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The Origin Recognition Complex in Silencing, Cell Cycle Progression, and DNA Replication

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This report describes the isolation of *ORC5*, the gene encoding the fifth largest subunit of the origin recognition complex, and the properties of mutants with a defective allele of *ORC5*. The *orc5–1* mutation caused temperature-sensitive growth and, at the restrictive temperature, caused cell cycle arrest. At the permissive temperature, the *orc5–1* mutation caused an elevated plasmid loss rate that could be suppressed by additional tandem origins of DNA replication. The sequence of *ORC5* revealed a potential ATP binding site, making Orc5p a candidate for a subunit that mediates the ATP-dependent binding of ORC to origins. Genetic interactions among *orc2–1* and *orc5 -1* and other cell cycle genes provided further evidence for a role for the origin recognition complex (ORC) in DNA replication. The silencing defect caused by *orc5–1* strengthened previous connections between ORC and silencing, and combined with the phenotypes caused by *orc2* mutations, suggested that the complex itself functions in both processes.

INTRODUCTION

Studies of yeast mating type have yielded numerous insights into the genetics of cell cycle regulation, DNA replication, signal transduction, combinatorial control of gene expression, and position effects (reviewed in Herskowitz *et al.*, 1992). In this study, we describe a gene that provides links among three of these phenomena: the cell cycle, DNA replication, and position-dependent repression of the mating-type genes.

Haploid strains of *Saccharomyces cerevisiae* are one of two mating types, known as **a** and α. The mating-type locus (*MAT*) determines whether a cell is of the **a** mating-type, α mating-type, or non-mating. **a** strains have the *MAT***a** allele, α strains have the *MAT*α allele, and *MAT***a**/*MAT*α diploid strains are non-mating because of simultaneous expression of both **a** and α genes. In addition to the alleles at *MAT*, most strains have unexpressed copies of either the **a** or the α genes at two other loci, *HML* and *HMR*. These additional copies of the mating-type genes possess all the regulatory sequences necessary for their successful transcription but are repressed by a process called silencing. Silencing requires a combination of *trans*-acting proteins as well as regulatory sequences flanking *HML* and *HMR* called silencers (Brand *et al.*, 1985; reviewed in Laurenson and Rine, 1992). By definition, silencing is gene nonspecific and results in a general occlusion of silenced chromosomal segments from site-specific protein-DNA interactions (Nasmyth, 1982; Gottschling, 1992; Loo and Rine, 1994). Thus one goal of silencing studies is to understand how silencers, which are relatively small sequence elements, can lead to the assembly of large (several kilobasepair) chromosomal domains that are functionally repressed.

Genetic screens for mutations that allow the inappropriate expression of mating-type genes from *HML* and *HMR* have defined several genes important in silencing. The four *SIR* (silent information regulator) genes, *SIR1*, *SIR2*, *SIR3*, and *SIR4*, are required for silencing. No biochemical function has been demonstrated for any of these genes and their primary amino acid sequences have emphasized their novelty. Mutations in other genes that affect silencing have been isolated (reviewed in Laurenson and Rine, 1992). In particular, the silencing defects caused by histones H3

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and H4 mutations support the idea that the assembly of a specialized chromatin structure is involved (Kayne *et al.*, 1988; Johnson *et al.*, 1990; Megee *et al.*, 1990; Park and Szostak, 1990; Thompson *et al.*, 1994).

A number of observations suggest a role for DNA replication in the silencing process. The first of these observations was the discovery that all four silencers contain a match to the highly conserved ARS consensus sequence and are capable of acting as autonomous replication sequences (Abraham *et al.*, 1984). Subsequent analysis of a synthetic *HMR*-E silencer revealed a close correlation between its silencing ability and its ability to act as an ARS or as a chromosomal origin of replication (McNally and Rine, 1991; Rivier and Rine, 1992), supporting the idea that the initiation of DNA replication at the silencer origins is an important event in silencing. *CDC7*, which encodes a protein kinase required for DNA replication (Culotti and Hartwell, 1971; Hollingsworth and Sclafani, 1990), antagonizes silencing (Axelrod and Rine, 1991); studies using temperature-sensitive alleles of *SIR* genes show that passage through S phase is required for the establishment of silencing (Miller and Nasmyth, 1984). A prediction based upon these observations is that a protein required for DNA replication or the act of DNA replication itself is important for silencing.

Proteins implicated in yeast chromosomal DNA replication have been identified by several different methods. Genetic studies have identified genes encoding proteins that function at the replication fork, including DNA polymerase α (*CDC17*) and DNA polymerase δ (*CDC2*) catalytic subunits (reviewed in Campbell and Newlon, 1991). Other genes, including *CDC7*, *DBF4* *CDC45*, and the MCM related genes (*CDC46*, *CDC47*, *CDC54*, *MCM2*, and *MCM3*), have phenotypes that suggest function early in DNA replication (Dalton, 1995 and reviewed in Rowley *et al.*, 1994). In addition, many of these genes show genetic interactions with one another, suggesting that they function in the same or closely related steps in the replication process (Hennessy *et al.*, 1991). Biochemical studies using assays that recapitulate subsets of the initiation process have also identified proteins and their corresponding genes involved in template-primer recognition and single stranded DNA binding (Heyer *et al.*, 1990; Brill and Stillman, 1991; Fien and Stillman, 1992). Despite these efforts, the key events leading to origin recognition and the subsequent initiation of DNA synthesis remain poorly understood.

The identification of proteins that bind to yeast replicators, the genetic determinants of initiation of DNA replication, has also contributed to our understanding of the replicative process. Like their cousins the silencers, replicators each contain an essential match to the highly conserved ARS consensus sequence (ACS). A six protein complex referred to as the origin recognition complex (ORC) specifically recognizes the ACS (Bell and Stillman, 1992). The effect of point mutations in the ACS on ARS function is closely mirrored by their effect on ORC DNA binding. Further evidence for an in vivo replicative role for ORC is provided by the properties of temperature-sensitive mutations in the *ORC2* gene, encoding the second largest subunit of the complex (Bell *et al.*, 1993; Foss *et al.*, 1993; Micklem *et al.*, 1993). At the nonpermissive temperature, *orc2–1* cells initially arrest with unreplicated DNA and a single large bud. After more prolonged incubation, new DNA synthesis is observed that appears to be linked to cell death. Reciprocal-shift experiments indicate that the essential function of the *ORC2* gene is performed early in S phase as expected for a protein involved in the initiation of DNA replication (Bell *et al.*, 1993). Even at the permissive temperature, *orc2–1* cells show reduced plasmid stability suggesting a defect in the replication of these extrachromosomal elements as well. The *orc2–1* cells also exhibit defects in transcriptional silencing (Foss *et al.*, 1993). This finding, as well as the ability of ORC to bind specifically to the ARS consensus sequence at each of the four silencers (Bell *et al.*, 1993), suggests that ORC plays an important role in mediating silencer function.

We report here the genetic isolation and cloning of *ORC5*, the gene encoding the 53-kDa subunit of the origin recognition complex. Like recently described *ORC2* mutations, the *orc5–1* mutation results in silencing and cell cycle defects, thus supporting the view that a functioning ORC is required for silencing. Phenotypes conferred by the *orc2–1* and *orc5–1* mutations establish that ORC has some of the predicted genetic properties of an initiator of chromosomal replication.

MATERIALS AND METHODS

General

Genotypes of strains and plasmids used in this work are presented in Tables 1 and 2.

Standard yeast media, genetic, and recombinant DNA methods were as described (Rose *et al.*, 1989; Sambrook *et al.*, 1989). Yeast transformations used the lithium acetate procedure (Ito *et al.*, 1983). Some strain constructions in this work required crosses between *MAT*α strains and strains lacking functional *MAT***a** alleles, which form diploids that are incapable of sporulation. To promote sporulation in crosses of this type, *MAT***a** was introduced on plasmids that were subsequently lost during mitotic growth.

For quantitative mating assays, cells grown into log phase in liquid rich medium (YPD) supplemented with adenine were mixed with 1.2×10^7 cells of mating-type tester strains (JRY2726 or JRY2728) in 0.3 ml YPD. The cell suspensions were plated onto selective minimal (YM) plates and grown at 23°C. Dilutions of the tested strain were also plated onto fully supplemented YM plates to determine the number of viable cells, and mating efficiencies were calculated as the number of prototrophic diploid colonies formed per viable cell. All mating efficiencies were normalized to a wild-type strain JRY3009, which mated with an efficiency near 1.0.

Yeast genomic DNA was isolated using glass bead lysis of cells in detergent followed by organic extractions (Hoffman and Winston, 1987). Total RNA was isolated by hot phenol extractions. In this method, each sample of 2 to 4×10^8 cells was harvested from a

logarithmic phase culture grown in rich medium and frozen in a dry ice-ethanol bath. Cells were thawed by adding 0.7 ml of 0.3 M NaCl, 20 mM Tris-HCl, pH 8.0, 10 mM EDTA. Phenol (0.6 ml of 65°C) was then added and the suspension was vortexed for 30 s before incubating for 4 min at 65°C. The aqueous phase was recovered by centrifugation and the 65°C phenol extraction was repeated, followed by two phenol-chloroform extractions at 4°C. Total RNA was precipitated by the addition of 2.5 volumes of ethanol.

For RNA blot hybridizations, 40 μg of total RNA was separated by formaldehyde-agarose gel electrophoresis (Sambrook *et al.*, 1989). DNA and RNA blots onto Zeta-Probe membrane (Bio-Rad, Richmond, CA) and hybridizations were done according to the membrane manufacturer's instructions. Probe DNA was labeled using Amersham's Multiprime kit (Arlington Heights, IL). Blots were exposed to Kodak XAR (Rochester, NY) or DuPont (Wilmington, DE) film at −80°C in the presence of DuPont Cronex Lightning-Plus intensifying screens. RNA blots were quantified using a Molecular Dynamics PhosphoImager (Sunnyvale, CA) and ImageQuant software.

Identification of the orc5–1-complementing Clones

The wild-type *ORC5* gene was cloned from a genomic library provided by F. Spencer (Spencer *et al.*, 1990). Approximately 3000 Leu^+ transformants of JRY4039 were screened for their ability to grow at 37°C. Candidate plasmids recovered in *Escherichia coli* were retested and of these, two were capable of complementing the temperature sensitivity of JRY4039 upon retransformation. Restriction analysis of the two complementing plasmids (pJR1565 and pJR1566) showed that they contained overlapping inserts. Both of these plasmids also restored mating ability to JRY4039, which had the *hmr*-Δe331–324 allele and hence was derepressed by the *orc5–1* mutation.

Identification and Mapping of the ORC5 Gene

ORC was purified from yeast whole cell extracts as previously described (Bell and Stillman, 1992). Purified ORC (~75 μg) was separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and stained with Coomassie brilliant blue G (Sigma, St. Louis, MO; >90%). The stained bands were cut from the gel and eluted in 0.1 M Tris-HCl (pH 9.0), 0.1% SDS including 100 ng of lysyl endopeptidase (Wako, Richmond, VA). After incubating overnight at 30°C, the gel slice containing the 53-kDa subunit was removed and the peptides in the aqueous phase were separated by reverse phase chromatography and sequenced by automated Edman degradation (Applied Biosystems model 470, Foster City, CA). The longest of the four peptides sequenced was used to design two degenerate oligonucleotides to amplify the region of the *ORC5* gene that encoded that peptide by polymerase chain reaction (PCR). A product of the expected size was isolated, cloned, and found to encode the predicted amino acid sequence. This sequence was then used to probe a lambda library of yeast genomic DNA (a gift of R. Young, Massachusetts Institute of Technology, Cambridge, MA) and a single class of clones was identified. This clone was then ligated into pSK^+/Bluescript (p53.11) and sequenced completely on both strands.

Crosses were performed to determine whether a single mutation was causing both temperature-sensitive growth and silencing defects. In these crosses, the actual number of temperature-sensitive *MATα* segregants that were also poor maters was 25. We inferred that the second mutation required to make the *MATα orc5–1* strains non-maters was the sensitized *HMR***a** allele because the map distance between *MAT* and *HMR***a** predicted a total of 24 *MATα orc5–1* segregants containing compromised *HMR* silencers from the 40 complete tetrads that were analyzed. This calculation assumed 40% recombination between *MAT* and *HMR***a** and no linkage between either *MAT* or *HMR***a** with *orc5–1*. Further evidence that the mating and temperature-sensitive growth phenotypes were linked was obtained by examining the mating ability of an *orc5–1 MATα hmr*-Δe331–324 strain (JRY4039) after reverting its temperature sensitivity. Mating was restored in two of six independent temperature-resistant revertants. The co-reversion of the temperature-sensitive growth and mating defects was strong evidence that the two phenotypes were due to the same mutation.

Because the sequence next to *ORC5* matched the previously mapped *POL2* gene, we predicted that *ORC5* was on chromosome XIV. This prediction was tested by a cross in which a triply heterozygous *met2 ORC5::URA3 RAP1/MET2 ORC5 rap1–12::LEU2* diploid (JRY4507/YLS371) was sporulated and tetrads dissected. No tetrads of the nonparental ditype class were recovered between the *MET2* and *ORC5* loci (93 tetrads examined, 76PD:17T:0NPD) or between the *ORC5* and *RAP1* loci (99 tetrads examined, 63PD:36T:0NPD). This cross demonstrated that *ORC5* mapped between *MET2* and *RAP1*, 9 cM from *MET2* and 18 cM from *RAP1*. These distances were in good agreement with the published map position of *POL2* (Mortimer *et al.*, 1992).

Deletion of ORC5 and Recovery of the orc5–1 Allele

A deletion of the N-terminal three-quarters of the *ORC5* protein-coding sequence was constructed in pJR1568 and removed nucleotides between unique *Dra*I and *Hpa*I sites, nucleotides 463-1626 (Figure 1), resulting in pJR1569. After two-step gene replacement into a diploid strain (JRY4108), the structure of the resulting chromosomal deletion was confirmed by DNA blot hybridization.

The mutant *orc5–1* allele was recovered by homologous recombination and repair of a gapped plasmid bearing the wild-type *ORC5* gene. A 1-kbp *Sph*I-*Eco*NI deletion was made in pJR1567 and the vector-containing fragment introduced into an *orc5–1* strain by transformation. Ura^+ transformants were selected, and plasmids were recovered in *E. coli* from temperature-sensitive transformants. The insert containing the presumptive *orc5–1* lesion, a *Xho*I-*Sac*I fragment, was then moved to an integrating vector (pRS406), generating pJR1621. The integration of pJR1621 into JRY3935 was directed by digesting the plasmid with *Hpa*I, which cuts within *ORC5*. The chromosomal structure of the Ura^+ transformants that had integrated pJR1621 at the *ORC5* locus was confirmed by restriction analysis and DNA blot hybridization. These were then grown on YPD to allow homologous recombination between the tandem *ORC5* and *orc5–1* alleles before selecting and screening for 5-FOA-resistant, temperature-sensitive, nonmating derivatives. Allelism of the newly integrated *orc5–1* allele was confirmed by a cross to a known *orc5–1* strain.

Determination of Plasmid Loss Rates

Transformants were grown into early stationary phase in liquid YM lacking leucine before inoculating into liquid YPD that was further supplemented with adenine, histidine, leucine, lysine, tryptophan, and uracil at standard concentrations (Rose *et al.*, 1989) to ensure maximum number of doublings. The initial fraction of cells that contained the plasmid (F_i) was determined by plating dilutions of the new culture onto solid YM either containing or lacking leucine. After approximately 13 doublings at 23°C, the final fraction of cells that contained the plasmid (F_f) was determined in the same way. The loss rate (L) was calculated as $1–10^m$, where $m = [\log(F_f) - \log(F_i)]$/number of cell divisions.

Cell Cycle Analyses

Cells used to photograph the cell cycle phenotypes of *orc5–1* were grown to mid-log phase in liquid YPD at 23°C. The cultures were then divided and one-half placed at 37°C for 6 h. Cells were harvested by low speed centrifugation, washed once with water, and fixed with 70% ethanol in a dry ice bath for 10 min. After two more washes with water, the cell pellet was suspended in 100 μl of 1 μg/ml 4′6-diamidino-2-phenylindole (DAPI), immediately washed twice with water, and photographed.

Table 1. Strains used in this work

Strain	Genotype (source)
W303-1A	*MAT***a** *ade2-1 his3-11,15 leu2-3, 112 trp1-1 ura3-1 can1-100* (R. Rothstein)
W303-1B	*MATα ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100* (R. Rothstein)
H6c1a5	*MAT***a** *cdc-6-1 can1 his7 hom3 sap3* (D. Koshland)
H14c1a5	*MAT***a** *cdc14-1 can1 his7 hom3 sap3* (D. Koshland)
DBY1101	*MATα cdc45-1 his4* (D. Botstein)
DBY1103	*MATα cdc46-1 his4* (D. Botstein)
DBY2029	*MATα cdc47 ade2-1 leu2-3,112 lys2-801 ura3-52* (D. Botstein)
DBY2035	*MATα cdc54 his4-619 ura3-52* (D. Botstein)
2780-12b	*MATα cdc9-1 can1 his7 hom3 leu2-3,112 ura3-52* (L. Hartwell)
JT249	*MATα cdc28-4 met tyr ural* (J. Thorner)
YAB62	W303-1B; *hmr*-Δe358-303 (Brand *et al.*, 1987)
YAB102	W303-1B; *hmr*-Δe331-324 (Brand *et al.*, 1987)
YLS371	W303-1A; *hmr*-ΔA::*TRP1 rap1-12*::*LEU2* (L. Sussel and D. Shore)
JRY 3009	W303-1A; *MATα*
JRY3689	*MATα orc2-1 ade2 leu2 trp1 ura3*
JRY3934	JRY3009; *HMR*-E ΔI
JRY3935	JRY3009; *HMR*-SS ΔI
JRY3966	*MATα orc5-1 ade2 leu2-3,112 lys2-801 ura3*
JRY4039	*MATα hmr*-Δe331-324 *orc5-1 ade2 leu2-3,112 lys2-801 ura3*
JRY4108	W303-1A/JRY3935; *ORC5*/*orc5*Δ
JRY4125	W303-1B; *orc2-1*
JRY4135	*mat***a***1 ste14 ade2 leu2-3,112 lys2-801 ura3*
JRY4186	W303-1A; *mat***a**Δp *hmr*Δ::*URA3*
JRY4249	JRY3008; *orc5-1*
JRY4253	JRY3009; *HMR*-SS ΔI *orc5-1*
JRY4257	*MATα orc5-1*::*URA3*::*ORC5 ade2-1 his3-11,15 leu2-3,112 trp1 ura3*
JRY4315	W303-1A; *mat***a**Δp *hmr*Δ::*URA3 orc2-1*
JRY4319	JRY3009; *HMR*-E ΔI *orc5-1*
JRY4362	YAB102; *orc5-1*
JRY4378	W303-1A; *mat***a**Δp *hmr*Δ::*URA3 orc5-1 lys2*Δ
JRY4402	JRY4135; *orc5-1*
JRY4413	*MAT***a** *orc5-1 ade2 leu2-3,112 lys2-801 ura3*
JRY4456	JRY3009; *HMR*-SSacs$^-$ ΔI
JRY4507	*MATα his3-11,15 leu2-3,112 met1 trp1-1 ura3-1 orc5-1*::*URA3*::*ORC5*
JRY4553	W303-1A; *ADE2 lys2*Δ *cdc7-1*
JRY4884	JRY3009; *HMR*-SS ΔI ρ^0
JRY4885	JRY3009; *HMR*-SS ΔI *orc5-1* ρ^0
ySPB5.15	W303-1A; *orc5*Δ::*HIS3*, pSPB56
ySPB5.28	W303-1A; *orc5*Δ::*HIS3*, pSPB5.20

To avoid interference by mitochondrial DNA, the cells used for DNA content analysis were isogenic ρ^0 derivatives of wild-type and *orc5–1* strains (JRY4884 and JRY4885, respectively). These strains were made ρ^0 by growth in liquid YM containing 25 μg/ml ethidium bromide (Fox *et al.*, 1991). Each sample of approximately 7.5×10^7 cells was harvested from liquid YPD, washed once in water, and fixed in 70% ethanol overnight at 4°C. To maintain the cultures in log phase, fresh medium was added after cells were removed at every time point. The fixed cells were washed twice with 0.2 M Tris-HCl (pH 7.5), 20 mM EDTA (20× TE), then treated with 0.2 ml of 1 mg/ml RNAse A (Sigma) in 20× TE for 4 h at 37°C. After RNAse treatment, the cells were washed twice with phosphate-buffered saline (PBS) and stained overnight at 4°C with 100 μg/ml propidium iodide (Sigma) in PBS. Stained cells were sonicated, diluted 20-fold with PBS, and their DNA contents were determined with a Becton Dickinson FACScan flow cytometer (Mountain View, CA). At least 10,000 cells were counted per time point.

To determine the cell cycle phase at which *orc5–1* cells were temperature sensitive, wild-type (W303–1A) and *orc5–1* (JRY4245) cells in log phase growth were suspended in YPD alone or YPD containing 24 μM α-factor (Sigma), 0.16 M hydroxyurea (Sigma), or 40 μg/ml benomyl (DuPont). After 4 h of incubation at 23°C, one-half of each culture was placed at 37°C. At this time, >95% of the cells showed uniform cell cycle morphology upon microscopic examination. At 2-h intervals, a portion of each culture was diluted and spread on solid YPD medium and incubated at 23°C for 2–3 days. The fractions of viable cells (colony-forming units) reported here were normalized to the fraction of viable cells in cultures that were treated with cell cycle inhibitors but not placed at 37°C. In this regard, *orc5–1* cells were sensitive to hydroxyurea even when incubated at the permissive temperature (20% viability after a 12-h exposure to hydroxyurea).

ORC Purification

ORC was partially purified from *orc5–1* cells (JRY4249) or *ORC5* cells (W303–1B) as described previously (Bell *et al.*, 1993). Briefly, 4 liters of the appropriate strain were grown to a density of 10^7 cells per ml. Extracts were prepared by bead beating of the yeast cells and the crude extract was fractionated over S Sepharose. Peak fractions of anti-ORC cross-reacting material were detected by protein blots and assayed for DNA binding activity. No DNA binding activity was detected in any fraction containing ORC cross-reacting proteins derived from *orc5–1* cells. Proteins were transferred onto nitrocellulose by electroblotting and antigen-antibody complexes were detected with horseradish peroxidase-conjugated secondary antibodies and a chemiluminescent substrate.

RESULTS

Identification of the ORC5 Gene

To identify the genes encoding the different subunits of ORC, protein sequence was obtained by direct se-

Table 2. Plasmids used in this work

Plasmid	Markers (source)
pRS316	*CEN6*/*ARS H4*/*URA3* (R. Sikorski and P. Hieter)
pRS406	*ARS H4*/*URA3* (R. Sikorski and P. Hieter)
pRS415	*CEN6*/*ARS H4*/*LEU2* (R. Sikorski and P. Hieter)
pRS416	*CEN6*/*ARS H4*/*URA3* (R. Sikorski and P. Hieter)
YIp5	*URA3* (R. Davis and D. Botstein)
YCp50	*CEN4*/*ARS1*/*URA3* (M. Johnston and R. Davis)
pSB32	*CEN4*/*ARS1*/*LEU2* (Spencer *et al.*, 1990)
pDK273	*CEN*/*ARS1*/*ARS H4*/*LEU2*/*ADE3* (Hogan and Koshland, 1992)
pDK368-7	pDK273; 7 *ARS H4* (Hogan and Koshland, 1992)
pJR157	YCp50; *MATα*
pJR1263	pRS316; *ORC2*
pJR1425	pRS316; *HMRα*-E-RAP1-10
pJR1565	pSB32; *ORC5* (library clone)
pJR1566	pSB32; *ORC5* (library clone)
pJR1567	pRS316; *ORC5*
pJR1568	YIp5; *ORC5*
pJR1569	YIp5; *orc5*Δ
pJR1621	pRS406; *orc5-1*
p53.11	pSK$^+$; *ORC5*
pSPB56	pRS416; *ORC5*
pSPB5.20	pRS415; *orc5-K40E*

quencing of peptides derived from each subunit. The amino acid sequence of the longest peptide derived from the fifth largest ORC subunit was used to design the synthesis of degenerate oligonucleotides that served as PCR primers. A PCR product of the predicted size and sequence was obtained and used to probe a yeast genomic library. A single class of clones was identified that contained a single large open reading frame (Figure 1). The sequences upstream of the putative initiator ATG include stop codons in each reading frame, several strong matches to the TATA box promoter element, and two weak matches to the *Mlu*I cell-cycle box. This open reading frame contained all four peptides derived from the 53-kDa subunit of ORC, indicating that the identified gene encoded this subunit of ORC.

The predicted amino acid sequence of the *ORC5* gene encoded a protein of 479 amino acids and a molecular mass of 55.3 kDa, in approximate agreement with the relative molecular mass determined by SDS-polyacrylamide gel electrophoresis. Comparison of the *ORC5* coding sequence to the GenBank and

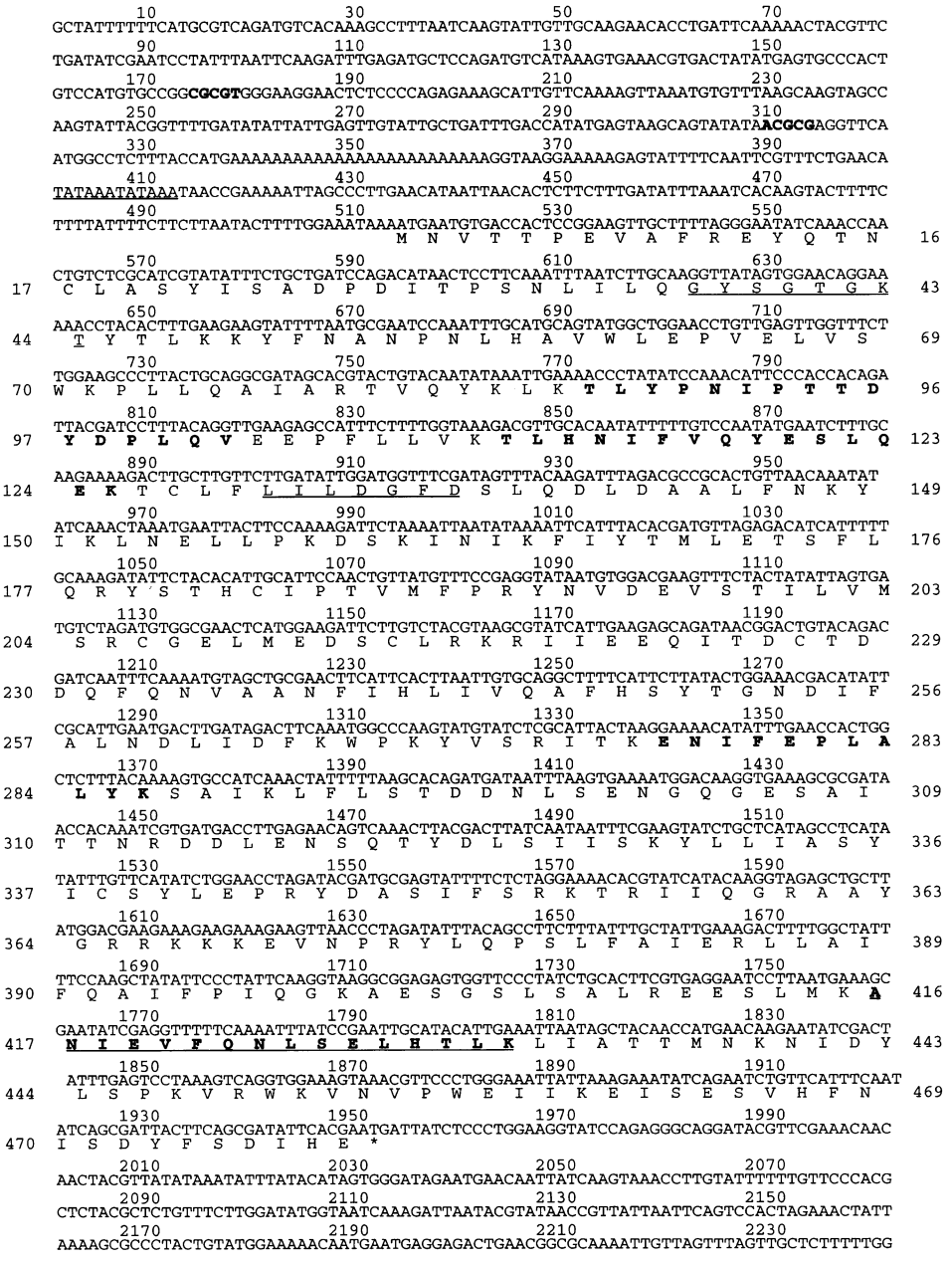

Figure 1. The nucleotide and peptide sequences of *ORC5* (GenBank accession number U24187). In the DNA sequence, the sites of two weak matches to the *Mlu*I cell-cycle box are shown in bold and two strong matches to the TATA consensus sequence are underlined. The bold regions of the protein sequence indicate the peptides sequenced (the underlined peptide in bold was used to design PCR primers for cloning), each of which was identical to the amino acid sequence obtained by translating the gene sequence. The underlined amino acid regions (not in bold) indicate the match to the consensus purine nucleotide binding site P-loop (amino acids 37–44) and A-loop (amino acids 130–136) in *ORC5* (Koonin, 1993).

EMBL databases revealed no close homologues of *ORC5*. Unlike the previously cloned *ORC2* and *ORC6* genes, the predicted amino acid sequence of *ORC5* contained no matches to the consensus phosphorylation site for the cell cycle regulated *CDC28* kinase ($^{S}/_{T}PX^{K}/_{R}$). Comparison of the *ORC5* coding region to libraries of known protein motifs identified a strong match to the P-loop (GYSGTGKT, amino acids 37–45) and the A-loop (LILDGFD, amino acids 130–136) of the consensus ATP/GTP binding site (Koonin, 1993). To address the importance of this motif for *ORC5* function, a site-directed mutant was constructed that changed lysine 43 to a glutamate. Mutations altering the equivalent highly conserved lysine in other ATP/GTP binding proteins alter ATP binding and/or hydrolysis (Haber and Walker, 1991; George *et al.*, 1994). Expression of the mutant gene in cells lacking the *ORC5* gene (ySPB5.28) resulted in cells that grew well at 24°C but much more slowly at 37°C. This finding suggested that the putative ATP binding motif plays an important but nonessential role in Orc5p function.

ORC5 was essential for cell viability. A deletion of the N-terminal two-thirds of the *ORC5* gene was made in one homologue of a diploid cell by the two-step gene replacement method (Scherer and Davis, 1979). Meiotic analysis of the haploid progeny from the diploid (JRY4108) revealed that none of the tetrads had more than two viable spores unless the diploid was first transformed with a plasmid containing the *ORC5* gene (pJR1567) before dissection. Segregants recovered from the cross that contained the deletion of *ORC5* and the complementing plasmid could not grow at temperatures ranging from 23–37°C on medium selecting for the loss of the plasmid, establishing that *ORC5* was essential for cell division.

Isolation of a Silencing-defective Allele of ORC5

In screens for essential genes involved in transcriptional silencing at *HMR*, four alleles of *ORC2* were identified (Foss *et al.*, 1993). One additional member in the collection of temperature-sensitive mutants from that screen exhibited phenotypes similar to *orc2–1* but did not contain a mutation in *ORC2*. Two phenotypes caused by this mutation, which was later identified as *orc5–1*, indicated that it deserved further study. First, these cells arrested at the nonpermissive temperature with large buds and undivided nuclei, a phenotype similar to that observed with *orc2–1* and suggestive of an S phase defect. Second, at the permissive temperature for growth, the temperature-sensitive mutation caused further derepression from *HMR* when combined with a partially compromised *HMR*-E silencer (see below).

To determine whether the phenotypes of the mutant strain (JRY4402) obtained from the genetic screen were due to a single mutation, this strain was crossed to a wild-type strain (W303–1A). The resulting *ORC5*/*orc5–1* heterozygous diploid grew well at the nonpermissive temperature (37°C), indicating that *orc5–1* was a recessive, loss-of-function mutation. Meiosis and sporulation require both **a** and α gene expression; therefore, to analyze the resulting *mat***a***1*/*MAT***a** diploid, it was transformed with a plasmid carrying *MAT*α (pJR157) and induced to sporulate. Tetrads from this cross were grown at the permissive temperature for *orc5–1* (23°C) and examined for temperature sensitivity by replica plating to 37°C. In each tetrad the temperature sensitivity segregated to two of the four haploid spores, indicating that *orc5–1* was a single, nuclear mutation.

The first evidence that *orc5–1* could derepress transcription at *HMR* came from the α-mating phenotype of the *mat***a***1 orc5–1* strain carrying the plasmid borne *HMR*α-E-RAP1–10 allele (Figure 2). This partially defective silencer contained the RAP1–10 mutation, a point mutation in the Rap1p binding site that blocks binding by Rap1p in vitro (Foss and Rine, 1993). By itself, the RAP1–10 mutation did not allow significant expression of the adjacent α genes. In addition, the parent strain (JRY4135) contained a *mat***a***1* mutation, which is completely recessive to expression of α genes (Kassir and Simchen, 1976). Therefore, any mutation affecting silencing would allow α genes to be expressed from the plasmid borne *HMR*α-E-RAP1–10 allele (pJR1425) and consequently cause the mutant to mate as an α strain.

To determine whether *orc5–1* was causing the α-mating phenotype by derepression of *HMR*α-E-RAP1–10 or in an unrelated way, two crosses were used to evaluate the effect of the *orc5–1* mutation on the chromosomal *HMR***a** locus, which encodes **a** genes rather than α genes. An *orc5–1* strain (JRY4413) was crossed to an *HMR***a**-containing strain with a mutation at the Rap1p binding site at

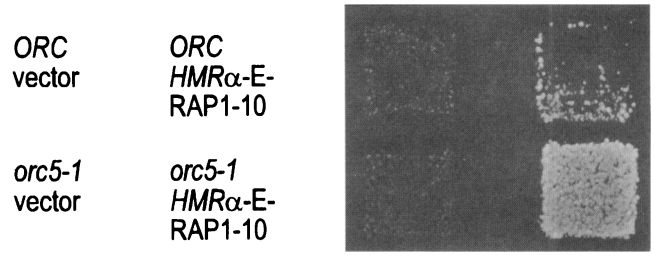

Figure 2. The *orc5–1* mutation completed the derepression of *HMR*α flanked by a crippled silencer. Isogenic *mat***a***1 ORC5* (JRY4135) and *orc5–1* (JRY4402) strains were transformed with either vector (pRS316) or a *HMR*α-E-RAP1–10–bearing plasmid (pJR1425) and replica plated onto a lawn of *MAT***a** cells at 23°C. Subsequent growth of prototrophic diploids formed by mating indicated the degree to which α genes were being expressed. Neither strain mated with the *MAT***a** lawn when transformed with vector, whereas the *orc5–1* mutation increased the mating efficiency of the strain carrying a plasmid with the *HMR*α-E-RAP1–10 allele.

HMR-E (*hmr*-Δe331–324, YAB102) and to an *HMR***a** strain with a synthetic *HMR*-E silencer and no *HMR*-I element (*HMR*-SS ΔI, JRY3935). The *hmr*-Δe331–324 mutation is an *HMR*-E allele functionally equivalent to the *HMR*-E-RAP1–10 mutation used in the genetic screen. Both of these *HMR***a** loci were silent but were sensitized to derepression by additional mutations that, on their own, would have little effect on repression. In a total of 40 tetrads (20 from each cross), temperature-sensitive growth segregated to two of four segregants in each tetrad. In addition, 25 of the *MATα* segregants were very poor maters or non-maters, and all of these segregants were also temperature sensitive, in good agreement with the predicted number of *MATα orc5–1* segregants with compromised *HMR* silencers (see MATERIALS AND METHODS). Thus the mutation causing the mating defect was linked to the temperature-sensitive *orc5–1* mutation.

To identify the gene responsible for the observed defects, the temperature-sensitive strain (JRY4039) was transformed with a centromere plasmid library. Two overlapping clones (pJR1565 and pJR1566) complemented the temperature-sensitive and mating-defective phenotypes. The similar phenotypes of this mutant strain to strains mutant in the *ORC2* gene suggested that the complementing clones might encode one of the other five ORC subunits. To test this possibility, the ability of the genes encoding the remaining five ORC genes to hybridize to these clones was determined. Both clones hybridized well to a DNA probe derived from the coding region of the *ORC5* gene, strongly suggesting that the temperature-sensitive mutation was within the *ORC5* gene. This hypothesis was confirmed by showing that the *ORC5* allele recovered from the temperature-sensitive strain could be used to replace the wild-type gene in a haploid yeast strain, and that the resulting strain (JRY4253) was temperature sensitive for growth and defective in silencing.

To determine more directly whether the nonmating phenotype of *MATα orc5–1* strains was due to derepression of *HMR***a**, the levels of **a***1* messenger RNA were measured in isogenic *MATα ORC5* and *orc5–1* strains that bore various alleles of the *HMR*-E silencer (Figure 3). Total RNA from wild-type and *orc5–1* strains grown at 23°C was analyzed by RNA-blot hybridization. The *orc5–1* mutation caused no detectable **a***1* transcript from *HMR* linked to the natural *HMR*-E silencer (Figure 3, lanes 2 and 7). However, in combination with either weakened *HMR*-E silencer, the *orc5–1* mutation caused an approximately twofold increase in transcript (Figure 3; compare lanes 3 and 4 with lanes 8 and 9). This increase was quantitatively similar to that caused by mutations in the ACS elements of *hmr*-Δe331–324 or of *HMR*-SS ΔI (Figure 3, lanes 5 and 10). Although the *orc5–1* mutation appeared to cause a silencing defect equivalent to that of an ACS mutation, a more sensitive measure of **a***1* gene expression, quantitative mating efficiency, indicated that the *orc5–1* mutation was not a null for its silencing function because the *orc5–1* mutation did not cause as severe a mating defect as mutations in the ACS (2.7×10^{-3} versus 2.3×10^{-4} [Figure 3, lanes 4 and 5] and 1.3×10^{-3} versus $<10^{-5}$ [Figure 3, lanes 9 and 10]).

The genetic screens that identified *orc2–1* and *orc5–1* used a sensitized *HMR*-E silencer and hence were biased for defects that affected silencing at *HMR* over *HML*. Such a screen could potentially uncover mutations whose effects were limited to *HMR*. Indeed, there was no obvious mating defect in any *MAT***a** *orc5–1* segregants from several crosses, indicating that the *orc5–1* mutation did not substantially derepress *HMLα*. However, the sensitivity of the mating assay can be increased by monitoring expression of *HMLα* in the absence of functional *MAT***a** genes, in a manner analogous to that used in the genetic screen. To determine whether *orc2–1* or *orc5–1* affected silencing of *HMLα*, strains that were *HMLα mat***a**Δp *hmr*Δ*::URA3* and either *orc2–1* or *orc5–1* were constructed. The *mat***a**Δp allele is an allele of *MAT***a** from which the promoter sequences have been removed and hence was transcriptionally inactive (Loo and Rine, 1994), and *HMR***a** was deleted in this strain to prevent interference with the mating assay by another potential

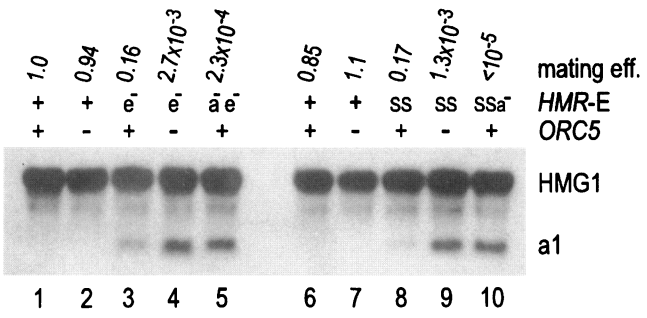

Figure 3. The *orc5–1* mutation caused a silencing defect. RNA was isolated from isogenic strains grown at 23°C and the amounts of **a***1* messenger RNA transcribed from *HMR***a** were quantified by RNA blot hybridization. The *HMR*-I silencer was present for lanes 1–5 but deleted for lanes 6–10. Although the *orc5–1* mutation alone (lane 2) or in combination with a deletion of the *HMR*-I silencer (lane 7) caused no detectable increase in **a***1* messenger RNA, the same mutation in combination with a deletion of the Rap1p binding site at *HMR*-E (lane 4, e^-) or in combination with a synthetic *HMR*-E silencer and a deletion of *HMR*-I (lane 9, SS) resulted in substantial derepression. The derepression caused by *orc5–1* was quantitatively similar to that caused by mutating the ACS of the RAP^- *HMR*-E silencer (lane 5, a^-e^-) or of the *HMR*-E synthetic silencer (lane 10, SSa^-). *HMG1* messenger RNA was used to control for the amount of RNA loaded. The mating efficiencies of these strains are presented above the lanes (W303–1B, JRY4249, YAB102, JRY4362, YAB62, JRY3934, JRY4319, JRY3935, JRY4253, and JRY4456, respectively).

source of **a** gene expression. Both the *orc2–1* (JRY4315) and *orc5–1* (JRY4378) segregants mated weakly as α cells at 23°C, whereas an isogenic wild-type strain (JRY4186) did not (Figure 4). Thus the function(s) of Orc2p and Orc5p in silencing was not limited to *HMR* and suitably weakened *HML* silencers may exacerbate the silencing defect to the level seen with, for example, *HMR*-SS ΔI or *hmr*-Δe331–324.

Plasmid Maintenance Defects in orc Mutants

In the course of this study we noticed that *orc5–1* strains exhibited an enhanced plasmid loss rate over similar wild-type strains. A cross was performed in which the *ORC5/orc5–1* heterozygous diploid carried a centromere plasmid (pJR157). Upon dissection of the diploid, no *orc5–1* segregant retained the plasmid. In contrast, 25 of 60 *ORC5* segregants retained the plasmid. This difference suggested that *orc5–1* cells had a plasmid maintenance defect. The elevated plasmid loss rate of *orc5–1* mutant cells was confirmed and quantified by measuring plasmid loss rates in isogenic wild-type, *orc2–1*, and *orc5–1* strains. Wild-type cells exhibited the low plasmid loss rate of centromere-containing plasmids, as reported by others (Clarke and Carbon, 1980). In contrast, the *orc2–1* and *orc5–1* mutations caused at least a 20-fold increase in plasmid loss rate (Table 3).

The increased plasmid loss rate suggested that both genes played a role in DNA replication; however, plasmids can be lost from a population of cells by several possible mechanisms, including a failure to initiate DNA replication efficiently, a failure in the elongation phase of DNA synthesis, or a failure of successfully replicated plasmids to segregate properly. It has been proposed that plasmid loss caused by a failure to initiate replication can be suppressed by increasing, in tandem, the number of potential origins on a plasmid, whereas plasmid loss caused by other defects cannot (Hogan and Koshland, 1992). Therefore, the loss rates of a plasmid containing seven tandem copies of *ARS H4* were determined in the same strains. The loss rates of the plasmid with multiple tandem origins in isogenic wild-type, *orc2–1*, and *orc5–1* strains were indistinguishable and similar to the loss rate of the plasmid with just a pair of origins in the wild-type strain (Table 3). The ability of multiple tandem origins to suppress the plasmid maintenance defect in these mutants strongly suggested that *ORC2* and *ORC5* both have a role in the initiation of DNA replication.

Cell Cycle Defects in orc5–1 Mutants

The terminal arrest phenotype of cells containing the *orc5–1* mutation suggested that the wild-type product was required for normal cell cycle progress through S phase. Approximately 6 h after an exponentially growing culture of *orc5–1* cells was shifted to 37°C, most had arrested growth with large buds (Figure 5). The continued growth in size of the arrested cells indicated that *ORC5* was not needed for cell growth but was instead required for cell cycle progression. Furthermore, staining with DAPI showed that the large-budded cells had failed to divide their nuclei. These phenotypes are consistent with a block in or near S phase of the cell cycle.

DNA content analysis of *orc5–1* mutants indicated that the mutant cells had completed most or all of DNA replication before arresting growth at the non-

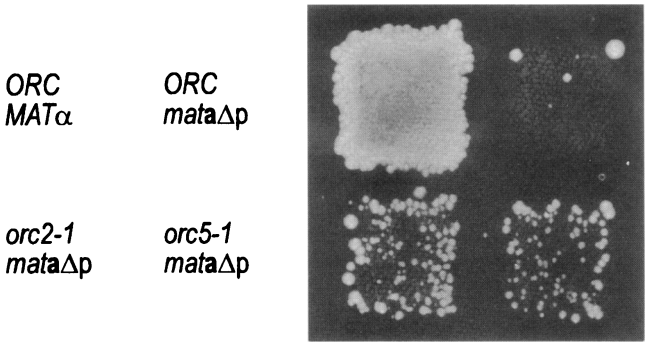

Figure 4. The *orc2–1* and *orc5–1* mutations reduced silencing of *HMLα*. Expression of *HMLα* was monitored by replica plating isogenic *HMLα mat***a**Δp *hmr*Δ::*URA3* strains that were either *ORC*, *orc2–1*, or *orc5–1* (JRY4186, JRY4315, and JRY4178, respectively) onto a lawn of *MAT***a** cells at 23°C followed by selection of the prototrophic diploids formed by mating. The ability to mate as an α cell indicated the degree to which *HMLα* was derepressed.

Table 3. Suppression of plasmid loss rates by tandem ARS elements

	Loss rate ± S.D.	
	1 ARS H4	*7 ARS H4*
ORC	0.005 ± 0.008	0.005 ± 0.006
orc2-1	0.100 ± 0.050	0.001 ± 0.001
orc5-1	0.211 ± 0.062	0.003 ± 0.004

orc2-1 and *orc5-1* mutants had a plasmid maintenance defect that was suppressed by increasing the number of ARS elements on a plasmid. Isogenic *ORC*, *orc2-1*, and *orc5-1* strains (W303-B, JRY4125, and JRY4249) were each transformed with two plasmids: pDK273, which bears one copy of *ARS1* and one copy of *ARS H4*, and pDK368-7, which bears one copy of *ARS1* and seven tandem copies of *ARS H4*. Four transformants of each strain were grown selectively at 23°C in liquid minimal medium lacking leucine before dilution into liquid rich medium. The loss rates, which represent one-half the fraction of cell divisions in which only one cell received plasmid(s), after 13–15 doublings were determined and the averages with S.D. were presented.

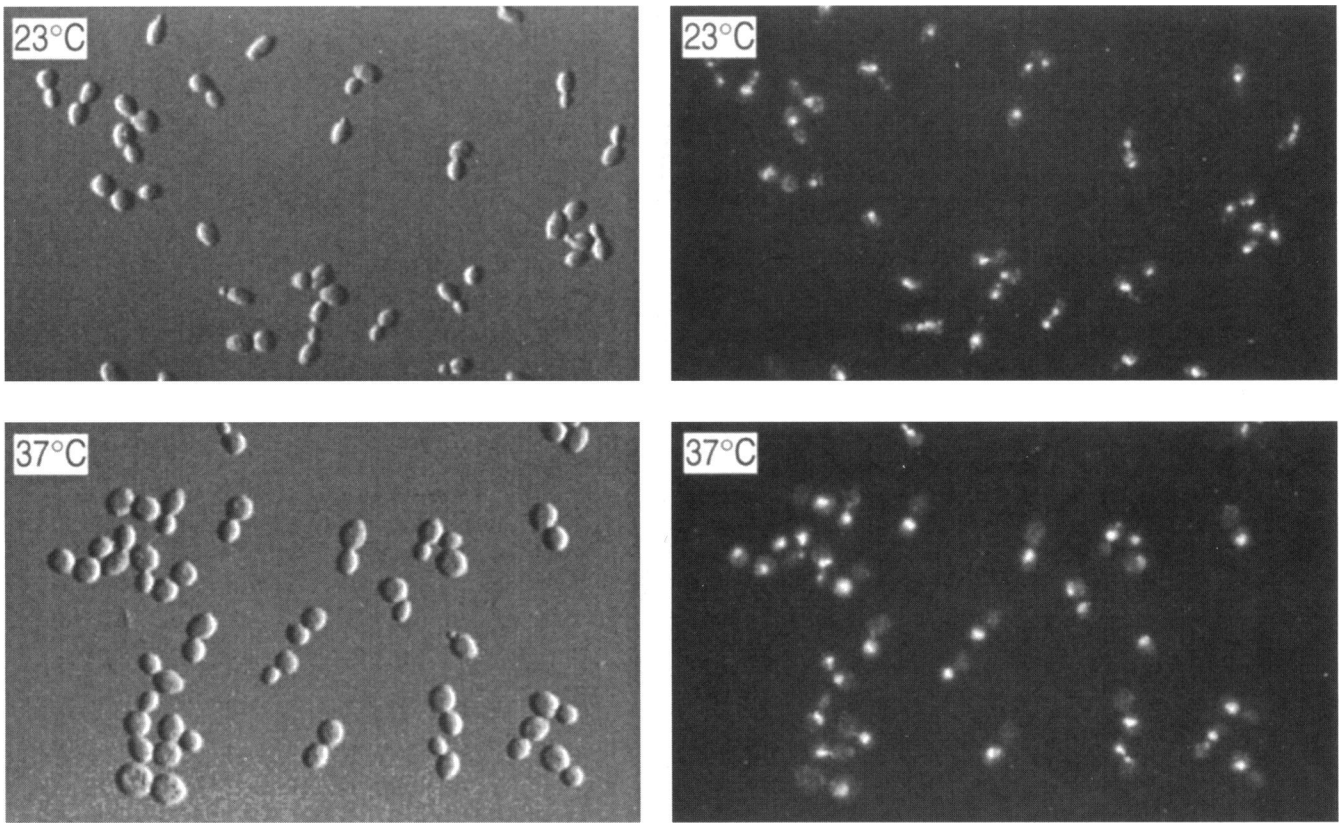

Figure 5. The *orc5–1* mutation caused cell cycle arrest. Isogenic strains that were *ORC* (top panels, W303–1B) and *orc5–1* (bottom panels, JRY4249) were grown to mid-log phase in rich medium at 23°C, after which the cultures were shifted to 37°C for 6 h. Cells were stained with DAPI and photographed using Nomarski optics (left) or under fluorescent illumination (right) to detect DAPI staining of nuclei.

permissive temperature. Cultures of an *orc5–1* strain (JRY4885) and a wild-type strain (JRY4884) were shifted from the permissive to the nonpermissive temperature for growth. At 2-h intervals, a sample from each culture was removed and the DNA content of cells was determined. Cell division in *orc5–1* cells shifted to the nonpermissive temperature stopped 6–8 h after the temperature shift, at which time the cells had divided approximately three times. During this interval, *orc5–1* cells with 1C DNA content were depleted in the culture, and there was a concomitant accumulation of cells with an approximately 2C DNA content (Figure 6).

Studies of cells arrested at different positions in the cell cycle indicated that the lethality caused by the *orc5–1* mutation was cell cycle dependent. In untreated cells, or in α-factor–treated cells (blocked in G_1), or in hydroxyurea-treated cells (blocked in replication elongation), incubation at the restrictive temperature resulted in a substantial loss of viability upon subsequent plating at the permissive temperature. In contrast, in benomyl-treated cells (blocked in mitosis), incubation at the restrictive temperature did not result in a significant loss of viability (Figure 7). Thus the effect of increased temperature on the *orc5–1* mutation could be overcome in cells arrested in M phase but not at other positions in the cell cycle.

The Subunit Composition of ORC in orc5–1 Mutants

To determine the effect of the *orc5–1* mutation on the biochemical properties of the ORC complex, ORC was purified from *orc5–1* cells. Mutant cells were first grown at the permissive temperature to log phase, then either shifted to the nonpermissive temperature or left at the permissive temperature for 4 h. Extracts were prepared from *orc5–1* cells grown under both conditions as well as an isogenic *ORC5* strain. Because the antibodies for ORC were unable to cleanly detect ORC protein in the crude extract, these samples were first partially purified using a single chromatographic step. To assess the polypeptide composition of ORC derived from *orc5–1* cells, these fractions were tested for the presence of each of the six ORC subunits using anti-ORC antibodies. The resulting immunoblots (Figure 8) showed that ORC derived from *orc5–1* cells was missing several subunits when compared with ORC purified from wild-type cells. Although the Orc1p and

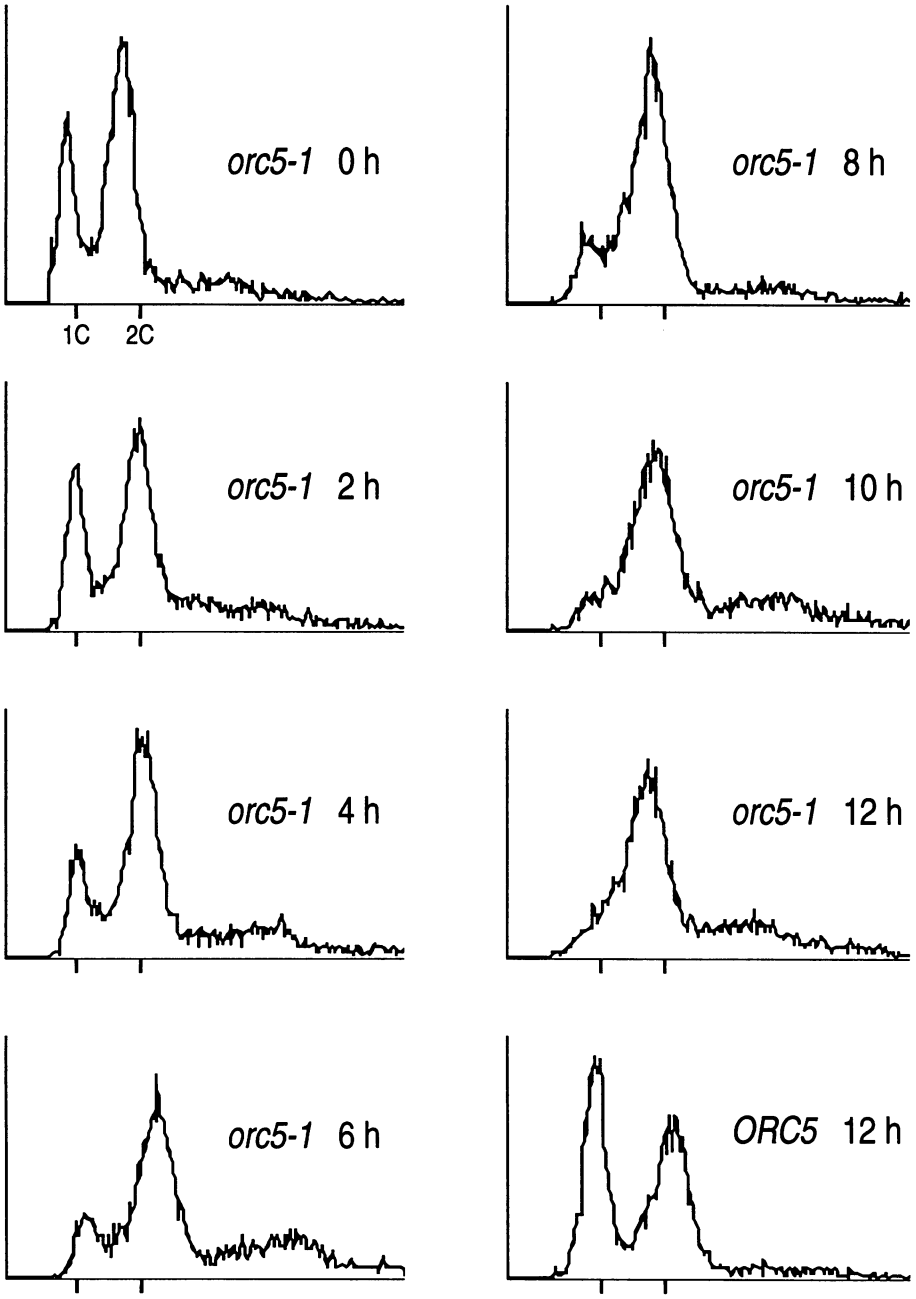

Figure 6. DNA content of isogenic wild-type (JRY4884) and *orc5–1* (JRY4885) strains after progressively longer incubation at the restrictive temperature (37°C). In each panel, the x-axis represents DNA content and the y-axis represents the number of cells. The length of time the cells have spent at the nonpermissive temperature is indicated as an inset in each panel.

Orc3p subunits of the complex were present in relatively high levels, the Orc2p, Orc4p, Orc5p, and Orc6p subunits were substantially reduced in abundance. Efforts to identify these subunits in other column fractions were unsuccessful. Consistent with the temperature-sensitive nature of the *orc5–1* mutation, ORC derived from mutant cells grown at the nonpermissive temperature showed a more significant reduction in each of the subunits, including Orc1p and Orc3p, and ORC DNA binding activity was not detectable in any fraction derived from the *orc5–1* cells. These findings clearly demonstrate that the *orc5–1* mutation altered the stability and DNA binding activity of ORC in vitro.

Interactions among ORC and other Cell Cycle Genes

The similar phenotypes caused by the *orc2–1* and *orc5–1* mutations and the presence of both proteins in the ORC suggested that they perform a related func-

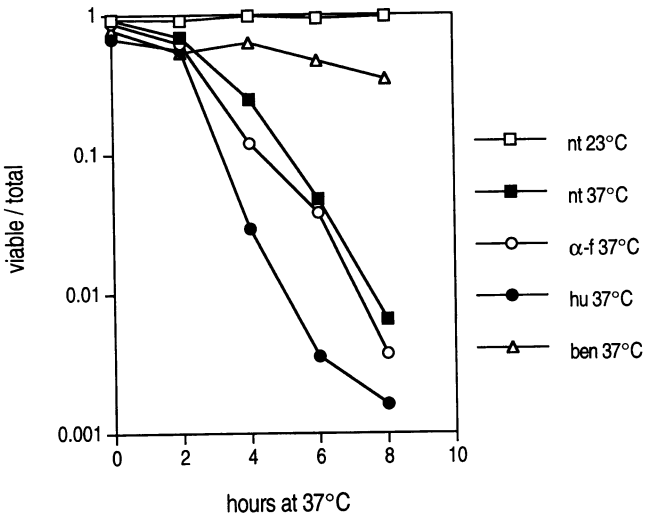

Figure 7. Viability of *orc5–1* cells grown at the restrictive temperature. Cultures of wild-type and *orc5–1* cells were treated with α-factor (α-f), hydroxyurea (hu), benomyl (ben), or not treated (nt) before shifting to 37°C. After various times at 37°C, cells were plated and incubated at 23°C to determine the number of viable cells in each culture. The viability at each time point was normalized to the viability of cultures that were treated with α-factor, hydroxyurea, or benomyl but were not shifted to 37°C to account for any toxicity of the treatment per se.

tion. Consistent with this prediction, *orc2–1 orc5–1* double mutants were inviable. Tetrads from an *ORC2 orc5–1/orc2–1 ORC5* double heterozygote (JRY3689/JRY4413) were dissected and the segregants were grown at 23°C. No double mutants were recovered from this cross (56 tetrads examined) unless the diploid was first transformed with a plasmid bearing the *ORC2* gene (pJR1263). The inferred *orc2–1 orc5–1* double mutants obtained from this second cross were unable to grow at 23°C on medium that selected against the plasmid, indicating that the inability to recover *orc2–1 orc5–1* double mutants was not due to a defect in germination.

To determine whether other cell cycle genes interact with the *orc2–1* and *orc5–1* mutations, a number of other double mutants were constructed. Mutations in *CDC6, CDC14, CDC7, CDC45, CDC46, CDC47,* and *CDC54* were chosen for this analysis because of their proposed roles in DNA replication (Hennessy *et al.*, 1991; Hogan and Koshland, 1992). At least 36 tetrads were analyzed from each cross between *orc2–1* or *orc5–1* mutant strains and *cdc6–1, cdc14–1, cdc7–1, cdc45–1, cdc46–1, cdc47,* and *cdc54* strains. In each case, tetrads were scored for both viability at various temperatures and for mating, to determine if synthetic silencing phenotypes existed (Table 4). Viable double mutants could not be recovered with *cdc7–1* and *orc2–1*, with *cdc54* and *orc2–1*, or with *cdc54* and *orc5–1*. Double mutants with *cdc6–1, cdc14–1, cdc46–1,* and *cdc47* with either *orc2–1* or *orc5–1* were rarely recovered and grew more slowly at 23°C than either of the single mutants. All other double mutants involving *orc2–1* and *orc5–1* grew no worse than the slower growing single mutant. In addition, all of the viable

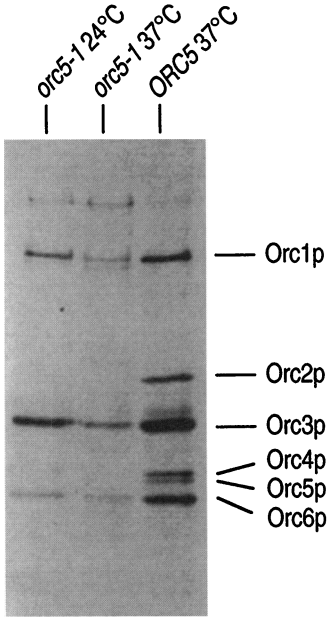

Figure 8. Polypeptide composition of ORC derived from *orc5–1* cells. Thirty micrograms of partially purified ORC derived from either JRY4249 (*orc5–1*) or W303–1B (*ORC5*) cells was separated on a 10% SDS-polyacrylamide gel and transferred onto nitrocellulose. The resulting protein blot was incubated with a polyclonal mouse sera raised against the entire ORC complex and a single monoclonal antibody specific to the Orc6p subunit (SB-49). ORC purified from the *orc5–1* cells was first grown at 24°C to log phase, then either left at 24°C or transferred to 37°C for 4 h.

Table 4. Genetic interactions among *ORC* and other cell cycle genes

	orc2-1	*orc5-1*
orc2-1	NA	Inviable
cdc6-1	Slow growth	Slow growth
cdc14-1	Slow growth/inviable	Slow growth
cdc45-1	No suppression	No suppression
cdc46-1	Slow growth	Slow growth
cdc47	Slow growth/inviable	Slow growth/inviable
cdc54	Inviable	Inviable
cdc7-1	Inviable	No interaction
cdc9-1	No interaction	No interaction
cdc28-4	No interaction	No interaction

Genetic interactions among *ORC* and other cell cycle genes. Crosses were performed between the indicated strains and at least 36 tetrads from each cross were scored for viability at various temperatures. Synthetic lethality was inferred when no viable double mutants were recovered and if there was no significant deviation from the 1PD:4T:1NPD ratio of tetrad types predicted for segregation of two unlinked genes. If tetrad segregants that contained both mutations formed small colonies that failed to restreak or restreaked poorly, they were described as "slow growth/inviable." In the instances where no interactions were observed between two mutations, segregants that were inferred to be double mutants were crossed to a wild-type strain and dissected to verify that both cell cycle mutations could be recovered. In crosses involving *cdc45-1*, a mutation that caused cold sensitivity, no suppression of either *cdc45-1* or the *orc* mutations was observed; other synthetic interactions between these genes could not be evaluated because of the nonoverlapping permissive temperature ranges of these mutations.

double mutants mated well, indicating no further enhancement of the *orc* mutant silencing defects. Double mutants between *cdc6–1*, *cdc14–1*, *cdc45–1*, *cdc46–1*, *cdc47*, or *cdc54* and sensitized silencer alleles such as *HMR*-SS ΔI and *hmr*-Δe331–324 did not result in significant silencing defects (Loo and Rine, unpublished observations). These data are consistent with other work that indicates separable roles for ORC in replication and silencing (Fox *et al.*, 1995).

DISCUSSION

This work describes the cloning of *ORC5*, the gene encoding the fifth largest subunit of the *S. cerevisiae* origin recognition complex, and the isolation of a temperature-sensitive allele by a genetic screen for silencing defects. This mutation, *orc5–1*, shared many characteristics with recently described *orc2* mutations isolated from similar screens (Foss *et al.*, 1993; Micklem *et al.*, 1993), including silencing and cell cycle defects. The *ORC5* gene was essential for life, and *orc5–1* was a partial loss-of-function allele.

The Role of ORC in Replication

One function of *ORC2* and *ORC5* appeared to be in initiation of DNA replication. Both the *orc2–1* and *orc5–1* lesions caused a substantial defect in plasmid maintenance (Table 4 and Foss *et al.*, 1993). In principle, this defect could be caused by inefficient initiation or inefficient elongation during DNA polymerization. The increased loss rate of an extrachromosomal plasmid caused by DNA ligase (*CDC9*) or DNA polymerase (*CDC17*) defects cannot be suppressed by adding extra tandem copies of an ARS to a plasmid (Hogan and Koshland, 1992); these mutations have been characterized as elongation mutants. Instead, Hogan and Koshland propose that providing additional tandem copies of an ARS increases only the probability of a successful initiation event in cells that are compromised for that function. Using this criterion, they suggest that *CDC6* and *CDC14* function during the initiation of DNA replication. If that model is correct, then the data presented here indicate that a role of *ORC2* and *ORC5* involves the proper initiation of DNA replication. This interpretation has received strong support from the discovery that *orc2–1* and *orc5–1* cause a substantial impairment of replication initiation from chromosomal origins (Fox *et al.*, 1995). Interestingly, the cell cycle phase in which *orc5–1* cells are least sensitive to increased temperature is at M phase (Figure 8). Of course, a trivial explanation for the lack of lethality of M phase–arrested *orc5–1* cells would be that for some reason the mutant protein was not thermolabile in M phase. However, that possibility is unlikely because incubation of benomyl-treated *orc5–1* cells at the restrictive temperature results in a loss of ORC's silencing function (Fox *et al.*, 1995). Thus it may be that a replication-competent ORC complex can be assembled after M phase but not in or after G_1. Indeed, ORC's DNA footprint undergoes changes in the G_2/M and G_1 cell cycle interval (Diffley *et al.*, 1994).

orc5–1 mutants arrest growth with 2C (or nearly 2C) DNA content and undivided nuclei, an observation that, at face value, would seem incompatible with ORC having only a single function in replication initiation. However, several observations indicate that *orc2–1* and *orc5–1* have principal defects in replication initiation. The suppression of plasmid loss by multiple tandem ARS sequences (Table 1) argues that replication initiation itself was compromised in *orc* mutants. Furthermore, analysis of chromosomal origins reveals that replication initiation at both *ARS1* and at the synthetic *HMR*-E silencer origin are reduced in *orc2–1* and *orc5–1* mutants (Fox *et al.*, 1995).

orc5–1 cells can undergo several cell divisions at the restrictive temperature, thus a possible explanation for the 2C content of arrested *orc5–1* cells may be that this mutation is "leaky." In this model, the few replication forks that are generated would be sufficient to replicate the bulk of the genome leaving only a small number of unreplicated regions. A subsequent mitotic checkpoint would prevent nuclear and cell division. This model makes the prediction that S phase would be longer in *orc5–1* cells than in wild-type cells because there would be fewer replication forks to replicate the entire genome.

Another possibility is that *ORC5* has two functions in cell cycle progression: one early in S phase and a second function during or after the completion of DNA synthesis. If so, the second process may require a higher level of ORC function, such that as ORC function becomes limiting after the temperature shift, the post-replicative process becomes rate limiting for cell cycle progression. Interestingly, the *orc5–1* mutation does not stand alone in this regard, and several other mutations used in this work share similar phenotypes. For example, the *cdc45–1* mutation can cause growth arrest with unreplicated or fully replicated DNA, depending on the restrictive temperature used (Hennessy *et al.*, 1991). The *cdc14–1* mutation causes a mitotic block but, like the *orc2–1* and *orc5–1* mutations, its plasmid maintenance defect can be suppressed by tandem ARS elements (Hogan and Koshland, 1992), implying an additional function early in S phase or a role in establishing a functional replication complex in mitosis of the previous cell cycle. Finally, both *ORC2* and *ORC5* are required continuously for silencing when cells are arrested in M phase (Fox *et al.*, 1995), again suggesting a function for the complex other than in replication initiation. At present, some evidence indicates that *ORC5* has roles both in replication initiation and at a later stage before mitosis. However,

until an *orc5* allele can be found that exhibits first cycle arrest at the restrictive temperature, it will be difficult to perform critical tests of this model.

Interactions among ORC and other Cell Cycle Genes

Mutations causing temperature-sensitive growth, when combined into the same cell, often cause a growth phenotype more severe than either single mutant alone. Synthetic lethality refers to those double mutant combinations that are lethal when neither single mutant is. It is often difficult to understand why two mutations would be synthetically lethal as lethality may occur for reasons unrelated to the process being studied. It is therefore noteworthy that the *orc2–1* and *orc5–1* mutations, which share many phenotypes by presumably weakening a single process, do not interact identically with mutations in cell cycle genes such as *cdc7–1*, and not at all with *cdc9* (encoding DNA ligase) or *cdc28* (encoding the *S. cerevisiae* cyclin-dependent kinase) mutants. Thus, the examples of synthetic lethality between *orc* mutants and only a subset of cell cycle progression genes are inconsistent with the lethality being due to a general weakening of the cell caused by weakening two unrelated processes.

Biochemical and sequence information of the cell cycle genes in this study offer some insight into the nature of the synthetic lethality. Because both Orc2p and Orc5p are subunits of the same protein complex, the synthetic lethality of *orc2–1 orc5–1* double mutants can easily be explained by the reduction in ORC activity caused by two partially defective subunits. The subunit composition of ORC purified from an *orc5–1* mutant also supported this hypothesis as the Orc2p subunit was already under-represented in an *orc5–1* mutant that has a wild-type copy of *ORC2* (Figure 8).

CDC7 encodes the catalytic subunit of a serine-threonine protein kinase that is required for DNA replication. The other subunit of this kinase is encoded by *DBF4* (Jackson *et al.*, 1993). The lethality of the *cdc7–1 orc2–1* double mutant is consistent with ORC being activated through phosphorylation by DBF4/CDC7 kinase either directly or in a kinase cascade. ORC is thought to be bound to yeast origins throughout most, if not all, of the cell cycle (Diffley and Cocker, 1992), implying that its S phase action is regulated by some mechanism other than controlling its binding activity. Phosphorylation would be an attractive mechanism for regulating the activity of bound ORC. An alternative interpretation of this synthetic lethality is suggested by the recent finding that Dbf4p is localized to the *ARS1* origin of replication in vivo (Dowell *et al.*, 1994). This localization is dependent on the presence of an intact ARS consensus sequence at *ARS1*, suggesting that it may be mediated through direct interactions between ORC and Dbf4p. If this is the case, the observed synthetic lethality may be the result of the disruption of a cooperative complex between ORC, Dbf4p, and Cdc7p during the G_1 to S phase transition of the cell cycle. Further understanding of these double mutant phenotypes will require determination of the substrates of the DBF4/CDC7 kinase and an understanding of the mechanism that localizes Dbf4p (and presumably Cdc7p) to origins of replication.

CDC14 encodes a protein that is likely to be a phosphoprotein phosphatase (Wan *et al.*, 1992; Sheng and Charbonneau, 1993). The synthetic lethality of *cdc14–1 orc2–1* double mutants would be consistent with ORC or a regulator of ORC being controlled by phosphorylation. By this hypothesis, phosphorylation may block the action of ORC and Cdc14p would be required to remove the phosphate residue from a critical residue to restore activity. Of course, double mutant analysis does not distinguish between direct and indirect interactions, hence it is possible that Cdc14p works on a different protein which, in turn, acts on ORC. Although DBF4/CDC7 is a kinase involved in replication, it is unlikely to be the target of Cdc14p because the mitotic instability of ARS plasmids in *cdc7* mutants cannot be suppressed with multiple tandem origins, whereas that defect in *cdc14–1*, *orc2–1*, and *orc5–1* mutants can (Hogan and Koshland, 1992 and Table 3).

The genetic interactions among *orc2–1*, *orc5–1*, and the group of genes represented here by *cdc54* integrates ORC into a larger set of proteins thought to be involved in the initiation process. Studies of *CDC46*, *CDC47*, and *CDC54* show that these genes form a group of genetically interacting components required early in yeast DNA replication (Hennessy *et al.*, 1991). Interestingly, like in *orc2–1* and *orc5–1* mutant cells, the *cdc46*, *cdc47*, and *cdc54* mutations used in this work all caused plasmid maintenance defects that were suppressible by tandem ARS sequences (Loo and Rine, unpublished observation). Together with the synthetic phenotypes observed between *orc2–1* or *orc5–1* and this class of mutants, these data suggest that these genes are involved in a related step during DNA replication.

The sequence of the *CDC46* gene shows that it encodes a member of a large class of proteins known as MCM proteins (Hennessy *et al.*, 1991). These include Mcm2p and Mcm3p (*MCM5* is identical to *CDC46*), Cdc47p, Cdc54p, and numerous homologues from other eukaryotes. The intracellular locations of Cdc46p, Mcm2p, and Mcm3p are regulated during the cell cycle, with each protein concentrated in the nucleus between late mitosis and the G_1 to S phase transition (Hennessy *et al.*, 1990; Yan *et al.*, 1993). During the remainder of the cell cycle these proteins are found to be excluded from the nucleus. This finding has led to the proposal that the entry and exit of these proteins from the nucleus is linked to either the as-

sembly of the DNA replication machinery or to the subsequent initiation of DNA synthesis. It is noteworthy that unlike the other mutants that show genetic interactions with *orc2–1*, *cdc14–1* arrests the cell cycle at mitosis. Taken together, the integration of ORC into the group of genes including *CDC46* and the interaction between *orc2–1* and *cdc14–1* suggest that this set of proteins is involved in the assembly of a DNA replication initiation complex that starts late in mitosis and is not activated until the transition to S phase. This hypothesis is supported by studies of in vivo DNase I footprints at origins of replication on plasmids, which indicate that there are substantial changes in the protein DNA interactions during this same cell cycle period (Diffley *et al.*, 1994). Clearly, the proteins directly involved in the formation of such a complex are likely to include ORC and the DBF4/CDC7 kinase.

The Role of ORC in Silencing

Previous work identified mutations in *ORC2* that blocked both initiation of DNA replication and silencing. Although those results were suggestive of a role for ORC itself in silencing, the data were also consistent with Orc2p playing a role in silencing that was independent of its role in the complex. The discovery of a mutation in a gene encoding a second ORC subunit strengthens the notion that the *orc2–1* and *orc5–1* silencing defects reflect a role for the complex itself in silencing. The absence of mutations affecting silencing in the genes encoding other subunits of ORC can still be explained on statistical grounds because the genetic screens that yielded *ORC2* and *ORC5* mutations have not yet been saturated.

How the *orc2–1* and *orc5–1* mutations reduce silencing is less clear. These two mutations on their own led to very little derepression unless they were coupled to a genetically compromised silencer. One explanation for the partial silencing defect of *orc* mutants is that the mutations identified to date do not reflect true null alleles for silencing, just as *orc5–1* was not a null allele for replication. At the other extreme, the role of ORC in silencing could be redundant, much as the roles of the ACS and the binding sites for Abf1p and Rap1p seem to be redundant at the wild-type *HMR*-E silencer (Brand *et al.*, 1987).

What role does ORC play in transcriptional silencing? Broadly, there are at least two views (reviewed in Dillin and Rine, 1995). In one, ORC's role in silencing may be inseparable from the act of replication initiation. In this model *HMR*-E must be a specialized origin of replication that, perhaps through the components at the replication fork, leads to the assembly of an inactive chromatin structure. This view is supported by the discoveries that *HMR*-E is a chromosomal origin of replication (Rivier and Rine, 1992) and that at least some *orc* mutations substantially reduce the firing of this origin (Fox *et al.*, 1995). However, the *HMR*-E origin initiates replication less than once per cell cycle (Rivier and Rine, 1992), yet *HMR* remains repressed in all cell cycles. Thus if ORC has a role in silencing that requires replication initiation, that role would be in establishment rather than in maintenance of the repressed state (Miller and Nasmyth, 1984; Pillus and Rine, 1989; Dubey *et al.*, 1991; Loo and Rine, 1995).

If replication initiation were required for silencing, mutations that affect initiation should lead to silencing defects. However, mutations in *CDC6*, *CDC45*, *CDC46*, *CDC47*, and *CDC54* do not cause silencing defects, even when they are paired with compromised *HMR*-E silencers such as those used in the screens that yielded *orc2–1* and *orc5–1*. If these mutations indeed cause initiation defects, then the failure of these mutations to cause a silencing defect would be inconsistent with initiation at the *HMR* silencers being an essential step in silencing. Alternatively, the mutations in *CDC6*, *CDC45*, *CDC46*, *CDC47*, and *CDC54* may be in the same class as mutations in *CDC7* that are able to suppress silencing defects (Axelrod and Rine, 1991).

In a different view, ORC's role in silencing may be independent of the initiation of replication per se. For example, ORC, Rap1p, and Abf1p bound to the *HMR*-E silencer may form a surface that recruits other silencing components and nucleates the assembly and spread of an inactive chromatin structure throughout *HMR*. In support of this model, a Gal4p DNA binding domain-Sir1p fusion, when targeted to the *HMR*-E silencer, can bypass the need for the ACS at *HMR*-E in silencing (Chien *et al.*, 1993). However, the ability of the fusion protein to bypass the ACS at *HMR*-E is not proof that replication initiation does not normally play a role in silencing as replication initiation might be an event required to recruit natural Sir1p (but not the $Gal4_{bd}$-Sir1p fusion) to *HMR*. Nevertheless, initiation of replication at the *HMR* silencers may simply be a coincidence associated with ORC binding. It is noteworthy that neither of the *HML* silencers, although requiring ORC function to silence, is an efficient origin (Dubey *et al.*, 1991). An indication that ORC might function in different initiation and silencing pathways may come from the analysis of *ORC* alleles that are defective in silencing but not in replication initiation, and, as discussed above, there is evidence that ORC is required for silencing outside of S phase (Fox *et al.*, 1995). The recovery of *orc* mutations that are defective only in replication initiation and not in silencing would lend even stronger support to models involving two distinct and separable roles for ORC in silencing.

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