak and nonspecific binding to DNA (Fig. 4, *A* and *B*, *lanes 2*, *11*, and *21*). In the absence of competitor DNA the addition of Cdc6 E224G and N263A formed an ORC·Cdc6·*ARS1* complex like wild type Cdc6 (Fig. 4*A*, compare *lanes 4–9*, *13–18*, and *22–27*). However, in the presence of competitor DNA, binding of the two mutant Cdc6 proteins were significantly impaired (Fig. 4*B*, compare *lanes 4–9*, *13–18*, and *22–27*; note that in the presence of competitor DNA 5-fold more ORC was used to detect ORC binding). This result indicates that Cdc6 ATPase mutants reduced DNA sequence-specific complex formation. The DNase I footprint assay showed that Cdc6 ATPase mutants did not influence ORC binding at the A and B1 elements of the *ARS1* origin; however, Cdc6-induced protection adjacent to the B1 element was lost (Fig. 4*C*, *dotted line*). More significant, the two Cdc6 mutants, but not the wild type Cdc6, induced significant nonspecific DNA binding in multiple regions outside the genetically defined origin (Fig. 4*C*, *arrows*). Because the Cdc6 mutants in the absence of ORC had no DNase I footprint, we suggest that the ORC·Cdc6 ATPase mutant stabilizes the ORC-mutant Cdc6 complex in regions outside the origin and, therefore, has reduced sequence specificity. Similar results have been seen when the ORC·Cdc6 complex was analyzed in the presence of ATPγS (38).

The Mechanism of Cdc6-induced Sequence Specificity—The DNA binding experiments established that the Cdc6 ATPase activity contributes to sequence specific DNA binding to origin DNA; however, the mechanism of how this activity contributes to origin selection remained unclear. Theoretically, Cdc6 ATPase can regulate DNA sequence specific binding in several ways, by 1) stabilizing ORC binding to the origin, 2) destabilizing ORC on non-origin DNA (37) or by regulating Cdc6 interaction with ORC that is not bound to origin DNA, 3) binding preferentially to ORC that is origin bound, or 4) release of Cdc6 from non-origin DNA. Because many biological regulatory interactions are controlled at the level of dissociation and the footprint experiments suggested a dissociation defect of the Cdc6 ATPase mutants when in a complex with ORC, we measured the dissociation rates of

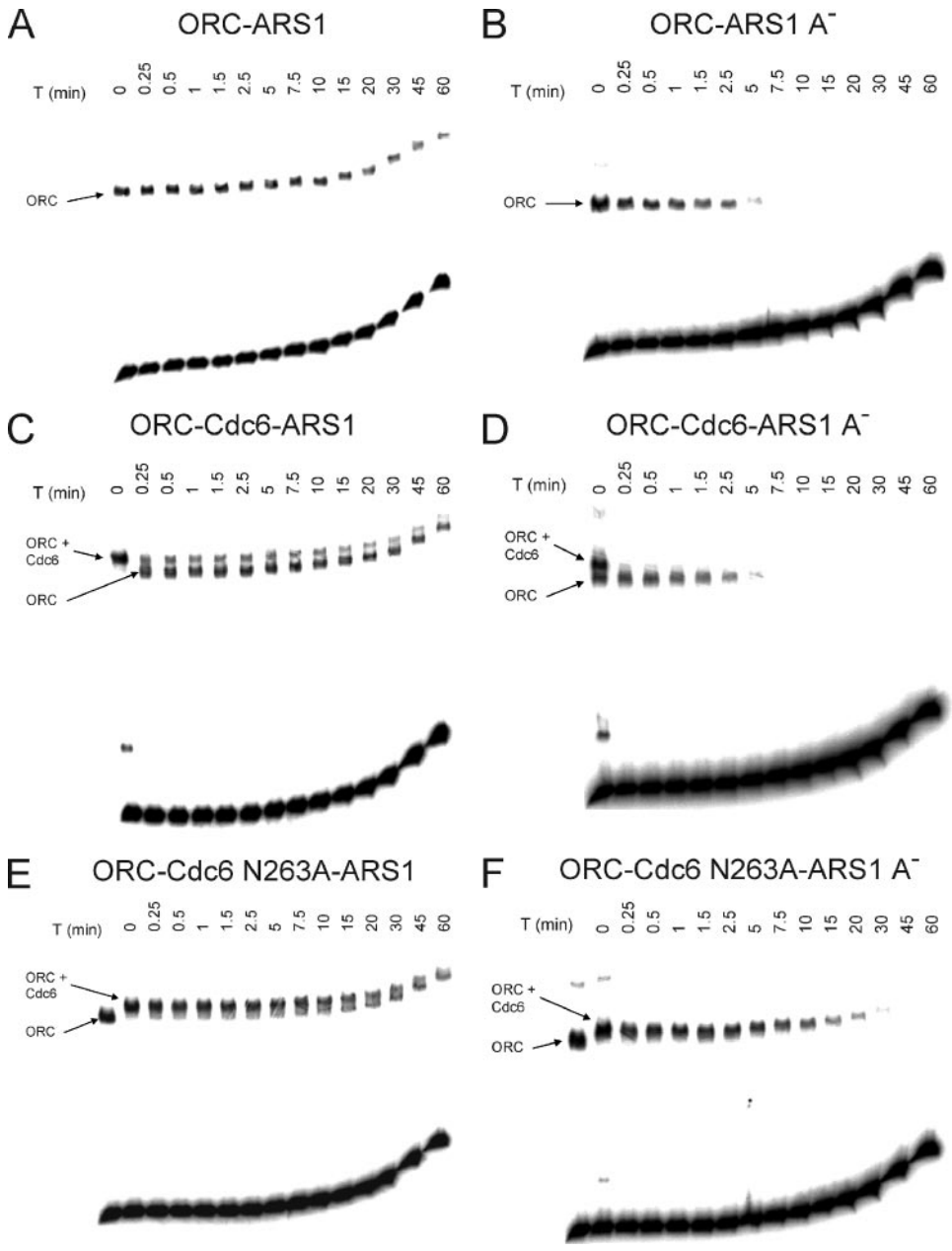

FIGURE 5. **Cdc6 ATPase is required for rapid dissociation of the ORC·Cdc6 complex from mutant origin DNA.** Equilibrated binding reactions (time point 0 min) were challenged with a 100-fold molar excess of unlabeled *ARS1* competitor DNA and then loaded onto a running native polyacrylamide gel at the times indicated. *A*, gel shift assay of ^{32}P-labeled *ARS1* DNA with ORC. *B*, gel shift assay of ^{32}P-labeled *ARS1* A$^-$ DNA with ORC. *C*, gel shift assay of ^{32}P-labeled *ARS1* DNA with ORC and Cdc6. *D*, gel shift assay of ^{32}P-labeled *ARS1* A$^-$ DNA with ORC and Cdc6. *E*, gel shift assay of ^{32}P-labeled *ARS1* DNA with ORC and Cdc6 N263A. In the *first lane* ORC and *ARS1* A$^-$ (no competitor DNA) were loaded as a reference. Note that ORC binds to A$^-$ DNA non-specifically in the absence of competitor DNA. *F*, gel shift assay of ^{32}P-labeled *ARS1* A$^-$ DNA with ORC and Cdc6 N263A. In the *first lane* ORC and *ARS1* A$^-$ (no competitor) were loaded as a reference. *A* + *B* were used to determine ORC dissociation rates from DNA, and *C–F* were used to determine Cdc6 and Cdc6 N263A dissociation rates from ORC·DNA complexes.

ORC, Cdc6, and Cdc6 N263A in the presence of ^{32}P-labeled *ARS1* or *ARS1* A$^-$ DNAs. ORC, Cdc6, and origin DNA were incubated in the absence of competitor DNA and then challenged by the addition of 100× wild type *ARS1* DNA. The reactions were loaded at time intervals onto a running native polyacrylamide gel, and the dissociation rates were determined from the autoradiogram. The $t_{½}$ of ORC on origin DNA was determined to be 64.9 min., indicating a very slow turnover (Fig. 5*A*, Table 1). The binding of ORC to *ARS1* A$^-$ displayed increased turnover, with a 17.5× decreased $t_{½}$ of 3.7 min (Fig. 5*B*, Table 1). Wild type Cdc6 showed a biphasic dissociation rate from origin DNA, one fraction of Cdc6 coming off DNA immediately upon the addition of competitor and another fraction with a $t_{½}$ of 62 min, similar to ORC (Fig. 5*C*, Table 1). Interestingly, 15 s after competitor DNA addition, all the Cdc6 was dissociated from an ORC·*ARS1* A$^-$ DNA complex; the $t_{½}$ was consequently below our detection limit (Fig. 5*D*, Table 1). The Cdc6 N263A mutant, incubated with *ARS1* and ORC, showed in contrast to wild type Cdc6, a dissociation rate with a $t_{½}$ of 29.7 min (Fig. 5*E*, Table 1). This lower $t_{½}$ for ORC·Cdc6 N263A compared with the wild type ORC·Cdc6 probably reflects the measurement of the combined dissociation of two separate complexes, 1) Cdc6 N263A that is nonspecifically bound to the DNA and 2) the Cdc6 N263A that is within the ORC·Cdc6 N263A·DNA complex. In contrast, for wild type Cdc6, the nonspecifically bound Cdc6 immediately dissociated from the origin DNA upon the addition of competitor DNA, leaving only the ORC·Cdc6 complex to dissociate.

In the case of *ARS1* A$^-$ the dissociation rate of Cdc6 N263A was measurable at $t_{½}$ of 9.9 min (Fig. 5*F*, Table 1), which is in stark contrast to Cdc6 that had a dissociation rate 100× faster. Interestingly Cdc6 N263A led to a 2.7-fold stabilization of ORC on *ARS1* A$^-$ (compare Fig. 5, *D* and *F*), indicating that the ORC·Cdc6 N263A·*ARS1* A$^-$ complex is overall more stable than the ORC·*ARS1* A$^-$ complex, indicating that Cdc6 promotes moderately higher ORC·Cdc6·DNA complex stability in the absence of Cdc6 ATPase. This result also fits with the increased nonspecific binding of the ORC·Cdc6 N263A·DNA complex seen in the DNase I footprint assay in regions outside the primary ORC binding site (Fig. 4*C*). Overall, these results indicate that Cdc6 ATPase regulates the disassembly of the ORC·Cdc6 complex on non-origin DNA. The major mechanism is the release of Cdc6 from ORC bound to non-origin DNA due to Cdc6 ATPase.

TABLE 1

Dissociation rates and $t_{1/2}$ of ORC-DNA and ORC·Cdc6·DNA complexes

Based on the data exemplified in Fig. 5, dissociation rates (k_{off}), *R* values, and $t_{1/2}$ were calculated using KaleidaGraph 4.0 (Synergy software). The rates were calculated for ORC or Cdc6 as indicated in bold. ORC·Cdc6·A$^-$ dissociation rates were too fast to be determined and, therefore, are labeled (ND). The $t_{1/2}$ was estimated to be lower than 0.125 min since this is the lower detection limit of this assay.

Complex	k_{off}	*R* value	$t_{1/2}$
	min^{-1}		*min*
ORC·ARS1	0.011	0.976	64.9
ORC·A$^-$	0.190	0.993	3.7
ORC·**Cdc6**·ARS1	0.112	0.945	63.0
ORC·**Cdc6**·A$^-$	ND	ND	<0.125
ORC·**Cdc6 N263A**·ARS1	0.024	0.988	29.7
ORC·**Cdc6 N263A**·A$^-$	0.070	0.999	9.9

DISCUSSION

In previous studies we have analyzed ORC·Cdc6 complex formation in the presence and absence of DNA and showed that Cdc6 confers increased sequence specificity to the ORC·Cdc6 complex (37, 38). We also showed that ORC and Cdc6 form a ring-shaped complex with a surface of similar dimension and shape to the ring-shaped surface of the MCM-hexamer, which likely functions as a MCM loading machine before initiation of DNA replication (38). In this study we concentrated on analyzing the mechanism by which Cdc6 increases DNA sequence specificity or origin recognition and stability of the ORC·Cdc6·DNA complex, both of which are relevant to understanding why origins of DNA replication locate to specific regions within chromosomes for pre-RC formation and MCM loading (31).

Six of the seven proteins within the ORC·Cdc6 complex are predicted to belong to the class of AAA+ proteins (38, 39). ATP binding and hydrolysis frequently regulate the function of AAA+ proteins (40). In our previous study we focused on the role of ORC ATP binding and ORC ATPase in regulating ORC·Cdc6 complex (38). In this study the contribution of Cdc6 ATPase on ORC·Cdc6 interaction and sequence specificity was investigated. Mutants interfering with Cdc6 ATPase have been characterized *in vivo*; Cdc6 E224G has a mutation in the highly conserved Walker B motif (31, 35) and is dominant lethal when overexpressed in yeast. Cdc6 N263A (42, 43) has a mutation in sensor 1 and is temperature-sensitive and lethal when overexpressed. In the absence of DNA we found that Cdc6 addition to ORC resulted in rapid Cdc6-induced ATP hydrolysis and dissociation from ORC. Cdc6·ADP, which is produced during ATP hydrolysis, is not capable of binding to ORC (38). Cdc6 mutants defective for ATPase did not show increased ATPase in the presence of ORC and resulted in stable complex formation. The Cdc6 E224G mutant actually inhibited ORC ATPase activity, perhaps explaining why this mutant is a dominant lethal. The biological function of ORC-induced Cdc6 ATPase in the absence of DNA is likely the forced disassembly of the complex to ensure that the ORC·Cdc6 complex only forms on origin DNA (38).

In the presence of origin DNA, a stable ORC·Cdc6 complex was formed (31, 38), and Cdc6 ATPase activity was induced, but this low level ATPase could originate from partial binding of the ORC·CDC6 complex to regions outside the origin that are unable to suppress Cdc6 ATPase. In the presence of non-origin DNA, however, the Cdc6 ATPase activity was much higher, and the complex was not stable on DNA. Mutations in conserved genetic elements of ARS resulted in activation of ATPase activity, which was dependent on Cdc6 ATPase. The A element represents the primary binding site for ORC (11), the sequence of A elements are most conserved within different ARS, and point mutants between the A-element have drastic phenotypes on plasmid stability (11, 12). Mutations in the A element have also been shown to reduce ORC binding (11). We suggest that Cdc6 activated ATPase, and consequently, disassembly of the ORC·Cdc6·DNA complex is a reason for the low plasmid stability of ARS A box mutants in addition to the reduced affinity of ORC for DNA. Similarly, mutations in the B elements are important for origin function; however, in this case only simultaneous deletion of at least two of the B elements results in nonfunctional origins (12). Mutations in the B1 element reduce slightly the affinity of ORC for origin DNA (13, 38), and mutations in the B2 element can be compensated by Cdc6 overexpression (15). We found that mutations in the B elements resulted in increased Cdc6 ATPase activity, which promotes disassembly of the complex. Cdc6 overexpression may be sufficient to force ORC·Cdc6 complex formation on the defective origin and, therefore, could compensate for B2 mutations.

The *ARS1 A838G* mutation is of particular interest because this mutant binds to ORC well but is partially defective in the Cdc6·dependent extended footprint on the *ARS1* origin DNA (38) and has reduced ability to suppress the Cdc6-induced ATPase activity of the ORC·Cdc6 complex. We, therefore, suggest that specific nucleotide sequences modulate origin utilization by targeting the ORC·Cdc6 complex rather than ORC alone, again suggesting that Cdc6 contributes to origin selection.

It is known that origins with weak ORC binding sites can still function efficiently (46). We suggest that these DNA sequences bind the ORC·Cdc6 complex more efficiently to promote pre-RC formation. Furthermore, when Cdc6 is overexpressed in certain circumstances, re-replication of only a subset of origins of DNA replication occurs (47, 48). Because Cdc6 contributes to origin selection and utilization, this may be one reason why not all origins are re-replicated when Cdc6 is present in excess.

Cdc6 ATPase within the ORC·Cdc6·DNA complex, first described by Randell *et al.* (31), regulates the stability of the ORC·Cdc6 complex. The function of the ORC·Cdc6 complex is to load in cooperation with Cdt1 the potential MCM helicase onto DNA to form a pre-RC complex (49). Based on our new findings that Cdc6 ATPase controls the stability of ORC·Cdc6 complex on DNA, we suggest that it is the stability of the ORC·Cdc6 complex on origin DNA, but not on non-origin DNA, that regulates directly the location in chromosomes and amount of MCM loading. Cdc6 ATPase contributes to the selection of DNA sequences that can promote MCM loading and, hence, formation of a pre-RC. The ORC·Cdc6 complex stability depends on several factors, many of which are hardwired into the DNA sequence at origins, namely the A and B elements, but we envision that chromatin structure might also affect complex stability.

It has been shown that Cdc6 availability is further regulated by association with cyclin (50), and Cdc6 destruction is regu-

lated by ubiquitin-mediated proteolysis (51–53). Cyclin-Cdc6 interaction and proteolysis of Cdc6 regulate the overall availability of Cdc6 during the cell division cycle, but when Cdc6 is available to bind to ORC before pre-RC assembly, DNA sequence information is a significant determinant in modulating Cdc6 activity and, hence, where on the DNA pre-RCs are formed. Because subtle changes in the DNA sequence influence Cdc6 ATPase activity, Cdc6·ORC complex stability can also be represented as having a specific probability at any given DNA sequence and, therefore, contributes to selection of functional origins. This fits well with the recent data in fission yeast in which origins of DNA replication are selected in a stochastic manner dependent on DNA sequence and do not function every cell cycle (54–56). We suggest that Cdc6 (cdc18 in *S. pombe*) ATPase activity influences those DNA sequences that are selected as origins of DNA replication and even the stochastic firing of origins in each S phase. Such a scenario may exist for the selection of sites of DNA replication in the chromosomes of plant and animal cells where ORC binding is not known to be sequence specific. In fact, Cdc6 is a rate-limiting component for the initiation of DNA replication in vertebrate cells (36, 57), and we suggest that it is the interaction between ORC and Cdc6 and the subsequent ATPase activities of these proteins that determines when and where in chromosomes initiation of DNA replication takes place.

Cdc6 ATPase activity within the ORC·Cdc6 complex is suppressed by origin DNA when compared with the activity in the absence of DNA and activation of Cdc6 ATPase, due to non-origin DNA, leads to disassembly of Cdc6 from DNA. It is possible that ORC bound to non-origin DNA has an altered conformation that stimulates Cdc6 ATPase upon complex formation. This results in ADP·Cdc6, which cannot interact with ORC and is released from the ORC·DNA complex. Once ADP·Cdc6 is in solution, it has to be recharged with ATP. This could be a passive reaction or actively modulated by a protein. On the other hand a specific protein present during pre-RC assembly might modulate Cdc6 ATPase to promote several rounds of MCM loading. Alternatively, a protein may stimulate yeast Cdc6 ATPase activity on origin DNA to promote dissociation of Cdc6 from the origin before its destruction at the G_1/S phase transition and, hence, hinder re-replication in a single cell cycle.

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