Analysis on Origin Recognition Complex containing Orc5p with defective Walker A motif*

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Running title: ATP-binding to Orc5p
Orc5p is one of six proteins which make up the Origin Recognition Complex (ORC), a candidate initiator of chromosomal DNA replication in eukaryotes. To investigate the role of ATP-binding to Orc5p in cells, we constructed orc5-A, a strain of *Saccharomyces cerevisiae* having a mutation in the Walker A motif of Orc5p (K43E). The strain showed temperature-sensitive growth. Incubation at a non-permissive temperature (37°C) caused accumulation of cells with nearly 2C DNA content. Over-production of Orc4p, another subunit of ORC, suppresses this temperature-sensitivity, but over-production of other subunits did not. Over-production of Orc4p did not suppress the temperature-sensitivity of another orc5 mutant, orc5-1, whose mutation, L331P, is outside the ATP-binding motif. These results suggest that Orc4p is specifically involved in ATP-binding to Orc5p itself or its function in DNA replication. Immuno-blotting experiments revealed that in the orc5-A strain at a non-permissive temperature, all ORC subunits gradually disappeared, suggesting that ORC5-A becomes degraded at non-permissive temperatures. We therefore consider that ATP-binding to Orc5p is involved in efficient ORC formation, and that Orc4p is involved in this process.
The initiation of chromosomal DNA replication is tightly regulated to achieve genome replication just once per cell cycle. In chromosomal DNA replication, adenine nucleotides bound to initiator proteins are involved in this regulation both in prokaryotes and eukaryotes. In *E. coli*, the DnaA protein, the initiator of chromosomal DNA replication, has a high affinity for both ATP and ADP (1). The ATP-DnaA complex is active for DNA replication, but the ADP-DnaA complex and nucleotide-free DnaA, are inactive both *in vivo* and *in vitro* (1-3). DnaA has intrinsic ATPase activity, and ATP bound to DnaA can be hydrolyzed to ADP. This hydrolysis inactivates DnaA, suppressing re-replication, and thus over-initiation of DNA replication (4, 5). Acidic phospholipids, such as cardiolipin, interact with conserved basic amino acid residues of DnaA and stimulate the release of ADP from the ADP-DnaA complex, resulting in re-activation of the complex (6-9).

In eukaryotes, Origin Recognition Complex (ORC) is a possible initiator of chromosomal DNA replication (10-13). ORC was originally identified as a six-protein complex that specifically binds to *S. cerevisiae* origins of chromosomal DNA replication (14). ORC homologues have been found in various eukaryotic species, including humans (15). Following observations were reported for ORC in *S. cerevisiae*. ORC has at least two subunits (Orc1p and Orc5p) which bind to ATP. Orc1p, but not Orc5p has ATPase activity (16). ATP-binding to Orc1p is essential for specific ORC-binding to origin DNA (16). The ATPase activity of Orc1p may suppress re-replication, as is the case with DnaA (17, 18). On the other hand, plasmid shuffling experiments for a mutant ORC, ORC5-A (containing Orc5p with a defective Walker A motif), revealed that cells expressing ORC5-A showed temperature-sensitive growth (19). This suggests that ATP-binding to Orc5p is important for chromosomal DNA replication, but its precise role remains unknown. In this study, we examined the role of ATP-binding to Orc5p in chromosomal DNA replication in cells, by replacing the wild-type *ORC5* gene on *S. cerevisiae* chromosome with the *orc5-A* gene, to
construct the *orc5-A* strain. We confirmed that this strain shows temperature-sensitive growth, which is suppressed by over-production of the Orc4p subunit. Furthermore, we found that at high temperatures, the ORC5-A proteins become degraded. These results suggest that the binding of ATP to Orc5p is involved in the possibly stable association of Orc4p to the rest ORC subunits.
EXPERIMENTAL PROCEDURES

Strains, Plasmids and Medium—S. cerevisiae strains are listed in Table 1 (20, 21). Cells were cultured in synthetic complete (SC) medium or YPD medium (1% yeast extract, 2% Bacto peptone and 2% glucose).

To disrupt the chromosomal ORC5 gene, the LEU2 gene was inserted between flanking sequences (70 bp) of the ORC5 gene. This DNA fragment was introduced into the W303 diploid (resultant strain, YY101). We confirmed that all tetrads showed only two viable spores.

The ORC5 gene was amplified by PCR from chromosomal DNA of the W303-1A strain, and inserted into pRS416 (22), a low-copy number plasmid containing the URA3 gene, to create pRS416-ORC5, which was used for the plasmid shuffling experiments. Site-specific mutation was performed using a PCR-mediated method (Fig. 1).

The orc5-A gene (orc5K43E) was introduced into pRS406 (another plasmid containing the URA3 gene) (22) to create pRS406-orc5-A. This plasmid was transformed into W303-1A and transformed cells were selected on SC agar plates lacking uracil. The resultant strain was transferred to plates containing 5-fluoro orotic acid (5-FOA) (the two-step gene replacement method (23)). A colony whose growth was sensitive to high temperature was selected as the orc5-A strain (YY411). We confirmed that pRS416-ORC5 suppressed this temperature sensitivity.

To over-express each ORC subunit and Cdc6p in cells, the genes for these proteins were amplified by PCR from chromosomal DNA of the W303-1A strain and introduced to pRS426, a high copy-number plasmid containing URA3 (24), pRS416, a low copy-number plasmid containing URA3 (22), YEplac181, a high copy-number plasmid containing LEU2 (25) or pRS415, a low copy-number plasmid containing LEU2 (22).
**Plasmid Shuffling Analysis**—The YY101 strain was transformed with pRS416-ORC5, sporulated, and the spores were dissected. Ura+ Leu+ spores were selected (YY401) and used for plasmid shuffling experiments. Plasmid pRS414 (22), a low-copy number plasmid containing the TRP1 gene, and containing each mutant orc5 gene, was transformed into YY401 by the lithium-acetate method. The transformant was selected on SC agar plates, containing 5-FOA, without tryptophan.

**Fluorescence-activated Cell Sorter (FACS) Analysis**—Samples were prepared as previously described (26) with the following modifications. Cells were pelleted by centrifugation, washed with sterilized water, and fixed in 70% ethanol for 12 hr. Cells were again pelleted, re-suspended in 50mM sodium citrate, sonicated for 1 min, treated with 0.25 mg/ml RNase A for 1 hr at 50°C, and then with 1 mg/ml Proteinase K for 1 hr at 50°C. DNA was stained with 50 µg/ml of propidium iodide, and 20,000 cells from each sample were scanned with a FACSCalibur (Becton Dickinson).

**Pulse-Field Gel Electrophoresis**—Pulse-field gel electrophoresis experiments were as previously described (27, 28) with the following modifications. Cells were harvested by centrifugation and washed three times with solution I (50mM Tris-HCl, 1.2M sorbitol, 20mM EDTA), resuspended in solution II (50mM Tris-HCl, 1.2M sorbitol, 20mM EDTA, 5% β-mercaptoethanol) and incubated for 10 min at room temperature. Cells were then suspended in solution III (0.1M Na-citrate-citric acid, 1.2M sorbitol, 10mM EDTA) and mixed with 2.4% agarose (Bio-Rad, low melt preparative grade). The resultant solidified blocks of agarose were transferred to solution IV (0.1M Na-citrate/citric acid, 1.2M sorbitol,
10mM EDTA, 0.5% zymolyase) and incubated for 24 hr at 37°C. Blocks of agarose were washed with solution III and then with solution I once, and incubated with solution V (0.1M EDTA, 1% Na-lauroylsarcosine, 0.1% Proteinase K) for 24 hr at 50°C. After washing with 0.2M EDTA, the samples were applied to a 1.5% agarose slab and subjected to electrophoresis for 15.2 hr at 300V, 10°C with a 50-100 switch interval. Gels were stained with ethidium bromide and observed under a UV illuminator.

**Construction of a Yeast Genomic Library and Screening for a Multi-copy Suppressor Gene for the Temperature Sensitivity of the orc5-A Strain—**Total chromosomal DNA was extracted from W303-1A cells and partially digested by Sau3AI. DNA fragments of 4-10 kbp were purified by ultra-centrifugation in the presence of CsCl and ligated into the BamHI site of YEplac181 (a high-copy number plasmid containing the LEU2 gene). Resultant yeast genomic library was introduced into YY411 (orc5-A) cells by the lithium-acetate method, and temperature-resistant transformants were selected on SC agar plates at 37°C.

**Chromatin-binding Analysis—** Yeast spheroplasts were lysed with Triton X-100, and samples were processed into soluble (supernatant) and chromatin (insoluble precipitate) fractions by centrifugation, as previously described (26). Equivalent amounts (total protein) of chromatin fractions were subjected to electrophoresis on 7.5% or 10% polyacrylamide gels containing SDS, transferred to PVDF membrane, and probed with monoclonal antibodies against Orc3p (SB3) and Orc5p (SB5) (26, 29).
**ORC Purification**—Wild type ORC and ORC5-A were expressed in Sf9 cells infected with recombinant baculoviruses, and purified as described (16). A recombinant baculovirus for ORC5-A was donated by Dr. Stephan P. Bell (MIT).

**Filter-binding Assay for DNA-binding to ORC**—DNA fragments (290 bp) containing an origin of chromosome replication (ARS1) and its mutant (AB1') were prepared by PCR as described previously (29) and purified by polyacrylamide gel electrophoresis. DNA fragments were radio-labeled by T4 polynucleotide kinase and [γ-32P]ATP. The specific activity of each probe was about 4000 cpm/fmol DNA.

ORC (0.2 pmol) was incubated with ATP for 5 min at 37°C in buffer T (25 mM Tris-HCl pH7.6, 5 mM MgCl2, 70 mM KCl, 5 mM DTT and 5% glycerol) and further incubated with radio-labeled ARS1 DNA fragments (0.4 pmol) at 37°C for 15 min in the same buffer. Samples were passed through nitrocellulose membranes (Millipore HA, 0.45 µm) and washed with ice-cold buffer T. The radioactivity remaining on the filter was monitored with a liquid scintillation counter.
RESULTS

*Site-directed Mutational Analysis for ATP-binding to Orc5p—* *S. cerevisiae* Orc5p has a complete Walker A motif and an incomplete Walker B motif (Fig. 1). Based on sequence similarity to other ATP-binding proteins, in Orc5p, K43 in the Walker A motif seems to be important for ATP-binding (30) and this amino acid residue is conserved in Orc5p from various species (Fig. 1). To study ATP-binding to Orc5p, we constructed a mutant at the Walker A motif, the *orc5-A* gene (*orc5-K43E*; Fig. 1). *In vitro*, ORC containing this mutation (ORC5-A) showed no ATP-binding to Orc5p (16). The Walker B motif consensus sequence is DEXY (X, hydrophobic amino acid residue; Y, acidic amino acid residue) and the corresponding sequence of *S. cerevisiae* Orc5p is DGFD, which means that the motif is incomplete (Fig. 1). We also constructed two types of mutant *orc5* gene at this site: *orc5-B1* with AAFD and *orc5-B2* with DEFD (Fig. 1).

Each mutant *orc5* gene was inserted into pRS414 (22) and the resultant plasmid was transformed into yeast strain YY401, whose chromosomal *ORC5* gene had been deleted, but which had a wild-type *ORC5* gene on a plasmid with the *URA3* selectable marker. When the transformant was grown on agar plates containing 5-FOA, the *URA3* plasmid was selected against and lost, causing cells to rely solely on the mutant *orc5* gene (plasmid shuffling analysis). Cells that expressed the Orc5-Ap did not grow on agar plates at 37°C (Fig. 2A). At 24°C, they grew slowly, but could form colonies (Fig. 2A). Thus, the *orc5-A* mutation confers temperature-sensitive growth as reported previously (19). The sizes of colonies and doubling rates of cells that expressed Orc5-B1p or Orc5-B2p were indistinguishable from those of cells expressing wild-type Orc5p (Fig. 2A and data not shown). These results suggest that the incomplete Walker B motif of Orc5p is not important for the function of ORC in cells.
In order to examine the effect of ORC5-A on chromosomal DNA replication, we replaced the chromosomal wild-type ORC5 gene with the orc5-A gene (YY411 strain). As shown in Fig. 2B, this strain also showed temperature-sensitive growth on agar plates. After 8 hr incubation at 37°C, more than 80% of YY411 cells showed a large bud, suggesting that they were in late S phase or G2/M phase. We also determined the efficiency of colony formation in wild-type cells and in YY411 cells, both at 24°C and at 37°C. As shown in Table 2, in YY411 cells, the ratio of colony formation efficiency (37°C/24°C) was less than 1x10⁻⁵, confirming that this strain has temperature-sensitive growth.

**Cell Cycle Progression in orc5-A Cells**—To determine which phase of the cell cycle was blocked at high temperatures, YY411 cells were grown at 24°C, then shifted to 37°C, and their DNA content was determined by FACS analysis. Compared to wild-type, the proportion of cells with nearly 2C DNA content increased over time (Fig. 3), suggesting that most of cells were blocked in late S phase or G2/M phase.

To distinguish between these two possibilities, we performed pulse-field gel electrophoresis. Chromosomal DNA from cells in S phase does not enter the gel due to lack of condensation of chromatin and the presence of DNA replication intermediates, in contrast to DNA from cells in other phases of the cell cycle (31-33). We confirmed that chromosomal DNA from cells in S phase (hydroxyurea-treated cells) but not that from cells in G2/M phases (nocodazole-treated cells) remained in the well (Fig. 4, compare lanes 2 and 3).

In wild-type cells and YY411 cells cultured at 37°C, some chromosomal DNA entered the gel (Fig. 4), therefore, it is not the case that almost all of YY411 cells cultured at 37°C are in S phase. It is not clear that whether most of cells are in G2/M phase or they are mixture of cells in both S and G2/M phases (see Discussion).
Identification of a Multi-copy Suppressor Gene for orc5-A Temperature Sensitivity—To reveal the mechanism of temperature sensitivity (and hence the role of ATP-binding to Orc5p in chromosomal DNA replication), we constructed a yeast genomic library with a high copy-number plasmid (YEplac181) and searched for a gene which could suppress the temperature-sensitivity of YY411. We obtained 20 independent temperature-resistant colonies. Restriction enzyme mapping and direct DNA sequencing revealed that most had a plasmid containing the wild type ORC5 gene, and the rest had a plasmid containing full length ORC4 and TIF3 genes and a part of the MMS1 and SGV1 genes (pNT201; Fig. 5A). Of these, it is the ORC4 gene which suppressed temperature-sensitivity. A plasmid which contains only the ORC4 gene (pNT204) suppressed temperature-sensitivity, and deletion of the ORC4 gene from pNT201 (pNT205) diminished the activity of this plasmid for suppression (Fig. 5).

As shown in Table 2, in YY411, introduction of plasmid expressing the ORC4 gene restored the ratio of colony formation efficiency (37°C/24°C) to that of the wild-type strain. The ORC4 gene suppressed temperature-sensitivity when present in a high copy-number plasmid (YEplac181) and also to a lesser degree when present in a low copy-number plasmid (pRS415; Table 2). Furthermore, introduction of ORC4 restored cell cycle progression; FACS analysis revealed no accumulation of cells with nearly 2C DNA content after 37°C culture (Fig. 3). Although introduction of the vector (YEplac181) into YY411 caused a slight increase in cells with 1C DNA content after 10 hr incubation at 37°C, there is a significant difference between YY411 with the vector and YY411 with pNT203, in relation to the extent of accumulation of cells with nearly 2C DNA content (Fig. 3). We confirmed by immunoblotting analysis that introduction of pNT203 caused over-production of Orc4p (data not shown). Therefore, we concluded that over-production of Orc4p suppresses the temperature-sensitivity of YY411 strain.
To test whether the suppression is specific for the *ORC4* gene, we also examined the effect of over-production of each other ORC subunit. As predicted, the introduction of a plasmid containing *ORC5* also suppressed temperature-sensitivity, but introduction of plasmids expressing *ORC1*, *ORC2*, *ORC3* or *ORC6*, did not (Fig. 6). We confirmed by immuno-blotting analysis that each subunit was over-produced (data not shown). Therefore, suppression of temperature-sensitivity is specific for the *ORC4* gene, suggesting that Orc4p specifically affects the function of Orc5p.

*Comparison of the orc5-A Strain with another Temperature-sensitive orc5 Mutant, the orc5-1 strain*—The *orc5-1* strain is another well-known temperature-sensitive *orc5* mutant. As shown in Fig. 7A, introduction of a plasmid expressing the *ORC4* gene (pNT203) did not suppress the temperature-sensitivity of the *orc5-1* strain (JRY4249). We confirmed by immuno-blotting analysis that Orc4p was over-produced similarly both in YY411 and JRY4249 cells (data not shown).

Over-production of Cdc6p was reported to suppress the temperature-sensitivity of the *orc5-1* strain (21). Cdc6p directly binds to ORC in vitro (21, 29). We examined the effect of over-production of Cdc6p on the temperature-sensitivity of the *orc5-A* strain, using a high-copy number plasmid with *CDC6* gene (pRS426-*CDC6*). As shown in Fig. 7B, the introduction of pRS426-*CDC6* suppressed the temperature-sensitivity of the *orc5-1* strain (JRY4249), but not that of the YY411 strain. We confirmed by immuno-blotting analysis that Cdc6p was over-produced similarly in both strains (data not shown). In JRY4249, the extent of the suppression was much the same as reported previously (21). Thus the *orc5-1* mutation affects the function of Orc5p differently from the *orc5-A* mutation.

Although the *orc5-1* strain has been used in many previous studies, the position of its mutation was not known. We sequenced the *orc5-1* gene and found only one amino acid
substitution, L331P, which is located outside the ATP-binding region (Fig. 1). L331 is conserved among various species (34-36), suggesting that it is important for the function of ORC. Since the temperature-sensitivity of the orc5-1 strain was suppressed by over-production of Cdc6p, it seems that L331 is involved in ORC-binding to Cdc6p.

**Immuno-blotting Analysis of the Levels and Location of ORC in Cells**—The amount of chromatin-bound ORC5-A in YY411 cells at 37°C was examined by use of a chromatin-binding assay (26). As shown in Fig. 8A, the amount of Orc5p in chromatin decreased after the incubation temperature was shifted from 24°C to 37°C. The amount of another subunit of ORC, Orc3p, in chromatin also decreased after the temperature shift (Fig. 8A), suggesting that the entire ORC5-A was decreased. Introduction of the plasmid over-expressing ORC4 partially restored the levels of Orc5p and Orc3p in chromatin at 37°C (Fig. 8A).

To test whether the temperature shift translocates ORC5-A from chromatin to other locations, we measured the amounts of Orc5p and Orc3p in non-chromatin soluble fractions (the supernatants of centrifugation from chromatin precipitation). As shown in Fig. 8A, in YY411, these also decreased after the temperature shift, suggesting that the total amount of ORC protein decreased, possibly as it became sensitive to protein degradation. To test this possibility, protein synthesis in YY411 and the wild-type cells was blocked by cycloheximide before temperature shift, and then the levels of Orc5p and Orc3p in chromatin were monitored. As shown in Fig. 8B, the amounts of Orc3p and Orc5p in chromatin again decreased, suggesting that ORC5-A becomes degraded at non-permissive temperatures in cells. It was possible that instability of ORC-5A in YY411 at 37°C is due to that the cell cycle is arrested at late S or G2/M phase under the conditions (Fig. 3). For example, if ORC (not only ORC5-A but also wild-type ORC) becomes unstable at S or G2/M phase, results in Fig. 8A and B can be explained. In order to test this possibility, we examined the stability of wild-type ORC in...
W303-1A and ORC5-A in YY411 with pNT203 after blocking cell cycle by nocodazole (G2/M phase) or hydroxyurea (S phase). As shown in Fig. 8C, blocking of cell cycle by these chemicals did not affect the stability of wild-type ORC in W303-1A and ORC5-A in YY411 with pNT203 at 37°C. This result suggests that the instability of ORC5-A in YY411 at 37°C is not due to the cell cycle arrest under the conditions.

Biochemical Analysis on Stability of ORC5-A in vitro—Previous biochemical studies revealed that ORC5-A maintains its DNA-binding activity, and ATP-binding to Orc1p, but not ATP-binding to Orc5p (16), and we confirmed those findings here (data not shown). Here, we compare the stability (susceptibility to denaturation) in vitro of purified ORC5-A to that of purified wild-type ORC by a filter-binding assay. Based on results in Fig. 9A, we concluded that wild-type ORC and ORC5-A binds to wild-type ARS1 DNA fragments in a sequence-specific manner in the presence of 0.32 µg poly dI/dC (non-specific competitor DNA). We also confirmed that both wild-type ORC and ORC5-A did not bind to mutant ARS1 DNA fragments (A-B1-) even after 37°C incubation in the presence of 0.32 µg of poly dI/dC (data not shown). Both wild-type ORC and ORC5-A were incubated at 37°C and then remaining origin DNA-binding activity was measured by a filter-binding assay. As shown in Fig. 9B, both ORC and ORC-5A lost their DNA-binding activity at approximately same rate. We also compared the stability at 37°C of the complex of ORC5-A with DNA and the complex of wild-type ORC with DNA. As shown in Fig. 9C, both of complexes were very stable at 37°C.
DISCUSSION

In this study, we showed that YY411 strain expressing ORC5-A (ORC with Orc5pK43E, a mutation in the ATP binding domain) showed temperature-sensitive growth and we found that the amount of ORC is decreased at a non-permissive temperature (37°C). Experiments with cycloheximide suggested that at 37°C in cells ORC5-A is more sensitive to degradation than wild-type ORC. Based on these results we consider that ATP-binding to Orc5p is important to form the correct higher ordered structure of ORC.

Suppression of the temperature-sensitivity by over-production of Orc4p was specific for the *orc5-A* strain. Orc4p did not suppress the temperature-sensitivity of the *orc5-1* strain which has a mutation in L331, outside of the ATP-binding domain. Furthermore, over-production of other subunit of ORC (Orc1p, Orc2p, Orc3p or Orc6p) did not suppress the temperature-sensitivity of the *orc5-A* strain. Therefore, Orc4p seems to be specifically involved in ATP-binding to Orc5p itself or its function in DNA replication. There are two possible mechanisms. In ORC bound onto origin DNA, Orc4p and Orc5p either interact directly, or are closely located on origin DNA (37). One possibility is that Orc5p interacts with Orc4p depending on ATP-binding to Orc5p. Higher amounts of Orc4p (from a high copy number plasmid) may enable Orc4p to interact with Orc5p even in the absence of ATP-binding to Orc5p. However, in GST-pull down experiments for measuring the interaction of Orc5p with Orc4p, there was no difference between wild-type Orc5p and Orc5pK43E (Takahashi *et al.*, unpublished results). Therefore, some other factors seem to be involved in the Orc5p’s ATP-binding-dependent Orc5p-Orc4p interaction. In previous UV cross-linking experiments, Orc4p was cross-linked with radio-labeled ATP analogue depending on ATP-binding to Orc1p, suggesting that Orc4p is located close to the ATP-binding site of Orc1p and therefore that Orc4p interacts with Orc1p depending on ATP-binding to Orc1p (16).
Furthermore, we recently found that ATP-binding to Orc5p increases the affinity of ATP binding to Orc1p; the $K_d$ value of ORC5-A for ATP is much higher than that for wild-type ORC (Makise et al., submitted). Therefore, it is also possible that, in ORC5-A, the absence of ATP-binding to Orc5p may prevent ATP-binding to Orc1p, which in turn decreases the interaction of Orc1p with Orc4p, resulting in ORC degradation. Higher amounts of Orc4p (from a high copy number plasmid) may enable Orc1p to interact with Orc4p even in the absence of ATP-binding to Orc5p and Orc1p.

FACS analysis showed that incubation of the orc5-A strain at non-permissive temperatures caused accumulation of cells with nearly 2C DNA content. Pulse-field gel electrophoresis suggested either that most cells are in G2/M phase, or that there is a mixture of cells in S or G2/M. We think the latter more likely, for the following reasons. When YY411 cells were blocked at G2/M phase by nocodazole, incubated at 37°C for several hours, and then released to medium containing α-factor (G1 arrest), the loading of the six minichromosome maintenance proteins (MCM) onto chromatin was inhibited compared to the wild-type cells (data not shown). Mutants cells expressing Cdc6p with defective ATPase activity showed a defect in S phase progression, which was due to inefficient loading of MCM onto chromatin (32, 38). When MCM was loaded onto a smaller fraction of origins of DNA replication than normal, DNA duplication should be prolonged, resulting in the accumulation of cells in S phase. A similar mechanism may happen in YY411 cells at 37°C. It is also possible that S phase check point system is induced in YY411 cells at 37°C, resulting in cell cycle arrest at S phase. Therefore, we assume that some cells are in S phase under those conditions (YY411 cells at 37°C). On the other hand, most orc mutants show growth arrest at G2/M phase (31, 39). It has been suggested that in the orc2-1 mutant, growth arrest at G2/M phase is due to the DNA damage and spindle assembly checkpoint (40). Therefore, by analogy, it seems that the orc5-A strain may also have a defect in G2-M
progression by a similar mechanism and therefore, we assume that some cells are arrested in G2/M phase.
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REFERENCES


FIGURE and TABLE LEGENDS

Table 1. Yeast strains.

Table 2. Colony formation efficiency of YY411 strain.

Full growth suspensions of W303-1A (wild-type), YY411 (orc5-A), YY411 harboring pNT203 (YEplac181-ORC4), and YY411 harboring pNT213 (pRS415-ORC4) were diluted approximately, spread on SC agar plates and incubated at 24°C or 37°C for 2 days. The ratio of colony formation efficiency (37°C/24°C) was calculated.

Figure 1. Amino acid sequences of the Walker A and B motifs of Orc5p in various species, and the strategy for site-directed mutagenesis.

Sc, Saccharomyces cerevisiae (19); Sp, Schizosaccharomyces pombe (35); Dm, Drosophila melanogaster (41); Ms, mouse (36); Hs, human (34).

Figure 2. Site-directed mutational analyses on the Walker A and B motifs of Orc5p.

(A) Plasmid shuffling analysis. YY401 was transformed with pRS414 (a low copy-number plasmid with TRP1) containing each mutant orc5 gene or the wild-type ORC gene and spread on SC plates containing 5-FOA without tryptophan. The resultant strains were incubated on SC agar plates at 24°C or 37°C for 2 days.

(B) Growth phenotype. YY411 (orc5-A) and W303-1A (ORC5) cells were streaked on YPD plates and incubated at 24°C or 37°C for 2 days.
Figure 3. FACS analysis, showing the effect of the orc5-A mutation on cell cycle.

W303-1A (ORC5), YY411 (orc5-A), YY411 with YEplac181 (vector control) and YY411 harboring pNT203 (YEplac181-ORC4) were cultured in SC medium to logarithmic phase at 24°C, and then the temperature was shifted to 37°C. A small portion of culture was taken after the indicated periods and cellular DNA content was analyzed by FACS.

Figure 4. Pulse-field gel electrophoresis of chromosomal DNA, to determine chromatin state and phase of cell cycle.

W303-1A (ORC5) and YY411 (orc5-A) cells were grown to log phase at 24°C and then further incubated for 10 hr at 37°C. To obtain control DNA from cells in S or G2 phase, W303-1A cells were incubated with hydroxyurea (1 mM) or nocodazole (3 µg/ml), respectively. Chromosomal DNA was extracted from cells and analyzed by pulse-field gel electrophoresis.

Figure 5. Identification of the ORC4 gene as a multi-copy suppressor gene of the temperature sensitivity of the YY411 strain.

The lac promoter of YEplac181 is located on the right side of each insert. Activity of each plasmid for suppressing the temperature-sensitivity of the YY411 strain is shown on the right (+, positive; -, negative) (A). Each of these plasmids was introduced in YY411 and the resultant strains were incubated on SC agar plates at 24°C or 37°C for 2 days (B).

Figure 6. Effect of over-expression of each ORC gene on the temperature-sensitivity of YY411 strain.
YEplac181-ORC1, -ORC2, -ORC3, -ORC4, -ORC5, -ORC6 or vector only was introduced into YY411 cells and the resultant strains were incubated on SC agar plates at 24°C or 37°C for 2 days.

**Figure 7.** Comparison of the orc5-A strain with the orc5-I strain.

YY411 (orc5-A) and JRY4249 (orc5-I) cells were transformed with YEplac181-ORC4, YEplac181-ORC5 or YEplac181 (A). These cells were transformed with pRS426-CDC6, pRS416-ORC5 or pRS426 (B). The resultant strains were incubated on SC agar plates at 24°C or 37°C for 2 days.

**Figure 8.** Immuno-blotting analysis of ORC in cells.

W303-1A (ORC5), YY411 (orc5-A) and YY411 with pNT203 (YEplac181-ORC4) cells were cultured in SC medium to logarithmic phase at 24°C, and then the temperature was shifted to 37°C (A, B). Cells were treated with cycloheximide (200 µg/ml) for 30 min before the temperature shift (B, C). Cells were incubated with hydroxyurea (1 mM) or nocodazole (3 µg/ml) for 2 hr before the cycloheximide treatment (C). A small portion of culture was taken after the indicated periods. Chromatin fractions (precipitates) (A-C) and soluble fractions (supernatants) (A) were prepared and analyzed by immuno-blotting using monoclonal antibodies specific for Orc5p (SB5) and Orc3p (SB3). As loading controls, samples were stained with silver.

**Figure 9.** Stability of purified ORC5-A and the wild-type ORC at 37°C *in vitro.*
Purified ORC5-A and the wild-type ORC (0.2 pmol) were incubated with ATP (5 mM) in buffer T for 5 min at 37°C and further incubated in buffer T with 0.4 pmol of radio-labeled ARS1 DNA (origin DNA) (closed circle) or mutant ARS1 (A B1) DNA (open circle) fragments in the presence of indicated amounts of non-specific blocking DNA, poly dI/dC (A).

Purified ORC5-A (open circle) and the wild-type ORC (closed square) (0.2 pmol) were incubated at 37°C in buffer H (50 mM HEPES-KOH, pH 7.5, 0.2 M KCl, 1 mM EDTA, 1 mM EGTA, 5 mM MgOAc, 0.02% NP-40 and 10% glycerol) for the indicated periods. Samples were incubated with ATP (5 mM) in buffer T for 5 min at 37°C and further incubated in buffer T with 0.4 pmol of radio-labeled ARS1 DNA fragments (origin DNA) in the presence of 0.32 µg poly dI/dC (B).

Purified ORC5-A (open circle) and wild-type ORC (closed square) (0.2 pmol) were pre-incubated in buffer T at 37°C with 5 mM ATP for 5 min and then with radio-labeled 0.4 pmol ARS1 DNA fragments in buffer T at 37°C for 15 min in the presence of 0.32 µg poly dI/dC. Samples were further incubated at 37°C in buffer T for indicated periods (C).

The amounts of ARS1 DNA fragments bound to ORC (or ORC5-A) were determined by a filter-binding assay and shown relative to control (without dI/dC (A) or without 37°C incubation (B, C). For Fig 9A, these control amounts were 59.8 fmol (wild-type ARS1) or 45.5 fmol (mutant ARS1) for wild-type ORC and 81.1 fmol (wild-type ARS1) or 59.4 fmol (mutant ARS1) for ORC5-A; For Fig 9B, these control amounts were 58.5 fmol for wild-type ORC and 49.1 fmol for ORC5-A; and for Fig 9C, 50.9 fmol for wild-type ORC and 45.5 fmol for ORC5-A.
<table>
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<tr>
<th>Strain</th>
<th>Genotype</th>
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<td>W303-1A</td>
<td><em>Mata ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1</em></td>
<td>Thomas and Rothstein (1989)</td>
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<td>W303-1B</td>
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<td>Thomas and Rothstein (1989)</td>
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### Takahashi *et al.* Table 2

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<th>strain/plasmid</th>
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Takahashi et al. Figure 2A

\[ \text{24°C} \]

\[ \text{37°C} \]
Takahashi et al. Figure 2B

24°C

W303-1A

YY411

37°C

W303-1A

YY411
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<th>37°C incubation (hr)</th>
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Figure 4

Takahashi et al. Figure 4

lanes 1 2 3 4 5

asynchronous hydroxyurea (S) nocodazole (G2/M) W303-1A YY411
Takahashi et al. Figure 5A

suppressor activity

pNT201
pNT202
pNT203
pNT204
pNT205
pNT206
Takahashi et al. Figure 5B

24°C

37°C
Takahashi et al. Figure 6

24°C

37°C
Takahashi et al. Figure 7A

24°C

37°C
Takahashi et al. Figure 7B

24°C

37°C
Takahashi et al. Figure 8A
Takahashi et al. Figure 8B

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<th>( YY411 )</th>
<th>( YY411/pNT203 )</th>
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<td>chromatin fraction</td>
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<tr>
<td>loading control</td>
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Arrows indicate Orc5p and Orc3p.
Takahashi et al. Figure 8C

**W303-1A**

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**loading control**

**chromatin fraction**

- Orc5p
- Orc3p

**YY411/pNT203**

<table>
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<th>37°C incubation (hr)</th>
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<th>Hydroxyurea</th>
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<td>0 5 10</td>
<td>05 10</td>
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**loading control**

**chromatin fraction**

- Orc5p
- Orc3p
Takahashi et al. Figure 9A

wild-type ORC

ORC5-A
Takahashi *et al.* Figure 9B
Takahashi et al. Figure 9C
Analysis on origin recognition complex containing Orc5p with defective Walker A motif
Naoko Takahashi, Yoshihiro Yamaguchi, Fumiko Yamairi, Masaki Makise, Hitomi Takenaka, Tomofusa Tsuchiya and Tohru Mizushima

J. Biol. Chem. published online November 18, 2003

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