Saccharomyces cerevisiae Cdc6 is a protein required for the initiation of DNA replication. The biochemical function of the protein is unknown, but the primary sequence contains motifs characteristic of nucleotide-binding sites. To study the requirement of the nucleotide-binding site for the essential function of Cdc6, we have changed the conserved Lys114 at the nucleotide-binding site to five other amino acid residues. We have used these mutants to investigate in vivo roles of the conserved lysine in the growth rate of transformant cells and the complementation of cdc6 temperature-sensitive mutant cells. Our results suggest that replacement of Lys with Glu (K114E) and Pro (K114P) leads to loss-of-function in supporting cell growth, replacement of the Lys with Gln (K114Q) or Leu (K114L) yields partially functional proteins, and replacement with Arg yields a phenotype equivalent to wild-type, a silent mutation. To investigate what leads to the growth defects derived from the mutations at the nucleotide-binding site, we evaluated its gene functions in DNA replication by the assays of the plasmid stability and chromosomal DNA synthesis. Indeed, the K114P and K114E mutants showed the complete retraction of DNA synthesis. In order to test its effect on the G1/S transition of the cell cycle, we have carried out the temporal and spatial studies of yeast replication complex. To do this, yeast chromatin fractions from synchronized culture were prepared to detect the Mcm5 loading onto the chromatin in the presence of the wild-type Cdc6 or mutant cdc6(K114E) proteins. We found that cdc6(K114E) is defective in the association with chromatin and in the loading of Mcm5 onto chromatin origins. To further investigate the molecular mechanism of nucleotide-binding function, we have demonstrated that the Cdc6 protein associates with Orc1 in vitro and in vivo. Intriguingly, the interaction between Orc1 and Cdc6 is disrupted when the cdc6(K114E) protein is used. Our results suggest that a proper molecular interaction between Orc1 and Cdc6 depends on the functional ATP-binding of Cdc6, which may be a prerequisite step to assemble the operational replicative complex at the G1/S transition.
Roles of Nucleotide-binding Site of the Yeast CDC6 Gene

Xenopus (19). The conservation of Cdc6 among species implies an essential role for this protein.

Genomic footprinting has defined two cell cycle stages regarding the formation of replication complexes (RC): pre-RC in G1 phase and post-RC after S phase to M/G1 border (20–22). The post-RC footprint closely resembles that produced in vitro with purified ORC and Abf1, which protect A/B1 and B3 elements, respectively. The pre-RC is defined by its further protecting the B2 element of ARS1 in addition to the A/B1 and B3 elements. The data suggest that the initiation of DNA replication is not controlled by the binding of ORC and Abf1 to the origins; instead, modification of origins has to occur with the involvement of some additional factors. The Cdc6 protein is required for establishment and maintenance of pre-RC, because the arrested cdc6–1 mutant produces a post-replication footprint (23–25). The Cdc6 protein is also required for the loading of MCM proteins (26–30). In addition, the Cdc6 protein interacts with Cdc28 protein complex after p40

Roles of Nucleotide-binding Site of the Yeast CDC6 Gene

The roles of the Cdc6 protein are discussed in this report.

In Vitro Mutagenesis—Muta-Gene in vitro mutagenesis kit (Bio-Rad) was used to carry out the experiments as described below. The mutagenic (GTT CCG CCT GCC ACT GCT (GCC/GCT/GTG ACT)) and universal primers were synthesized (12, 37). T7 DNA polymerase instead of the Klence fragment of DNA polymerase I was used in the reaction (38). The mutagenized DNA is shown in Fig. 1.

Plasmid Stability Assay—Yeast cdc6–1 shows elevated chromosomal loss. The wild-type and mutant cdc6 genes were transformed into strain cdc6–1 to examine their plasmid stability. A colony grown on selective medium was resuspended in 0.2 ml of water. A 0.1-ml sample was used to inoculate 5 ml of nonselective media (eitherYPD or SD plus uracil), and cultures were grown at 30 °C with aeration for 5–10 generations. Dilutions of the initial suspension were plated on YPD plates, and colonies were counted to determine the initial concentration of cells. These plates were then replica-plated to SD-ura to determine the percentage of plasmid-bearing cells.

PFGE Labeling Method—The PFGE labeling method was used to investigate yeast chromosomal DNA synthesis (39). In this method, yeast cells are first labeled with 32P in vivo and chromosomal DNA is then resolved by pulsed field gel electrophoresis. Briefly, 20 ml of yeast cell culture was grown at room temperature to the early log phase (~108 cells/ml) and arrested by α-factor for 90 min. The cells were then washed with 20 ml of low phosphate medium (LPM) three times, and resuspended into the same volume of LPM. After heat treatment (37 °C), 50 μCi of radiolabeled [α-32P]dCTP was added. The culture was grown for another 1 h, and labeling was quenched with 50 mM cold phosphate. The labeled cells were harvested and then washed with phosphate-buffered saline buffer, and molecules were separated using the Bio-Rad CHEF-DR II Megabase DNA pulse field electrophoresis system. The resulting gel was dried and autoradiographed.

Preparation of Synchronous Chromatin Fractions—Yeast chromatin was prepared according to Lue and Kornberg (40) with slight modifications. Briefly, yeast strain K4055 cells harboring plasmid fusions with co-transformation of YEp-Mcm5-HA and YCP5N-Cdc6–1-T7, or YCP5N-cdc6–1-K114E–1-T7. The transformant cells were grown on the CSM medium in the presence of methionine (MET–) to shut off the endogenous CDC6. The cells were synchronized at G1 with α-factor at final concentration of 10 μg/ml at room temperature for 4 h. The cells were then released from α-factor block, grown on the fresh MET– medium, and collected at the indicated time points. Aliquots of cells were harvested at different time intervals to monitor the degree of synchrony by measurement of percentage of budded cells and FACS analysis (38). Yeast cells were washed once with 50 ml ice-cold EDTA and incubated in a solution containing 20 ml EDTA/2% β-mercaptoethanol at 30 °C for 30 min. After incubation, the cells were centrifuged and washed with 1 ml sorbitol. Spheroplasts were generated by digesting the cells with 100 μg/ml yeast lytic enzyme (ICN) in a solution containing 1 M sorbitol, 5 mM β-mercaptoethanol at 37 °C for 60 min. The spheroplasts were cooled on ice for 10 min, centrifuged at 3000 × g for 10 min at 4 °C, and washed once with the 1 M ice-cold sorbitol. The spheroplasts were collected by centrifugation and lysed in a buffer containing 20 mM (w/v)
Fig. 1. Site-directed mutagenesis of the CDC6 gene at its nucleotide-binding site. A, the consensus sequence of a nucleotide-binding domain is shown on the top. The Cdc6 protein sequence from position 108 to 115 is presented in the middle, and the corresponding CDC6 DNA sequence is lined up on the bottom. The mutagenic primer is identical to the wild-type CDC6 DNA sequence depicted above except that the nucleotide A is changed to G, C, and/or T, resulting in the Lys residue altering to other amino acid residues. B, alterations of the codon and the amino acid residue are summarized. C, the DNA sequences of mutated cdc6 genes are shown on the bottom.

Ficoll 400 (Sigma), 20 mM HEPES-KOH, pH 7.5, 20 mM KCl, 5 mM MgCl₂, 3 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1 mM EDTA with a Teflon/glass homogenizer. Cell debris were removed by four spins at 3000 × g for 5 min at 4 °C. Uniform white supernatants were recovered and were spun at 36,000 rpm and 4 °C for 30 min. Nuclear pellets were resuspended in 500 μl of the spermatophore lysis buffer described above and subjected to Ficoll 400 gradient centrifugation (20–45%) at 36,000 rpm and 4 °C for 30 min. Chromatin pellets were resuspended in a buffer H0.1 (50 mM HEPES-KOH, pH 7.5, 100 mM KCl, 5 mM magnesium acetate, 1 mM EDTA, 0.02% Nonidet P-40, 1 mM dithiothreitol, 1 mM PMSF, and 10% glycerol) and stored at −80 °C.

Western Blots to Detect Mcm5 and Cdc6 Proteins—10 μg of protein from each chromatin sample were mixed with 4X SDS loading buffer and boiled in a water bath for 5 min. The samples were resolved by SDS-polyacrylamide gel (8–10%) electrophoresis at 4 °C for 4 h, and proteins were blotted onto an Immobilon-P polyvinylidene difluoride transfer membrane (Millipore). The protein blots were probed either with 12CA5 monoclonal antibody (Boehringer Mannheim) for detecting HA-tagged proteins, or with anti-T7 tag monoclonal antibody (Novagen) for detecting T7-tagged proteins, followed by alkaline phosphatase-conjugated secondary antibody. The protein samples were detected with the Tropix chemiluminescent system.

In Vitro and in Vivo Interaction between Orc1 and Cdc6—500 ml of bacterial culture containing expressed 6xHis-T7-Orc1 tagged protein was harvested and resuspended into 15 ml of Buffer I (20 mM Tris-HCl, pH 8, 500 mM NaCl, 20 mM imidazole, 0.1% Triton X-100) in the presence of 20 mM PMSF and 20 mM benzamidine. 10 mg of lysozyme and 2 mg of DNase were added and incubated at 4 °C for 30 min. The mixture was centrifuged at 13,000 rpm for 20 min. The supernatant was collected (~15 ml) and mixed with 0.2 ml of Ni-NTA (Qiagene) at 4 °C for 60 min. The resulting slurry was packed onto a column and was harvested and resuspended into 15 ml of Buffer I (20 mM Tris-HCl, pH 8, 150 mM NaCl, 50 mM imidazole, 0.1% Triton X-100). The Orc1-charged matrix was ready for Cdc6 interaction assay. GST-Cdc6 fusions were prepared from 250 ml of culture. Crude extracts were made in Buffer II with 20 mM PMSF and 20 mM benzamidine. Aliquot was used for SDS-polyacrylamide gel electrophoresis to analyze their expression (Fig. 2B, lanes 4–6). The rest (~15 ml) was passed through Orc1-charged matrix 10 times for the interaction studies.

For Orc1 and Cdc6 co-precipitation experiments (Fig. 7C), yeast strain BJ5459 was first transformed with YEp181-His-T7-ORC1 (selection marker Leu2). The resulting strain was then transformed separately with either YEp123--CDC6-T7, or YEp-GK114E-T7, or YEp-K114P-cdc6–1-T7, with selection marker Trp1. 500 ml of culture was induced with 2% galactose for 4 h. The cell pellets were disrupted by Bead-Beater™ in Buffer II, supplemented with 20 mM benzamidine, 20 mM PMSF, 1 mg of DNase. Tagged Orc1 was purified by Ni-NTA matrix, concentrated 10-fold, and probed with anti-T7 antibody on protein blots. Monoclonal antibody against T7 tag can detect both His-T7-Orc1 and Cdc6-T7 on the same blot if Orc1 can bring down Cdc6-T7 proteins in the experiment shown in Fig. 7C.

RESULTS

Site-directed Mutagenesis of the Nucleotide-binding Site of the CDC6 Gene—In vitro site-specific mutagenesis is a powerful tool to probe the relationship between the structure and activity of proteins, because the amino acid residues responsible for a particular function can be identified directly (41). Previous analysis of the primary sequence (34) has shown that CDC6 contains a motif conserved among known ATPases, GXGXGKTL, ranging from residues 108–115 in CDC6 (Fig. 1A). This element plays a role in binding the pyrophosphate moiety of nucleotides, and the Lys residue is essential for electrostatic interaction between the proteins and the nucleotides (42). In order to examine whether the conserved Lys residue represents an essential amino acid residue as in other nucleotide-binding proteins, we have mutated the Lys residue to five other residues. The mutagenic primers were similar to the wild-type sequence except for one or two altered nucleotides at the first two bases in the lysine codon (Fig. 1A and B). The following substitutions were made at the conserved lysine: to glutamate to test the effect of introducing a negative charge, to proline to test the effect of a general disruption, to glutamine to test the effect of substituting an amide, to leucine to test the effect of introducing a neutral amino acid, and to arginine to test the effect of changing the configuration of the positive charge. Although both Lys and Arg are basic amino acids, none of the nucleotide-binding sites known so far contain Arg. Substitution of arginine for lysine results in a protein with partial function in some cases. For example, this substitution in yeast RAD3 was found to abolish its ATPase and DNA helicase activities, but not to change the ability to bind ATP (43). Mutagenesis was confirmed by DNA sequencing (Fig. 1C).

Functional Complementation of cdc6–1 Mutant Cells—To test the in vivo function of the nucleotide-binding site, we examined whether the mutant genes can complement the cdc6–1 temperature-sensitive growth defect. We transformed the cdc6–1 mutant strain with plasmids expressing the mutant genes. Transformants were selected at the nonpermissive temperature. As shown in Table I, the wild-type and K114L, K114Q, and K114R are able to complement the defective mutant cells at nonpermissive temperature. Mutant K114E, K114P, and vector alone, however, fail to transform. The few colonies observed for K114E and K114P are presumably due to the recombination between the episomal and chromosomal copies. These results imply that the Lys to Glu mutation may...
Table I

| Plasmids                  | 23 °C | 37 °C |
|---------------------------|-------|-------|
| YEp352-CDC6               | 4.8 x 10³ | 4.7 x 10³ |
| YEp352-K114E              | 4.9 x 10³ | 1     |
| YEp352-K114L              | 4.5 x 10³ | 3.5 x 10³ |
| YEp352-K114R              | 4.2 x 10³ | 4.0 x 10³ |
| YEp352-K114P              | 4.4 x 10³ | 2     |
| YEp352-K114Q              | 4.7 x 10³ | 3.2 x 10³ |
| Vector only               | 4.0 x 10³ | 0     |

Perturb the interaction between Cdc6 and nucleotides; the Lys to Pro mutation may alter steric structure resulting in the loss of CDC6 gene function. Thus, the result supports that the Lys residue is essential for CDC6 gene function.

A trivial explanation of the above findings is that the mutant proteins are not efficiently present. To ensure that failure of the mutant alleles to complement cdc6–1 was due to a defective Cdc6 protein, and not merely due to absence of protein, we compared the steady-state levels of wild-type Cdc6 and the cdc6(K114E) proteins. The cdc6(K114E) protein was chosen, because it had the most drastic defect in the complementation assay. Since Cdc6 is present at very low levels in yeast and is barely detectable with the polyclonal Cdc6 antibody, we cloned the wild-type and mutant alleles under the control of the GAL1,10 promoter and introduced this construct into yeast vectors marked with URA3 or LEU2 (YEp12 and YEp102, respectively). Protein blots of extracts of cells carrying the plasmids were probed with Cdc6 antibody. As shown in Fig. 2, both mutant and wild-type proteins were expressed at the same level, demonstrating that expression levels were not the cause of the lack of in vivo function of cdc6(K114E) protein.

Effects on the Growth Rate from cdc6–1 Transformant Cells—To further evaluate the effect of mutant alleles in the cdc6–1 background, cdc6–1 was transformed with the mutant plasmids, the wild-type plasmid and vector alone, and transformants were selected at room temperature. Transformant cells were grown to log phase at the permissive temperature (25 °C), then transferred to the nonpermissive temperature (37 °C) and incubated for another 8 h, during which growth rates were determined (Fig. 3A). Cells harboring the wild-type plasmid grew at a rate similar to wild-type strains in this medium (tₐ = 120 min). For mutants K114E, K114P, and vector alone, cells essentially ceased to divide after 2 h and totally arrested at 37 °C. The remaining strains grew more slowly than wild-type, with doubling times of 125–130 min for K114R, and 150–160 min for K114L and K114Q at 37 °C. Thus, all of the mutants generated show at least some impairment in growth rate, indicating that an intact lysine residue is critical for optimal CDC6 function. Similar results can be observed from the plates incubated at 25 °C (permissive temperature) and 37 °C (nonpermissive temperature). No colonies were formed in the K114E and K114P transformants (Fig. 3B). As the growth curves of the K114E and K114P transformants are similar to cdc6–1 cells transformed with vector only, it is clear that the K114E and K114P are loss-of-function proteins that cannot rescue the cdc6–1 mutation.

Plasmid Stabilities in cdc6–1 Cells Expressing Mutant cdc6 Genes—Temperature-sensitive mutation cdc6–1 shows enhanced plasmid loss at the nonpermissive temperature, but plasmids may be stabilized by the inclusion of a functional copy of CDC6 on the plasmid. We tested the above mutated alleles of cdc6 for their ability to confer stability on plasmids. As shown in Fig. 4, cdc6–1 cells bearing a plasmid with the wild-type CDC6 gene maintains ~85% of plasmid after 10 generations under nonselective conditions, a loss rate similar to wild-type backgrounds. The K114R plasmid confers slightly lower stability (~70%), and K114L and K114Q plasmids result in 45–50% plasmid-containing cells after 10 generations. K114E and K114P transformants, however, show dramatic plasmid loss, similar to that of YEp352 transformants. The loss of plasmid stability is most likely due to the defect(s) of DNA synthesis. To explore what mechanism affects the growth rate and plasmid DNA stability, we investigated the chromosomal DNA synthesis.

Yeast Chromosomal DNA Synthesis in cdc6–1 and Transformant Cells—The PFGE labeling technique is a novel method, which is highly specific to chromosomal DNA synthesis in yeast. In this method, yeast cells are first labeled by 32P in vivo and chromosomal DNA molecules are then resolved by pulsed field gel electrophoresis. DNA synthesis can be measured by the 32P incorporation into individual chromosomal DNA molecules. We have previously used the PFGE labeling technique to verify that cdc6–1 cells fail to synthesize chromosomal DNA molecules (39). In Fig. 5, we examined DNA synthesis by the PFGE method in cdc6–1 cells transformed with different cdc6 Lys114 mutants. The cultures were synchronized with α-factor (at START), 32P label was added, and cells were incubated at the non-permissive temperature. PFGE was used to resolve the labeled chromosomal DNA molecules. Cells transformed with wild-type CDC6 and vector YEp352 only, are used as the positive and negative controls, respectively. The DNA pattern (Fig. 5A, lanes 3 and 7) is the 32P incorporation (Fig. 5E, lanes 1 and 7) for the controls are as expected. In the K114E transformant (lane 6) and K114P transformant (lane 4), ethidium bromide staining revealed a similar chromosomal pattern, yet little radioisotope incorporation into chromosomal DNA was observed. However, in cells transformed with K114R, K114Q, and K114L plasmids, a nearly wild-type amount of 32P incorporation was observed at the nonpermissive temperature (lanes 2, 3, and 5). Taken together (Figs. 3–5), we conclude that the
K114E and K114P are loss-of-function mutations, K114Q and K114L are partially functional proteins, and K114R functions equivalently to wild-type. Since the only fully functional mutant represents a conservative change in the conserved ATP-binding site, we argue that a functional ATP-binding site in \textit{CDC6} is required for DNA synthesis in vivo.

Effect of Mutant K114E on the Assembly of the Replicative Complex—It has been shown that the Cdc6 is essential for establishment and maintenance of pre-replicative complexes (pre-RCs) and the loading of MCM proteins at G1 phase on chromatin is Cdc6-dependent. Since the initiation of DNA replication requires the prior assembly of pre-RCs, we further tested the hypothesis that the mutation of ATP-binding domain of Cdc6 would lead to the failure of the pre-RC assembly. We selected the mutant cdc6(K114E) for these studies, because it showed the most drastic effect on the \textit{CDC6} gene functions.

**FIG. 3.** Growth properties of the transformants carrying mutant cdc6 genes at the nonpermissive temperature (37 °C). A, yeast carrying the indicated plasmids were grown at SD-ura at room temperature until early log phase. The cultures were shifted to 37 °C (nonpermissive temperature) and harvested at different time points, and cells were counted using a hemacytometer. The value given represents an average number of several independent determinations. ● and solid line, YEp352-CDC6; ○ and thick dashed line, YEp352-K114R; ○ and thin dashed line, YEp352-K114Q; + and dotted line, YEp352-K114L. ● and dashed/dotted line, YEp352-K114P; ▲ and dashed line, YEp352-K114E; △ and solid line, YEp352 only.

**FIG. 4.** Plasmid stability in cdc6–1 and its transformant cells. Strain cdc6–1 was transformed with the mutant plasmids described in Fig. 1. YEp352-CDC6 (wild-type gene) and YEp352 (vector only) were used as positive and negative controls, respectively. Transformants were grown in nonselective media for 5–10 generations and plated in supplemented minimal medium or on YPD. The percentage of colonies containing plasmids was determined by replica plating on to medium lacking uracil. The value given represents an average number of several independent determinations. ● and solid line, YEp352-CDC6; ○ and thick dashed line, YEp352-K114R; ○ and thin dashed line, YEp352-K114Q; + and dotted line, YEp352-K114L. ● and dashed/dotted line, YEp352-K114P; ▲ and solid line, YEp352-K114E; △ and dashed line, YEp352 only.

**FIG. 5.** Chromosomal DNA synthesis in cdc6–1 and transformants. Yeast cdc6–1 transformants containing the wild-type CDC6 and mutant cdc6 genes were subjected to the PFGE-labeling protocol (see “Experimental Procedures”). The cultures (20 ml) were grown in the SD-ura at room temp to the early log phase (\( \times 10^7 \) cells/ml). Cells were washed with prewarmed LPM three times. They continued growing in the LPM for 3 h at the nonpermissive temperature (37 °C). The cultures were then synchronized by adding α-factor (10 units/ml) and incubating for 120 min (approximately one doubling time). The cultures were again washed three times with LPM to remove pheromone. The washed cells were resuspended into 20 ml of fresh LPM supplemented with 0.2 mCi of radioative \(^{32}\)P and incubating for 30 min. Labeling was quenched with 50 mM cold phosphate buffer before harvesting. Chromosomal DNA molecules were resolved by PFGE, and DNA was visualized by ethidium bromide staining (A). Incorporation of radiisotope was visualized by autoradiography (B). All samples are aligned. Lane 1, YEp352-CDC6; lane 2, YEp352-K114R; lane 3, YEp352-K114Q; lane 4, YEp352-K114P; lane 5, YEp352-K114L; lane 6, YEp352-K114E; lane 7, YEp352 only.
Fig. 6. Association of Cdc6 and Mcm5 proteins in the synchronized chromatin fractions. Yeast strain K4055 was transformed with either T7-tagged wild-type CDC6 gene (A) or mutated cdc6(K114E) gene (B), respectively. Both strains also contained the HA-tagged MCM5 gene. The cells were synchronized with α-factor arrest-and-release method. In the presence of methionine, the chromosomal CDC6 is shut off, and the plasmid carried T7-tagged Cdc6 was expressed. Aliquots of cultures were collected every 20 min, and subjected to FACS analysis, counting percentage of budded cell (upper panel), and protein blotting analyses (bottom panel). Based on the percentage of budded cells and FACS analysis, the first S phase is around 60 min in the wild-type Cdc6 cells. Panel A shows the expression of the wild-type T7-tagged Cdc6 and panel B shows the expression of the mutated cdc6(K114E) protein. The same blot was used to detect HA-tagged Mcm5 by the monoclonal antibody 12CA5.

We determined the association of the cdc6(K114E) mutant protein with chromatin and the loading of Mcm5 onto chromatin in chromatin-binding assay. Chromatin was prepared from the yeast cell released from G1-phase block at the indicated time points and probed either with anti-T7 tag (for detecting the association of Cdc6 with chromatin) or with monoclonal antibody 12CA5 (for detecting HA-tagged Mcm5 associated with chromatin). In the presence of methionine, the endogenous Cdc6 was shut off, so growth of the cell was totally dependent on Cdc6 produced from the plasmids harboring in the cell. The cultures were synchronized by the α-factor arrest-and-release method. Based on the percent of budded cells and FACS analysis, the peak of the S-phase is around ~60 min (Fig. 6A, upper panel) in the wild-type cells. Orc2, which was used as an internal control showed a constant level throughout the cell cycle (data not shown). The association of wild-type Cdc6 with chromatin was in a cell cycle-dependent manner, i.e. Cdc6 was detected when the cell was released from G1 phase block. It remained on chromatin during G1 phase, decreased at the start of S phase and disappeared from chromatin during G2 and M phases, and reappeared after M phase (Fig. 6A, bottom panel). The association of Mcm5 with chromatin showed a quite similar pattern to that of the Cdc6 protein, i.e. Mcm5 was loaded onto chromatin during G1 phase, replaced from chromatin during S phase and was not detected during G2 and M phases. The association of cdc6(K114E) mutant proteins with chromatin, however, was dramatically different from that of the wild-type Cdc6 (Fig. 6B). The cdc6(K114E) mutated proteins can associate with chromatin around 20 min, but then faded quickly from the chromatin. Under the same condition, the association of Mcm5 with chromatin was determined in the presence of the mutant cdc6(K114E) proteins. The loading of the Mcm5 protein onto chromatin was delayed for at least 60 min after the cell was released from the G1 phase block (Fig. 6B, lower panel). There was no second time associating or re-loading for Cdc6 and Mcm5, suggesting that the cell cycle was arrested and the cell ceased to grow. Consistent with these observations, atypical FACS pattern in the mutant transformants was observed. Presumably, this is due to defective DNA synthesis and/or abnormal morphology. Thus, the data suggested the improper chromatin assembly and the perturbation of the cell cycle progression in the cdc6(K114E) background.

Functional Orc1 and Cdc6 Interaction Depends on the Normal ATP-binding Site of Cdc6—During the process of Cdc6 purification, we consistently observed the co-fractionation of Orc1 with Cdc6. In the glycerol gradient centrifugation, both Orc1 and Cdc6 proteins co-sediment in a large 450–660-kDa protein complex. Due to their structural similarity, it has been proposed that the Cdc6 and Orc1 may be in a single gene family (44). This information led us to investigate the relationship between Cdc6 and Orc1 in vitro and in vivo.

In the in vitro interaction studies, bacterially expressed Orc1-His-tagged protein retained on the nickel-chelating matrix was used as a protein affinity column. The same vector without the Orc1 insert was used as negative control. Yeast extracts, as the Cdc6 protein source, were then passed through the Orc1-charged matrix. After extensive washing, the interacting components were analyzed by protein blots. The presence of Cdc6 protein can be detected by anti-Cdc6 polyclonal antibodies, indicating an interaction between Orc1 and Cdc6 proteins (Fig. 7A). To test whether this is a direct interaction between Cdc6 and Orc1, we used bacterially expressed wild-type GST-Cdc6 and mutant GST-cdc6 proteins for interaction studies. In the Escherichia coli background, one would assume that yeast adaptor protein(s), if any, can be avoided. In Fig. 7B (lane 1), the wild-type GST-Cdc6 was able to interact with Orc1 suggesting a direct interaction. Intriguingly, there was no detectable GST-cdc6(K114E) band (Fig. 7B, lane 2). This result suggests that Orc1 could not interact with mutated cdc6(K114E) protein. To avoid artifact, we used another mutant protein GST-cdc6–1 as control. The mutation point of the cdc6–1 protein has been determined as Gly260 to Glu260.

2 L. Feng and A. Y. Jong, unpublished data.
Fig. 7. Interaction between Orc1 and Cdc6. A, bacterially expressed 6xHis-T7-Orc1 tagged protein was retained on the nickel-chelating matrix used as a protein affinity column (lane 1). The same vector without the Orc1 insert was used as negative control (lane 2). Yeast extracts were prepared from strain BJ5459/ YGp123-CDC6 and used as the Cdc6 protein source. The extracts were then passed through the Orc1-charged matrix, washed extensively, and subjected to protein blotting experiments. The retention of Cdc6 protein, if any, was detected by anti-Cdc6 polyclonal antibodies. B, E. coli extracts containing GST-Cdc6 (lane 1), GST-Cdc6(K114E) (lane 2), and GST-cdc6–1 (lane 3), individually, were used for Orc1-matrix interaction as described under “Experimental Procedures.” As controls, aliquot of bacterial expressed GST-Cdc6 (lane 4), GST-cdc6(K114E) (lane 5), and GST-cdc6–1 (lane 6) was analyzed by anti-Cdc6 polyclonal antibodies, indicating that they were expressed about the same level. C, yeast strain BJ5459 was transformed with YGp181-His-T7-ORC1 (selection marker Leu2) first. The resulting strain was then transformed separately with either YGp123-Cdc6-T7 (lane 1), or YGp123-K114E-T7 (lane 2), or YGp123-cdc6–1-T7 (lane 3) with selection marker Trp1. 500 ml of culture was induced with 2% galactose for 4 h. The tagged Orc1 was purified by Ni-NTA matrix, concentrated 10-fold, and probed with anti-T7 antibody on the protein blots. The T7 monoclonal antibody can detect not only 6xHis-T7-Orc1 tag protein but also Cdc6-T7, if there is an interaction between Orc1 and Cdc6 proteins. The upper arrow indicates the predicted size of Orc1 and the lower arrow indicates the predicted size of Cdc6.

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1. **How do these observations reconcile with the in vivo function of initiation of DNA replication?** The Cdc6 protein is required for establishment and maintenance of pre-RC, and the Cdc6 protein is also required for the loading of MCM proteins. In addition, the Cdc6 protein interacts with Cdc28 protein complex after p40 kinase is degraded at late G1 phase. The Cdc28 protein kinase may then activate the initiation of DNA repli-
Lys114 in the nucleotide-binding site of in vivo clearly demonstrated that the studies (Fig. 6). This system is dynamic and useful for understanding the molecular events from late G1 phase (a stable protein-origin complex) into the S phase (a mobile DNA machinery). Indeed, we demonstrated that the impaired function of cdc6(K114E) leads to an abnormal assembly of the replicative complex. At this stage, the preparation of replicative complexes was still quite crude, preventing us from drawing a conclusive picture. Nevertheless, two major findings allowed us to unravel the possible molecular mechanism action of ATP binding. First, we have demonstrated that there is a direct interaction between yeast Orc1 and Cdc6 proteins. The information is consistent with observations from many other laboratories regarding the structure and functions of the ORC-origin complex, and the interaction between Cdc6 and Orc1 is conserved in eukaryotes (50, 51). Moreover, we demonstrated that the interaction between Orc1 and Cdc6 is defective in the cdc6(K114E) mutation. This observation is original. The simplest model is that the ATP-binding mutation fails to form Orc1/Cdc6 complex properly, which results in a deficiency in the formation of an operational pre-replicative complex. Thus, the requirement of ATP for Cdc6/ Orc1 interaction is involved in a quite early stage of DNA replication. This possibility is supported by our chromatin studies (Fig. 6).

In summary, with the availability of these mutants, we have clearly demonstrated that the in vivo function of the conserved Lys114 in the nucleotide-binding site of CDC6 is required for cell growth and DNA synthesis. We have also demonstrated that, at the molecular level, the mutant c6 at its nucleotide binding fails to interact with Orc1. The fact that the ATP-binding domain is highly conserved among Cdc6 and Orc1 protein families suggests their similar role in the initiation of DNA replication (18, 19, 47). Perhaps by sequentially contributing ATPase activity or ATP-governed conformational change to the ATP-dependent ORC/MCM functions result in the ordered events during initiation of replication (18, 30, 44). The highly conserved central domain of the MCM and RF-C family proteins also contains a predicted consensus motif for DNA-dependent ATPase (49). It is tempting to speculate that the MCM proteins of the pre-replicative complex, driven by the Cdc6 and Orc1 ATPase or ATP-governed conformational change, start to unwind the origin sequence during initiation of replication. ATP binding in the MCM and RF-C families may follow a similar mechanism. Further studies of the roles of ATP in Cdc6/Orc1, MCM, and RF-C families may reveal this complicated, yet extremely important regulation in DNA replication and cell cycle progression.

Acknowledgment—We thank Dr. Zhou Chen for help with the mutagenesis study.

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