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Functions of Sensor 1 and Sensor 2 Regions of *Saccharomyces cerevisiae* Cdc6p *in Vivo* and *in Vitro**

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Cdc6p is a key regulator of the cell cycle in eukaryotes and is a member of the AAA$^+$ (ATPases associated with a variety of cellular activities) family of proteins. In this family of proteins, the sensor 1 and sensor 2 regions are important for their function and ATPase activity. Here, site-directed mutagenesis has been used to examine the role of these regions of *Saccharomyces cerevisiae* Cdc6p in controlling the cell cycle progression and initiation of DNA replication. Two important amino acid residues (Asn^{263} in sensor 1 and Arg^{332} in sensor 2) were identified as key residues for Cdc6p function *in vivo*. Cells expressing mutant Cdc6p (N263A or R332E) grew slowly and accumulated in the S phase. In cells expressing mutant Cdc6p, loading of the minichromosome maintenance (MCM) complex of proteins was decreased, suggesting that the slow progression of S phase in these cells was due to inefficient MCM loading on chromatin. Purified wild type Cdc6p but not mutant Cdc6p (N263A and R332E) caused the structural modification of origin recognition complex proteins. These results are consistent with the idea that Cdc6p uses its ATPase activity to change the conformation of origin recognition complex, and then together they recruit the MCM complex.

To coordinate DNA replication with cell division, initiation of chromosomal DNA replication must be strictly regulated. Initiation of eukaryotic chromosomal DNA replication is achieved by the stepwise assembly of various protein complexes at origins of DNA replication (1–5). *In vivo* footprinting analysis suggests that a protein complex, called a prereplication complex (pre-RC),[1] is formed in the G_1 phase on each origin of chromosomal DNA replication (6). At the G_1/S boundary, cyclin-dependent protein kinases activate the pre-RC to form a preinitiation complex that then initiates DNA replication in a temporally specific manner (1, 2, 5–8). Thus, the molecular mechanism of formation of the pre-RC and its regulation are important for understanding how the cell cycle regulates initiation of chromosomal DNA replication.

Studies in the yeast *Saccharomyces cerevisiae* have shown that at least four kinds of proteins are components of the pre-RC, including the origin recognition complex (ORC), Cdc6p, and the six minichromosome maintenance (MCM) proteins (9–18). Genetic analysis using mutant *S. cerevisiae* strains suggested that Cdc6p first binds to ORC, which is bound to origins of chromosomal DNA replication throughout the cell cycle, and then MCM is recruited onto the origins to form the pre-RC (13, 15, 17, 19). But the precise molecular mechanism of pre-RC formation, including how the MCM complex is recruited on chromatin that is already occupied by ORC and Cdc6p, is unknown. Recently, Cdt1 was identified in *Schizosaccharomyces pombe* and *Xenopus laevis* as another component required for formation of the pre-RC (20, 21), and the yeast Tah11p protein is likely the *S. cerevisiae* ortholog of Cdt1,[2] but its biochemical role in DNA replication is not certain.

In yeast, MCM loading onto chromatin and pre-RC formation depends on Cdc6p (13, 14, 18, 19). In the fission yeast *S. pombe*, the Cdc18 protein, the ortholog of *S. cerevisiae* Cdc6p, controls the initiation of DNA replication (22–24). The amount of Cdc6p fluctuates during cell cycle, but ORC and MCM remain constant (6, 10, 16, 25). Thus, Cdc6p is thought to be a key regulator of pre-RC formation and, thus, of the initiation of chromosomal DNA replication in eukaryotic cells.

Cdc6p belongs to the AAA$^+$ (ATPases associated with a variety of cellular activities) family (26, 27). The ATPase activity of an AAA$^+$ family protein is important for its function (28–31). There are at least two possibilities for the function of Cdc6p ATPase in pre-RC formation; one is contribution to its binding to ORC, and the other is to recruit the MCM protein complex onto origins of DNA replication. We recently established an *in vitro* system reconstituted from purified *S. cerevisiae* proteins and DNA fragments containing origins of replication to study ORC and Cdc6p interactions (32). In this system, we demonstrated that the ATP binding activity (and most likely the ATPase activity) of Cdc6p is not necessary for its binding to ORC. We found, however, that Cdc6p used its ATPase activity to alter the conformation of ORC. Based on these results, we suggested that Cdc6p changes the conformation of ORC using its ATPase activity, and together, this remodeled complex cooperates, perhaps with Cdt1 (Tah11p), to recruit the MCM

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[1] The abbreviations used are: pre-RC, prereplication complex; ORC, origin recognition complex; MCM, minichromosome maintenance; SC, synthetic complete; FACS, fluorescence-activated cell sorter; GST, glutathione *S*-transferase; ATPγS, adenosine 5′-*O*-(thiotriphosphate); SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors.

[2] C. Speck and B. Stillman, unpublished observation.

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complex (32). This model is supported by genetic evidence showing that Cdc6p with a defective Walker A motif could not recruit MCM proteins onto chromatin *in vivo* (18, 33). However, because this mutation may affect its ATP binding activity, we could not be certain that the ATPase activity was involved in loading MCM onto chromatin *in vivo*.

The sensor 1 and 2 regions are thought to be important for the ATPase activity of AAA^+ family proteins (27). X-ray structure analysis of *Escherichia coli* HslU protein, which also belongs to AAA^+ family, showed that a conserved arginine (Arg^{393}) in sensor 2 region can interact with the γ phosphate of ATP (34). Recently, the x-ray structure of *Pyrobaculum aerophilum* Cdc6p was reported (35), and the data suggested that the sensor 1 region interacts with the γ phosphate of ATP (35). These sensor regions are conserved in AAA^+ proteins and putatively detect the status of ATP *versus* ADP in the nucleotide-binding site and may regulate ATPase activity of these proteins (36, 37). Based on these previous findings, we searched for highly conserved amino acid residues in the sensor 1 and 2 regions of *S. cerevisiae* Cdc6p and found that Asn^{263} and Arg^{332} located in sensor 1 and sensor 2 regions, respectively, are important for its function *in vivo*. Genetic analysis of yeast strains that express mutant Cdc6p (N263A and R332E) showed that each mutant prolonged S phase because of inefficient loading of MCM onto chromatin. Biochemical analysis using purified mutant Cdc6p (N263A and R332E) showed that they were unable to change the conformation of ORC. These data further support the suggestion that Cdc6p recruits MCM onto DNA by changing the conformation of the ORC by using its ATPase activity.

EXPERIMENTAL PROCEDURES

Strains and Medium—*S. cerevisiae* strains are listed in Table I. In all experiments, the cells were cultured in synthetic complete (SC) medium with or without methionine as indicated.

Site-directed Mutagenesis and Plasmid Constructions—Site-specific mutation was performed using the method of Kunkel (38). Uracil-containing single-stranded DNA of M13 phage, bearing the *Sph*I-*Xba*I fragment of the wild type *CDC6* gene, was hybridized with an oligonucleotide primer that contained the desired mutation. The complementary DNA strand was synthesized *in vitro*, and the resultant double-stranded DNA was introduced into JM109. The mutation was confirmed by DNA sequencing. The *Sph*I-*Xba*I DNA fragment of the double-stranded DNA was used to replace that of pMW71 (18), which contained *CDC6* and *LEU2* genes (derivatives of pRS415). The resultant plasmids, named pNT11, pNT12, pNT13, and pNT14, contain mutant *cdc6* genes with N263A, K322E, R332E, and R339E, respectively. To construct plasmids for integration into yeast chromosomes, the *Hin*dIII-*Sac*I DNA fragment of pNT11 or pNT13 was introduced into the *Hin*dIII and *Sac*I sites of pRS405. The resultant plasmids were digested by *Hpa*I and introduced in the K4055 strain to construct NT101 or NT102 (see Table I).

Plasmid Shuffling—YB209 strain was used for plasmid shuffling experiments as described (39). pMW71, pNT11, pNT12, pNT13, and pNT14 were transformed into YB209 by the lithium-acetate method (40). The transformant was purified, spread on plates containing 5-fluoro-orotic acid without leucine, and incubated at 30 °C for 2 days.

Cell Synchronization and Repression of the Wild Type CDC6 Gene—The cells were grown at 30 °C in the absence of methionine to an A_{600} of 0.3, and then methionine (final concentration, 20 mM) was added to repress expression of the wild type CDC6 gene on the chromosomes of K4055, YB502, NT101, and NT102. In the G_2 block and α-factor release experiment, nocodazole was twice added (final concentration, 3 μg/ml) with an interval of 1 h. Methionine (final concentration, 20 mM) was added to the culture, and incubation was continued for 2 h in the presence of nocodazole. Finally, the cultures were washed with water and resuspended in SC medium containing α-factor (5 μg/ml) and 20 mM methionine for further incubation.

Fluorescence-activated Cell Sorter (FACS) Analysis—The samples were prepared as previously described (15) with the following modifications. The cells were pelleted by centrifugation, washed with water, and fixed in 70% ethanol for 12 h. Then the cells were again pelleted, resuspended in 50 mM sodium citrate, sonicated for 1 min, treated with 0.25 mg/ml RNase A for 1 h at 50 °C, and then treated with 1 mg/ml proteinase K for 1 h at 50 °C. DNA was stained with 50 μg/ml of propidium iodide, and then 20,000 cells from each sample were scanned with a FACS Calibur (Becton Dickinson).

Pulse-Field Gel Electrophoresis—Pulse-field gel electrophoresis experiments were done as described (41, 42) with the following modifications. The cells were harvested by centrifugation and washed three times with solution I (50 mM Tris-HCl, 1.2 M sorbitol, 20 mM EDTA), resuspended in solution II (50 mM Tris-HCl, 1.2 M sorbitol, 20 mM EDTA, 5% β-mercaptoethanol), and incubated for 10 min at room temperature. The cells were then suspended in solution III (0.1 M sodium citrate/citric acid, 1.2 M sorbitol, 10 mM EDTA) and mixed with 2.4% agarose (Bio-Rad; low melt preparative grade). The resultant solidified blocks of agarose were transferred to solution IV (0.1 M sodium citrate/citric acid, 1.2 M sorbitol, 10 mM EDTA, 0.5% zymolyase) and incubated for 24 h at 37 °C. Blocks of agarose were washed with solution III, then washed with solution I once, and incubated with solution V (0.1 M EDTA, 1% sodium lauroylsarcosine, 0.1% proteinase K) for 24 h at 50 °C. After washing with 0.2 M EDTA, the samples were applied to a 1.5% agarose slab and subjected to electrophoresis for 15.2 h at 300 V, 10 °C with a 50–100 switch interval. The gels were stained with ethidium bromide and observed under an UV illuminator.

Chromatin Binding Analysis—The samples were processed into soluble and insoluble (chromatin) fractions after Triton X-100 lysis of yeast spheroplasts, as previously described (15). Equivalent amounts (total protein) of insoluble fractions were subjected to electrophoresis on 7.5% polyacrylamide gels containing SDS, transferred to polyvinylidene difluoride membrane, and probed by using monoclonal antibodies against Mcm2p (Mcm2–18), Orc3p (SB3), and Cdc6p (9H8/5) (32).

Protein Preparation—Wild type ORC was expressed in Sf9 cells infected with a recombinant baculovirus and was purified as described (43, 44). Wild type and mutant glutathione *S*-transferase (GST)-Cdc6p fusion proteins were purified from *E. coli* cells (DH5α) transformed with overproducing plasmids[3] by use of glutathione-agarose column chromatography (45). The cells were lysed by digestion with lysozyme and centrifuged, and the supernatant was subjected to glutathione-agarose chromatography as specified by Sigma. GST-Cdc6p was eluted with 10 mM reduced glutathione, and the protein extract was dialyzed to remove glutathione (buffer A: 20 mM HEPES-KOH, pH 7.6, 0.1 M KCl, 1 mM EDTA, 1 mM dithiothreitol, and 20% glycerol).

Glutathione Beads Precipitation Assay (Pull-down Assay)—The pull-down assay for the detection of ORC and Cdc6p interaction was performed as described (32). ORC, GST-Cdc6p, and ARS1 DNA fragments (290-base pair fragments generated by PCR) were incubated at 30 °C for 10 min in a 25-μl reaction mixture containing 50 mM HEPES-KOH, pH 7.5, 0.2 M KCl, 1 mM EDTA, 1 mM EGTA, 5 mM MgOAc, 0.02% Nonidet P-40, 10% glycerol, 2 mg/ml bovine serum albumin, and 1 mM ATP. The mixture was diluted with the same buffer but without bovine serum albumin, and then 40 μl of glutathione agarose beads (Sigma, 50% slurry) were added. After 2 h of rotation at 4 °C, the beads were collected, suspended in SDS sample buffer, applied to 7.5% polyacrylamide gels containing SDS, and subjected to electrophoresis, and then proteins were immunoblotted with the anti-Orc5p monoclonal antibody, SB5 (32).

Trypsin Digestion Assay—The trypsin digestion assay for detecting conformational changes in the ORC was done as previously described (32). After incubation of ORC, Cdc6p, and ARS1 DNA fragments for 10 min at 30 °C, trypsin was added and samples were further incubated for 10 min at 30 °C. Samples were analyzed by SDS-polyacrylamide gel electrophoresis as above, and immunoblotted with anti-Orc6p monoclonal antibody, SB 49 (32).

RESULTS

Strategy for Site-directed Mutagenesis and Plasmid Shuffling Analysis—Sensor 1 and sensor 2 regions of *S. cerevisiae* Cdc6p were mapped to Thr^{253}–Asp^{266} and Gly^{329}–Pro^{366}, based on sequence similarity to other proteins belonging to the AAA^+ family (27). These regions are essential for ATPase activities in this family of proteins (27, 36), and therefore conserved and essential amino acid residues in these regions (including their proximal regions) of *S. cerevisiae* Cdc6p were identified. Of particular interest, asparaginine and threonine in the sensor 1 region and basic amino acid residues in the sensor 2 region are

[3] M. Weinreich and B. Stillman, unpublished observation.

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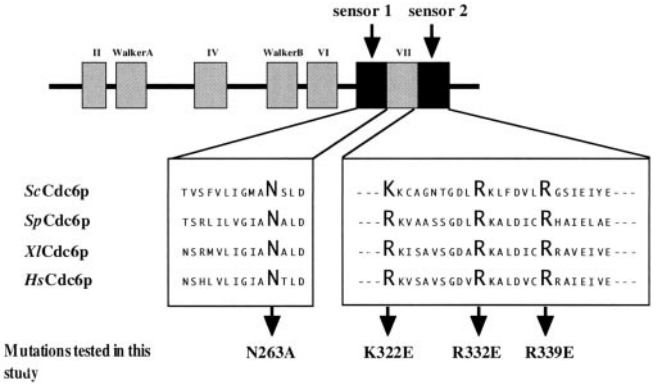

FIG. 1. **Amino acid sequences of Cdc6p and the strategy for site-directed mutagenesis.** The position of various domains of Cdc6p (27, 33) and amino acid sequences of sensor 1 and 2 regions of Cdc6p from various species (22, 53, 63, 65) are shown. *Sc*, *S. cerevisiae; Sp*, *S. pombe; Xl*, *X. laevis*; *Hs*, human.

important for the ATPase activity of AAA^+ family proteins (27, 36). Residues Asn^{263}, Lys^{322}, Arg^{332}, and Arg^{339} of *S. cerevisiae* Cdc6p are conserved among Cdc6p from various species (Fig. 1).

Using site-directed mutagenesis, four mutant *cdc6* genes were constructed (*cdc6N263A*, *cdc6K322E*, *cdc6R332E*, and *cdc6R339E*). Each mutant gene was inserted into a plasmid that contained the *LEU2* gene, and the plasmid was transformed into strain YB209 that contained a chromosomal *CDC6* gene deletion and an alternative wild type *CDC6* gene on a plasmid with the *URA3* selectable maker (46). When the transformant was grown on agar plates containing 5-fluoro-orotic acid, the *URA3* plasmid was selected against and lost, causing cells to rely solely on the mutant *cdc6* gene (plasmid shuffling analysis). Cells that expressed Cdc6N263Ap or Cdc6R332Ep grew slowly and formed small colonies only after 4 days of incubation (Fig. 2*A* and data not shown). The size of colonies and doubling rate of cells that expressed Cdc6pR322E or Cdc6pR339E were indistinguishable from those of cells containing wild type Cdc6p (data not shown). These results showed that Asn^{263} and Arg^{332}, but not Arg^{322} and Arg^{339}, were important for the function of Cdc6p *in vivo,* and suggested that the ATPase activity of Cdc6p is involved in some essential steps of eukaryotic DNA replication.

Cultures of cells expressing both the wild type Ccd6p and Cdc6N263Ap (YB209 transformed with pNT101) showed slow growth, with a doubling time about twice that of the wild type strain. Even slower growth was seen when the mutant *cdc6* gene was introduced into cells on a high copy number plasmid (data not shown), suggesting that N263A has a weak dominant negative phenotype. In a recent publication, a similar dominant negative phenotype was reported for this mutant when the protein was over expressed from a strong promoter (47). We did not observe this phenotype for other mutations, including R332E.

Effect of Mutant Cdc6p on Cell Cycle Progression—To examine the function of Cdc6N263Ap and Cdc6R332Ep *in vivo*, a system in which the expression of Cdc6p could be regulated was necessary. In the K4055 strain, the original *CDC6* gene is deleted, and another *CDC6* gene under the control of the *MET3* promoter is integrated into the chromosome. Thus, in this strain, the wild type *CDC6* can be repressed by adding methionine to the culture medium.

cdc6N263A and *cdc6R332E* expressed from the endogenous *CDC6* promoter were integrated into the *LEU2* locus of the K4055 strain to construct strains NT101 and NT102, respectively (Table I). NT101 and NT102 did not form colonies on

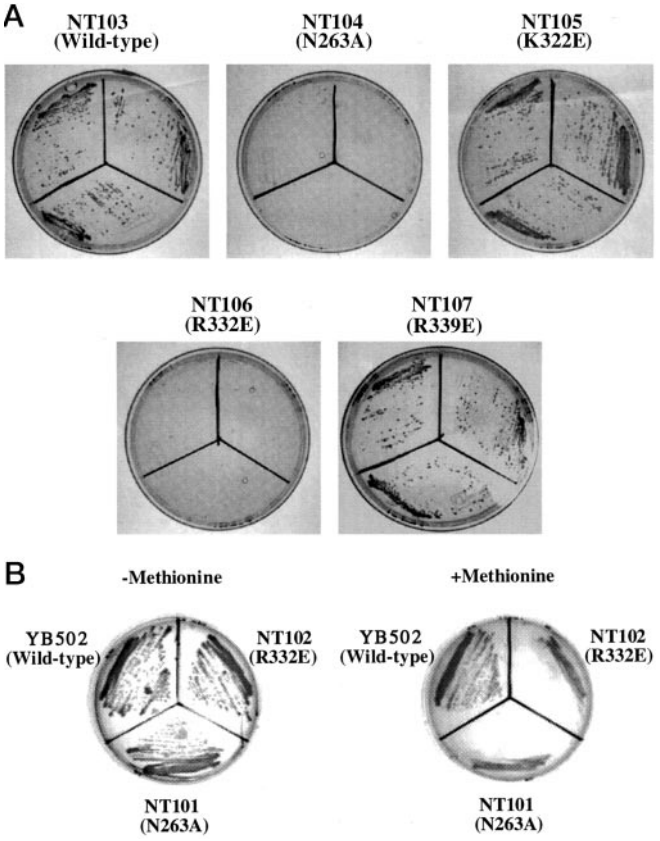

FIG. 2. **Identification of essential amino acid residues in Cdc6p.** *A*, plasmid shuffling analysis. YB209 strain was transformed with pMW71, pNT11, pNT12, pNT13, and pNT14, which contain genes encoding the wild type and mutants N263A, K322E, R332E, and R339E Cdc6p, respectively. The resultant strains were incubated on SC plates containing 5-fluoro-orotic acid at 30 °C for 2 days. Triplicate isolates are shown for each strain. *B*, methionine repression of *CDC6* gene. YB502, NT101, and NT102, which contain *CDC6*, *cdc6N263A*, and *cdc6R332E* genes, were streaked on SC plates with or without methionine (20 mM) and incubated at 30 °C for 2 days.

agar plates containing 20 mM methionine after 2 days of incubation (Fig. 2*B*). However, these two strains formed small colonies on methionine-containing plates after a further 2 days of incubation (data not shown). Thus, in the presence of methionine, NT101 and NT102 grew more slowly than the wild type control strain (YB502), possibly because of prolongation of a distinct phase of cell cycle.

To determine whether a phase of cell cycle was prolonged in NT101 and NT102 in the presence of methionine, exponentially growing YB502, NT101, and NT102 cells in methionine-free SC medium were transferred to SC medium containing 20 mM methionine, and the cellular DNA content was determined periodically by FACS analysis. Compared with wild type cells, in NT101 and NT102, the proportion of cells with an approximate 2 C DNA content at first increased over time (Fig. 3*A*), and eventually, a small but significant number of cells accumulated with an approximate 1 C DNA content (data not shown). These data suggest that cell cycle did not stop at a distinct stage but that some stages were prolonged.

FACS analysis was also performed on cells that were blocked at the G_2 phase by nocodazole and then released. The cells were incubated with methionine in the presence of nocodazole and then released into nocodazole-free SC medium containing 20 mM methionine. The results showed that NT101 and NT102 strains could be arrested in G_2 with nocodazole, but when released they entered the G_1 and S phase and then gradually accumulated with nearly 2C DNA content (Fig. 3*B*).

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TABLE I
S. cerevisiae strains used in this study

Strains	Genotype	Source
W303–1a	*MAT*a *ade2–1 ura3–1 his3–11,15 trp1–1 leu2–3, 112 can1–100*	Ref. 64
K4055	W303 *MAT*a *cdc6*Δ::*hisG pMET3-CDC6*::*TRP1*	Ref. 10
YB209	W303 *MAT*a *cdc6*Δ/*pRS426-CDC6*	Ref. 46
YB502	K4055 *CDC6*::*LEU2*	Ref. 18
NT101	K4055 *cdc6* N263A::*LEU2*	This work
NT102	K4055 *cdc6* R332E::*LEU2*	This work
NT103	YB209/pMW71	This work
NT104	YB209/pNT11	This work
NT105	YB209/pNT12	This work
NT106	YB209/pNT13	This work
NT107	YB209/pNT14	This work

Pulse-Field Gel Electrophoresis Analysis—The FACS analysis (Fig. 3) suggested that S or G_2 phases of the cell cycle were prolonged in NT101 (N263A) and NT102 (R332E) cells when methionine was present to repress expression of the wild type Cdc6p. In other words, the proportion of cells in S or G_2 was increased by expression of the mutant Cdc6p. To distinguish between these two phases, we performed pulse-field gel electrophoresis analysis. Chromosomal DNA from S phase cells does not enter this type of gel because of lack of condensation of chromatin and the presence of DNA replication intermediates, in contrast to DNA from other phases of the cell cycle (48). We confirmed that chromosomal DNA from S phase cells (hydroxyurea-treated cells) but not that from other phases of cell cycle (α-factor treated cells or nocodazole treated cells) remained in the wells of the gel even after very long (15.2 h at 300 V) electrophoresis (Fig. 4, compare *lane 3* with *lanes 1*, *2*, and *4*).

YB502, NT101, and NT102 cells were incubated in SC medium containing 20 mM methionine for 10 h, and identical amounts of their chromosomal DNA were analyzed (Fig. 4, *lanes 5–7*). Compared with the wild type YB502 strain, a smaller proportion of chromosomal DNA from NT101 (N332A) and NT102 (R332E) cells entered into the gel. The data suggested that the proportion of cells in S phase was higher in NT101 and NT102 than in YB502. We conclude that cells expressing Cdc6N263Ap or Cdc6R332Ep instead of the wild type protein accumulate in the S phase and that these mutant proteins slow the progress of the cell through the S phase. DNA from some cells of these two strains entered the gel, suggesting that a fraction of the cells were in the G_2 phase and had completed the S phase.

Chromatin Binding Analysis—MCM loading onto chromatin depends on Cdc6p *in vivo* (14, 16, 18, 19). Inefficient MCM loading may prolong S phase because the number of origins activated would be less. Thus, the prolonged S phase in NT101 and NT102 cells in the presence of methionine could be due to inefficient MCM loading by Cdc6N263Ap and Cdc6R332Ep. We tested this hypothesis using a chromatin binding assay (15).

Because MCM is loaded onto chromatin in the early G_1 phase (before the α-factor arrest point) (16), we synchronized cells with nocodazole and then released them into SC medium containing both α-factor and methionine. As shown in Fig. 5*A*, less Mcm2p was bound onto chromatin in NT101 and NT102 cells compared with YB502 cells. At 90 min after the nocodazole release, compared with the data in YB502, only about 20 or 4% of Mcm2p was loaded on chromatin in NT101or NT102, respectively, based on the densitometric scanning of Fig. 5*A*. Because the amounts of chromatin bound Orc3p were nearly constant in all samples (Fig. 5*A*), the results showed that expression of Cdc6N263Ap or Cdc6R332Ep instead of the wild type Cdc6p caused inefficient MCM loading onto chromatin. We detected two bands using anti-Mcm2p antibody. The size of the upper band is much the same as that deduced from the amino acid sequence. The lower band may be a degradation product as reported in other papers (15, 18).

Next, we examined the loading of Cdc6p (wild type, Cdc6N263Ap, and Cdc6R332Ep) onto chromatin. Because Cdc6p is expressed at a very low level compared with ORC and MCM proteins, the protein was difficult to detect. At 90 min after the nocodazole release, compared with the data in YB502, about 50 or 30% of Cdc6p was loaded on chromatin in NT101or NT102, respectively, based on the densitometric scanning of Fig. 5*A*. Therefore, comparing data between *panels A* and *B* of Fig. 5, it is not clear whether the inefficient loading of MCM onto chromatin in NT101 and NT102 cells was due to the inefficient loading of Cdc6p on chromatin. At least, we can say that the slow growth and the prolonged S phase phenotypes of mutations N263A or R332E may be caused by inefficient recruitment of MCM onto chromatin. Because we measured the binding of Orc3p, Mcm2p, and Cdc6p to bulk chromatin in Fig. 5, it is not certain whether these proteins bound to origins of DNA replication in cells.

Biochemical Analysis of Cdc6N263Ap and Cdc6R332Ep—We recently established an *in vitro* system to study the interaction between ORC and Cdc6p on an origin of chromosomal DNA replication in yeast (*ARS1*) (32). This system was reconstituted with ORC purified from recombinant baculovirus-infected insect cell extract, GST-Cdc6p fusion protein purified from *E. coli* cell extract, and *ARS1* DNA fragments amplified by PCR (32). Using this system, we here examined biochemical properties of Cdc6N263Ap and Cdc6R332Ep *in vitro*.

GST-Cdc6N263Ap and GST-Cdc6R332Ep were prepared by the same methods used for purification of the wild type protein (32), with similar purities and recoveries. The wild type and mutant fusion proteins were incubated with ORC and *ARS1* DNA fragments. Complexes were recovered by using glutathione-coated beads to bind the GST fusion proteins, and coprecipitated ORC was detected by immunoblotting with a monoclonal antibody directed against one of the Orc5p subunit. GST-Cdc6p with either a defective sensor 1 or sensor 2 region (GST-Cdc6N263Ap or GST-Cdc6R332Ep) bound to ORC to a similar extent as the wild type protein (Fig. 6).

In the previous paper, we showed, using partial protease digestion, that wild type GST-Cdc6p altered the higher order structure of ORC (32). This alteration was inhibited by ATPγS, an analog of ATP that is difficult to hydrolyze, suggesting that ATPase activity was required. The conformational change of ORC was specific for subunits Orc1p, Orc2p, and Orc6p. Within ARS1, Orc2p and Orc6p are located adjacent to the B2 element, which is also the most likely site of binding of MCM (49), a replication helicase (50–52). These results led to the proposal that the alteration in ORC conformation was required to recruit MCM onto chromatin. To further examine this proposed mechanism, we here tested whether GST-Cdc6N263Ap and GST-Cdc6R332Ep could induce the conformational change in ORC.

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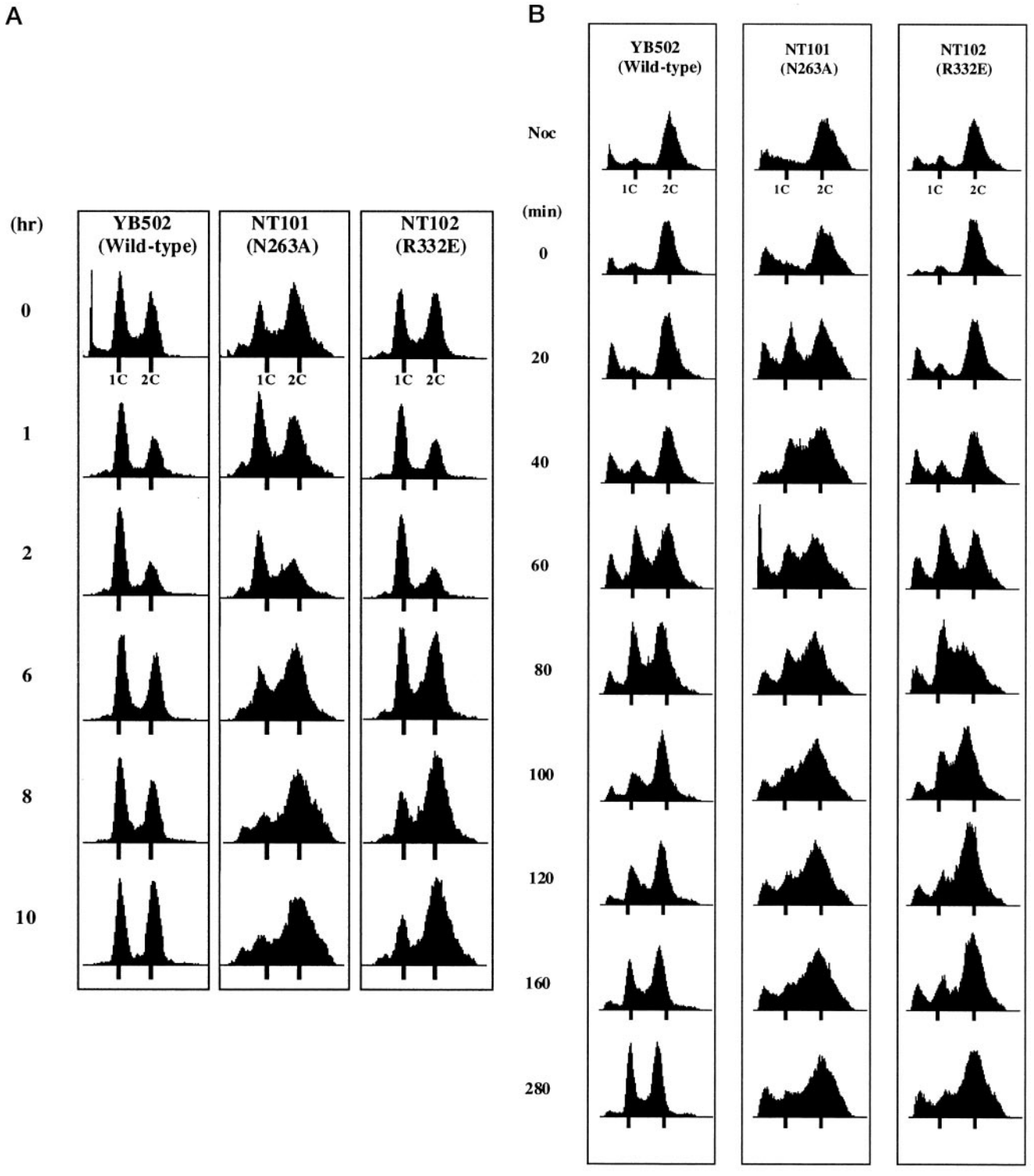

FIG. 3. **Effect of the mutant Cdc6p on cell cycle.** *A*, YB502, NT101, and NT102 cells were cultured to logarithmic phase, and then methionine was added to repress the expression of the wild type *CDC6* gene. *B*, YB502, NT101, and NT102 cells were arrested in G_2/M phase using nocodazole, and then expression of wild type Cdc6p was repressed by incubation with 20 mM methionine for 2 h. The cells were released into medium with methionine (20 mM). A small portion of culture was taken after indicated periods and analyzed by FACS.

Purified GST-Cdc6p, GST-Cdc6N263Ap, and GST-Cdc6-R332Ep were incubated first with ORC and the *ARS1* DNA fragment and then with 15 μg/ml trypsin. Degradation of Orc6p was monitored by immunoblotting. The Orc6p subunit was rendered more protease-sensitive by wild type GST-Cdc6p, compared with a reaction with no GST-Cdc6p, as described previously (32) (Fig. 7). Because the Orc6p subunit became more sensitive to trypsin in the presence of wild type GST-Cdc6p, this alteration in the pattern of trypsin digestion is not due to the Cdc6p-dependent protection of ORC from trypsin digestion but Cdc6p-dependent conformation change of ORC, which causes higher sensitivity of Orc6p subunit to trypsin. Interestingly, GST-Cdc6R332Ep did not increase the protease sensitivity of Orc6p (Fig. 7). GST-Cdc6N263Ap slightly increased the protease sensitivity of Orc6p; however, this increase required higher concentrations of GST-Cdc6N263Ap

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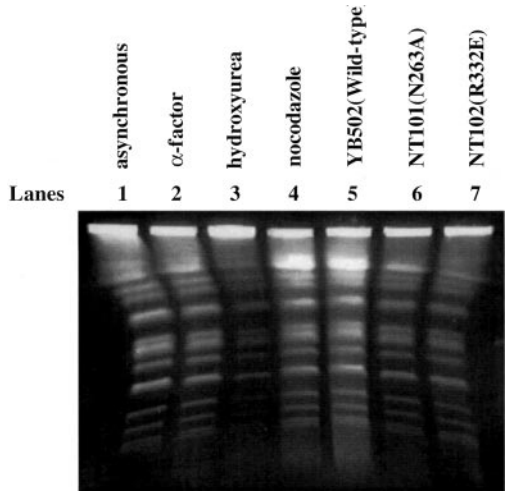

FIG. 4. **Pulse-field gel electrophoresis of DNA to determine chromatin state.** YB502, NT101, and NT102 cells, which express the wild type, N263A, and R332E Cdc6p, respectively, were grown to the log phase in the absence of methionine and then further incubated for 10 h in the presence of methionine (20 mM). To obtain the control DNA from G_1, S, and G_2 phases cells, W303 cells were incubated with α-factor (5 μg/ml), hydroxyurea (1 mM), and nocodazole (3 μg/ml), respectively. Chromosomal DNA was extracted from cells and analyzed by pulse-field gel electrophoresis.

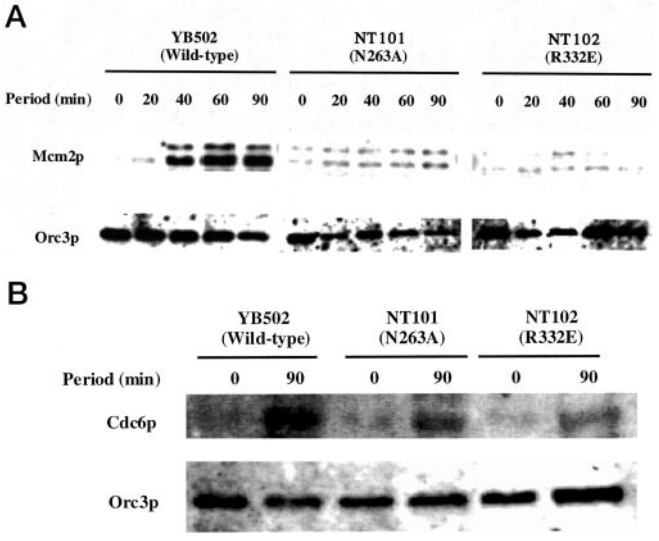

FIG. 5. **Chromatin binding analysis.** YB502, NT101, and NT102 cells were arrested in G_2/M phase using nocodazole, and then expression of wild type Cdc6p was repressed by incubation with 20 mM methionine for 2 h. The cells were released into medium with methionine (20 mM) and α-factor (5 μg/ml), and small portions of culture were taken after the indicated periods. Chromatin fractions were prepared and analyzed by immunoblotting using monoclonal antibodies specific for Orc3p (SB3) (*A* and *B*), Mcm2p (Mcm2–18) (*A*), and Cdc6p (9H/8) (*B*).

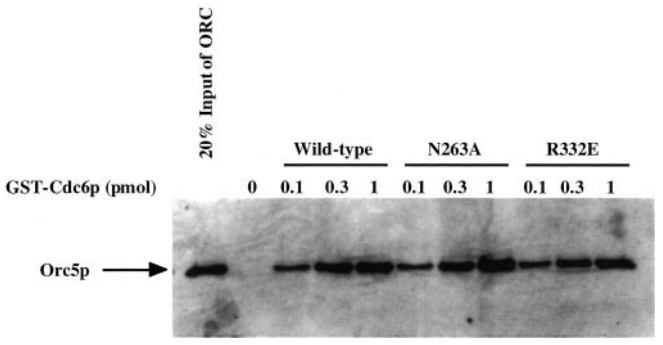

FIG. 6. **Interaction of Cdc6N263Ap and Cdc6R332Ep with ORC *in vitro*.** The indicated amount of purified GST-Cdc6p (wild type, N263A, and R332E) was incubated with 0.3 pmol of ORC and 0.3 pmol of *ARS1* DNA fragments (290 bp) for 10 min. Glutathione-agarose beads can precipitate the complexes containing GST-Cdc6p and ORC in precipitates was visualized by immunoblotting with anti-Orc5p antibodies (SB5).

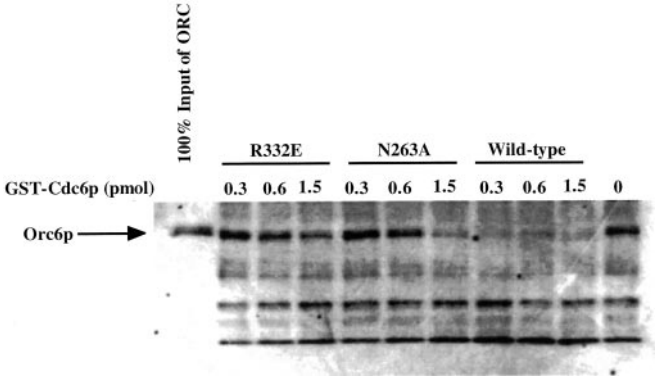

FIG. 7. **ORC trypsin digestion assay for Cdc6N263Ap and Cdc6R332Ep.** ORC (0.3 pmol) and 0.3 pmol of wild type *ARS1* DNA fragment were incubated with the indicated amounts of Cdc6p (wild type, N263A, and R332E) for 10 min. Then, trypsin was added (final concentration, 15 μg/ml), and incubation was continued for 10 min at 30 °C. The samples were separated by electrophoresis on an SDS-polyacrylamide gel (7.5%), followed by immunoblotting with anti-Orc6p monoclonal antibody (SB49).

than the wild type GST-Cdc6p (Fig. 7). The data suggested that GST-Cdc6N263Ap and GST-Cdc6R332Ep were less active for the change of the conformation of ORC.

DISCUSSION

The amount of Cdc6p fluctuates throughout the cell cycle, suggesting that Cdc6p is a key regulator of the cycle progression, particularly the establishment of a competent prereplication complex and entry into S phase. Cdc6p seems to have a number of different functions. The null mutant of *cdc6* was unable to enter S phase, but it also allowed progression through mitosis in the absence of DNA replication (10, 18). The former function mapped to the essential domains of Cdc6p that are structurally related to Orc1p and other AAA^+ proteins (18, 33, 43, 54). The latter function mapped to the amino terminus, suggesting that Cdc6p controls the G_1/S transition and also the G_1/M checkpoint (18, 55, 56). Genetic analysis in *S. pombe* suggested that it restricts the initiation of DNA replication to once per cell cycle (22–24). Thus, Cdc6p is a critical component of many aspects of DNA replication and its control.

In this study, we investigated whether the domains that are known to be required for ATPase activity of AAA^+ proteins are also involved for the function of Cdc6p in cell cycle progression and DNA replication. Mutants in *CDC6* that cause a defect in either of the sensor 1 or sensor 2 regions (Cdc6N263Ap or Cdc6R332Ep, respectively) were used because they were predicted to be important for regulation of ATPase activity. Both FACS and pulse-field gel electrophoresis analyses demonstrated that these mutant proteins prolonged S phase. Since completion of this work, a recent report appeared demonstrating that overproduction of Cdc6N263Ap or Cdc6R332Ap caused a prolonged S phase and a temperature-sensitive phenotype (47). The results of Schepers and Diffley (47) and those described herein suggest that the ATPase activity of Cdc6p is involved in S phase progression. This idea is consistent with conclusions from previous reports. Overproduction of a mutant Cdc6p with a defective Walker B motif, which is thought to be involved in the ATPase activity, prevented S phase progression in *S. cerevisiae* (33). In *S. pombe,* triple mutations in the sensor 2 region caused a cell cycle arrest at S phase (35). Furthermore,

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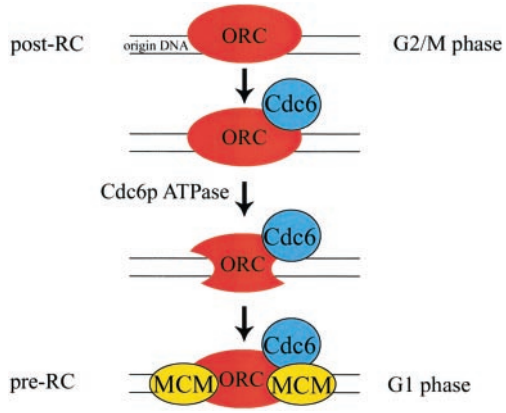

FIG. 8. **A proposed model of the recruitment of MCM onto origin of DNA replication by Cdc6p ATPase.** In the late M phase, Cdc6p binds to ORC, which is bound to origins of DNA replication throughout the cell cycle. Then Cdc6p changes the conformation of ORC using its ATPase activity. This conformation change stimulates MCM loading onto chromatin at G_1 phase.

mutant human Cdc6p with a defective Walker B motif and weak ATPase activity caused slow progression in S phase when transferred to *S. pombe* cells (57). Thus, the ATPase activity of Cdc6p probably plays an important role in S phase progression in all eukaryotes.

In cells expressing Cdc6N263Ap or Cdc6R332Ep, the observed slow progression through S phase was probably due to inefficient of loading MCM proteins onto chromatin. If MCM proteins were loaded onto a smaller fraction of origins of DNA replication than normally loaded in wild type cells, the period required to duplicate chromosomal DNA completely may be prolonged, resulting in slow progression of S phase. It has been reported recently that overproduction of Cdc6N263Ap or Cdc6R332Ap caused inefficient MCM loading onto chromatin (47). Perkins and Diffley (33) reported that overproduction of a mutant Cdc6p with a defective Walker B motif inhibited both MCM loading onto chromatin and also pre-RC formation. We showed here that mutant Cdc6p (N263A or R332E) bound normally to ORC *in vitro*. Therefore, the ATPase activity of Cdc6p is presumably not involved in Cdc6p binding to chromatin but in the later step of recruiting MCM.

Cdc6p was known to control the frequency of initiation of DNA replication. In an *orc5-1* mutant at the nonpermissive temperature, the frequency of initiation at each origin was considerably lower than in wild type cells, and cells could not proliferate. But when Cdc6p was overexpressed, the frequency of initiation at each origin increased so that cells were viable (46). Such suppression of the ORC defect is consistent with observations that Cdc6p interacts with ORC at origins and affects ORC function in a process that requires ATP (32, 46). Cdc6p not only interacts with ORC, but in the presence of a hydrolyzable form of ATP, ORC structure changes so that ORC is more susceptible to proteolysis (32). Mutations in the Cdc6p Walker A motif that should block ATP binding prevent MCM loading and alteration of ORC structure (32, 33). Here we demonstrate that mutations in the sensor 1 and sensor 2 motifs of Cdc6p also affect both of these functions of Cdc6p, further strengthening the suggestion that Cdc6p must alter ORC structure for both proteins to cooperate to load the MCM proteins onto origins of DNA replication (Fig. 8).

The sensor 1 and sensor 2 motifs were predicted to interact with ATP based on the crystal structures of a number of AAA^+ proteins, including *E. coli* DNA polymerase δ' subunit, *P. aerophilum* Cdc6p, the *N*-ethylmaleimide-sensitive factor D2 domain, and p97 chaperone proteins required for recycling the SNARE proteins during membrane fusion (27, 35–37, 58). The two regions of these proteins exist in the characteristic domains I and II that form the core structure of these ATPase and are conserved in all AAA^+ proteins (27). Sensor I and II interact with the γ-phosphate of the bound ATP and can therefore sense whether the nucleotide-binding domain of the proteins is occupied by ATP or ADP (36). In a number of AAA^+ proteins, including *N*-ethylmaleimide-sensitive factor, p97, and the recently determined structure of the *E. coli* clamp loading complex ($\delta'\gamma_3\delta$) (59), a number of proteins with the conserved AAA^+ domain structure combine to form a complex in which the sensor region in one subunit interacts with the γ-phosphate of ATP in an adjacent protomer. For example, the sensor 2 Arg^{359} residue in the p97 ATPase (in a similar position to Arg^{332} in Cdc6p) interacts with the γ-phosphate of ATP in an adjacent subunit (58). This allows ATP binding and hydrolysis in one subunit to influence the structure and function of a neighboring subunit. In this way, a ratchet mechanism driven by ATP, often involving many subunits, induces a significant structural change in the protein complex, as proposed for the *E. coli* γ-complex as it opens the β-clamp ring prior to loading the clamp onto DNA (59). Hence AAA^+ proteins can perform ATP-driven work and modify the structure of other protein complexes.

We suggest that Cdc6p might interact with ORC in a similar manner. Because three of the ORC subunits are AAA^+ proteins and are therefore predicted to have a structure similar to Cdc6p (27, 60), it is possible that Cdc6p sensor motifs influence the ATPase activity of ORC. Alternatively, one of the ORC subunits regulates the ATPase activity of Cdc6p via the sensor region. ORC has at least two ATP-binding proteins (Orc1p and Orc5p) and an ATPase activity that is regulated by origin DNA and requires ATP to bind to its double-stranded DNA recognition site (9, 44, 61, 62). Only the Orc1p subunit is known to hydrolyze ATP, but we suggest that the reason why the Orc5p subunit binds ATP is that it participates in the ratcheting mechanism by presenting an ATP-γ-phosphate to another protein in the ORC-Cdc6p complex. By this mechanism, the concerted action of ORC (the Orc1p, Orc5p, and possibly the Orc4p subunits) and Cdc6p, driven by ATP and with DNA as an anchor, may facilitate the loading of the hexameric structure of the MCM protein complex onto DNA to form the pre-RC (Fig. 8). Interestingly, the six MCM proteins are also AAA^+ ATPases and a DNA helicase, and if such a concerted ratchet mechanism exists for this hexamer, it may facilitate DNA unwinding at the origin upon activation by cyclin-dependent protein kinases and the Cdc7p-Dbf4p protein kinase (17). Clearly further detailed biochemistry of pre-RC formation on origin DNA will determine whether this model explains why many of the initiation proteins are members of the AAA^+ class.

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