Origin Recognition Complex (ORC) in Transcriptional Silencing and DNA Replication in *S. cerevisiae*

Margit Foss, Francis J. McNally,* Patricia Laurenson, Jasper Rine

In *Saccharomyces cerevisiae*, the *HMR-E* silencer blocks site-specific interactions between proteins and their recognition sequences in the vicinity of the silencer. Silencer function is correlated with the firing of an origin of replication at *HMR-E*. An essential gene with a role in transcriptional silencing was identified by means of a screen for mutations affecting expression of *HMR*. This gene, known as *ORC2*, was shown to encode a component of the origin recognition complex that binds yeast origins of replication. A temperature-sensitive mutation in *ORC2* disrupted silencing in cells grown at the permissive temperature. At the restrictive temperature, the *orc2-1* mutation caused cell cycle arrest at a point in the cell cycle indicative of blocks in DNA replication. The *orc2-1* mutation also resulted in the enhanced mitotic loss of a plasmid, suggestive of a defect in replication. These results provide strong evidence for an in vivo role of ORC in both chromosomal replication and silencing, and provide a link between the mechanism of silencing and DNA replication.

The expression of the genes that determine mating type in *Saccharomyces cerevisiae* is controlled, in part, by the position of the genes in the genome. When present at the mating type locus, MAT, the genes are expressed and determine the mating type of cells. The **a** cells have the MAT**a** allele and α cells have the MATα allele. Both MAT**a** and MATα encode regulatory proteins that control the expression of cell type–specific genes elsewhere in the genome (*1*). Yeast cells have two additional copies of the mating type genes. These additional copies are at the *HML* and *HMR* loci, which are located on the left and right arms of chromosome III, respectively. Most strains have a copy of the MAT**a** genes at *HMR* (*HMR***a**) and a copy of the MATα genes at *HML* (*HML*α) although *HML***a** and *HMR*α alleles are also known. The expression of mating type genes at *HML* and *HMR* are repressed because of a general inhibition of gene expression in the vicinity of these loci. This inhibition is referred to as silencing. Silencing requires sequence elements, known as silencers, that flank both *HML* and *HMR*. Both *HML* and *HMR* have a silencer immediately to the left of the locus, known as the *E* silencer, and a silencer immediately to the right of the locus, known as the *I* silencer (*2*). In addition to the silencers, proteins encoded by several genes scattered throughout the genome are also required for silencing. These genes encoding these proteins include *SIR1*, *SIR2*, *SIR3*, and *SIR4* (*3*), *RAP1* (*4*), *NAT1*, *ARD1* (*5*), and either of the two genes encoding histone H4 (*6*). The site of action of most of these proteins is unknown. The ability of mutations in histone H4 to cause derepression of *HML* and *HMR* indicates that the mechanism of silencing likely involves the assembly of a particular structure of chromatin (*7*).

Among silencers, the *HMR-E* silencer has been the most thoroughly studied. Genetic experiments have identified three functional domains within *HMR-E* (*8*). One domain consists of the binding site for the *RAP1*-encoded protein (Rap1p), a protein that binds the control regions of a large number of different yeast genes and also binds the $(C_{1-3}A)_n$ repeats of the telomeres (*9*, *10*) reviewed in (*11*). Mutations in the *RAP1* gene that interfere with silencing have been isolated, providing proof of its role in silencing (*4*). A second domain consists of the binding site for the *ABF1*-encoded protein (Abf1p), another sequence-specific DNA binding protein (*9*, *12*, *13*). In contrast to that of Rap1p, the evidence for the involvement of Abf1p in silencing is less direct. The third functional domain includes the ARS consensus sequence, a sequence found at yeast origins of DNA replication (*14*). The ARS consensus sequence is bound by a six-subunit protein complex known as ORC, for origin recognition complex (*15*). Bell *et al.* have shown that ORC binds the ARS consensus sequence of *HMR-E* as well as the ARS consensus sequence of the other silencers (*16*). A synthetic silencer consisting of only these three domains is capable of functionally substituting for the wild-type *HMR-E* silencer, an indication that a detailed understanding of these three elements would be sufficient to learn how silencers function (*17*).

DNA replication has been suggested to have a role in transcriptional silencing (*2*, *18*). The strongest evidence connecting silencing to replication is that *HMR-E* is a chromosomal origin of replication and that a mutation in *HMR-E* that blocks its action as an origin of replication also blocks its ability to function as a silencer (*19*). Despite the extent of this correlation, there are other explanations for even the strongest evidence. For example, the ARS consensus sequence might be bound by different proteins at different times in the cell cycle, one of which is required for replication but not for silencing and the other for silencing but not for replication. Mutations in the ARS consensus sequence would then affect both processes despite the lack of a mechanistic connection between them. One way to test the link between replication and silencing would be to isolate silencing-defective mutations that affect the protein acting through the ARS consensus sequence. If such mutations identify genes that have no role in replication, then the correlation between replication competence and silencer function would be broken. Alternatively, if these mutations identify proteins intimately connected with DNA replication, then the evidence linking replication and silencing would be substantially strengthened.

ORC2, a gene required for viability and silencing. In a mutant screen, a temperature-sensitive (ts) mutation called *orc2-1* was isolated that, at the permissive temperature, resulted in derepression of *HMR***a** flanked by the synthetic silencer and did not cause derepression of *HMR***a** flanked by the wild-type silencer (*20*). Because the *orc2-1* mutant was temperature sensitive and silencing defective, it merited further analysis. The temperature resistance of a heterozygous *orc2-1*/*ORC2* diploid (JRY2903 crossed to JRY2640) established that the mutation was recessive. The diploid was transformed with a plasmid containing *HMR***a** flanked by a mutant silencer (pJR1212), to provide MAT**a**/α function required for sporulation. The temperature-sensitive growth phenotype segregated 2 ts:2 wild type in each of 23 tetrads, indicating that it was caused by a single nuclear mutation. The gene was named *ORC2* for reasons that become apparent as shown below.

Genetic crosses were used to determine which features in the wild-type silencer distinguished it from the synthetic silencer

The authors are in the Division of Genetics, Department of Molecular and Cellular Biology, 401 Barker Hall, University of California, Berkeley, CA 94720.

*Present address: Department of Pharmacology, 0450, University of California at San Francisco, San Francisco, CA 94143.

with respect to derepression by *orc2-1*. A *mata1* *HMRα* strain (JRY3683) containing the *orc2-1* mutation was mated to a *MATα* strain containing a mutation in the RAP1 binding site of *HMR-E* flanking *HMRa* [the *HMRa-e-rap1-10* allele; 5401-1a (*21*)] to determine whether *orc2-1* could derepress *HMRa* in the absence of a functional RAP1 binding site. It was not possible to infer the complete genotype of all the segregants of the 48 tetrads analyzed from this cross. Nevertheless, all 29 of the 96 *MATα* segregants that had little or no mating ability were temperature sensitive for growth. Nineteen of the *MATα* temperature-sensitive segregants were mating competent, an indication that the *orc2-1* mutation per se was insufficient to disrupt mating ability, and suggesting that the *HMRa-e-rap1-10* allele was required in combination with *orc2-1* to block mating ability of α strains.

As further evidence that *orc2-1* in combination with *HMRa-e-rap1-10* blocked the mating ability of *MATα* strains, a somewhat unusual cross was used to simplify the previous cross by having *orc2-1* as the only relevant heterozygous marker. Two *MATα* *HMRa-e-rap1-10* strains (JRY4133 and JRY4132) had complementary auxotrophic markers, allowing for the selection of the rare *MATα/MATα* diploid formed by a mating event between these two strains. This diploid was able to sporulate as a result of the low expression of *HMRa* in the diploid caused by the RAP1-site mutation in the *HMR-E* silencer (*21*). One of these strains had the *orc2-1* mutation (JRY4133) and the other did not. As expected, the temperature sensitivity segregated 2:2 in each of 34 tetrads. All of the temperature-resistant segregants (two per tetrad) exhibited the α mating phenotype, and all of the temperature-sensitive segregants were either very weak α maters or were unable to mate at all. The absence of any recombinants between the temperature sensitivity and mating phenotype placed the gene (or genes) responsible for the temperature sensitivity and the mating defect less than 1.5 centimorgans (cM) apart, providing evidence that a lesion in a single gene was responsible for both phenotypes. This result was in agreement with the co-reversion of the ts and mating phenotypes.

Isolation of multiple alleles of ORC2. On the basis of the information from this analysis of *orc2-1*, we used a second screen to identify additional mutations in essential genes with a role in silencer function (Fig. 1). This second screen produced 50 mutants that were temperature sensitive for growth and in which *HMRα* (flanked by a mutation in the RAP1-binding site) was derepressed at a semipermissive temperature. Complementation tests both for growth at 37°C and for mating phenotype were performed between *orc2-1* and the collection of ts mutants from the second screen. The collection of ts mutants had the *mata1* *ste14* genotype, but were able to mate as α's because of the derepression of *HMRα*. These mutants were mated to a *mata1* *orc2-1* strain (JRY3683), and the diploids were tested for growth at 37°C and mating ability at 30°C. Three of the diploids failed to grow at the nonpermissive temperature and mated as α's, indicating that the three mutants were unable to complement either the temperature sensitivity or the derepression phenotype of the original *orc2-1* mutation. The new mutations (in strains JRY4136, JRY4137, and JRY4138) were designated *orc2-2*, *orc2-3*, and *orc2-4*.

To investigate the possibility that the new mutations were in a gene other than *ORC2* yet still failed to complement *orc2-1*, we tested the allelism between *orc2-1* and *orc2-3*. The original *mata1* *orc2-3* *ste14* mutant was cured of its *HMRα* plasmid, creating JRY4137, transformed with a *STE14-URA3* plasmid (pSM186) to allow it to mate as an **a**, and mated with a *MATα* *HMRa-e-rap1-10* *orc2-1* strain (JRY3685), transformed with an *ORC2-LEU2* plasmid (pJR1416). The diploid was cured of its plasmids and sporulated. In 24 tetrads from this diploid, all segregants were temperature-sensitive for growth, indicating strong linkage between *orc2-1* and *orc2-3* (<2 cM). All further studies were performed with the *orc2-1* allele, which provided the stronger mutant phenotypes.

Linkage between *ORC2* and *LYS2*, on chromosome II, was evident in crosses between two *lys2* strains (JRY2640 and PSY152) and the original *orc2-1* isolate (JRY2903), and placed *ORC2* approximately 24 cM from *LYS2*. A third cross (JRY4130 × JRY4134) tested the linkage between *sec18* (which is centromere proximal to *LYS2*) and *ORC2*. Because both *orc2-1* and *sec18* are temperature sensitive, an *ORC2* allele marked by *URA3* (from pJR1423) was used to determine that *SEC18* and *ORC2* were separated by 6.6 cM (Table 1). No previously mapped genes involved in silencing map near *SEC18*.

The ORC2 mutants arrested with a cell cycle terminal phenotype. If *ORC2* encoded a protein that acted through the ARS consensus sequence, it was possible that the function of Orc2p was related to DNA replication. Because mutations in known DNA replication proteins exhibit a specific cell cycle arrest in yeast, the effect of the *orc2-1* mutation on the cell division cycle was explored. Mutant *orc2-1* strains were grown in liquid medium at 23°C, the per-

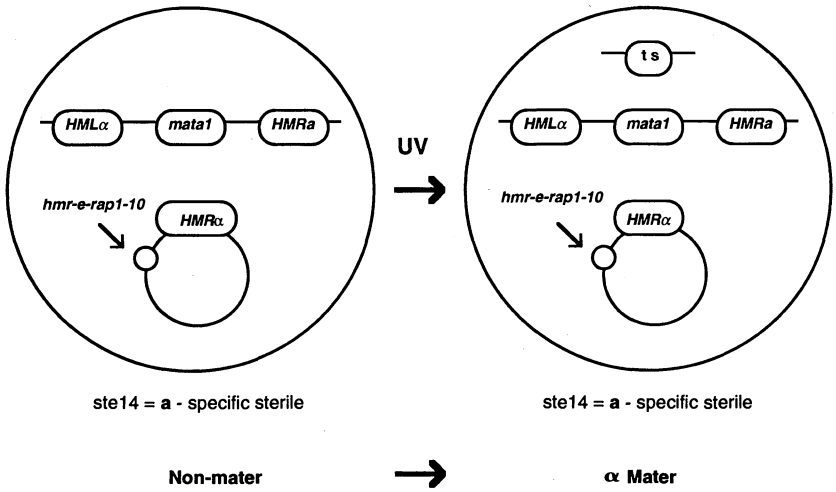

Fig. 1. Representation of one of the screens for *ORC* mutations. On the left of the scheme is the genotype and mating phenotype of the unmutagenized parent strain, JRY4135. The strain contains the *mata1* locus on the chromosome, and carries the *HMRα* locus on a plasmid, coupled to the *hmr-e-rap1-10* mutation. The strain is also *ste14* (*29*) which prevents **a** cells from being able to mate. On the right is a representation of the genotype and mating phenotype of a mutant in which a mutation (labeled ts) has caused the transcriptional derepression of *HMRα*, resulting in the α mating phenotype. The parent strain was mutagenized and screened for mutant colonies with the α mating phenotype at permissive temperatures and temperature-sensitive growth at the restrictive temperature (*30*).

Table 1. Linkage of *ORC2* to *LYS2* and *ORC2* to *SEC18*.

Cross	Tetrad types			Map distance (cM)
	PD	T	NPD	
ORC2 vs. *LYS2*	10	14	0	29
ORC2 vs. *LYS2*	20	14	0	21
ORC2 vs. *LYS2* total	30	28	0	24
ORC2 vs. *SEC18*	46	7	0	6.6

missive temperature, and then shifted to 37°C to test whether the cells arrested with a single terminal morphology. Nearly all (90 percent) of the cells arrested as large budded cells, which is characteristic of the arrest point caused by mutations in genes necessary for DNA replication. In contrast, parallel cultures maintained at the permissive temperature contained cells at all points in the cell cycle (Fig. 2, A and B).

ORC2 cells harvested either after continuous growth at the permissive temperature or after a shift to the nonpermissive temperature were fixed and stained with DAPI allowing visualization of DNA with fluorescence microscopy. The cells grown permissively displayed a range of morphologies from small unbudded cells to cells with single buds of various sizes. The cells shifted to the nonpermissive temperature looked very different: the majority arrested as large budded cells, and for the most part, each mother-daughter pair contained only a single brightly staining region, often at or near the neck (Fig. 2). These data indicated that *orc2-1* mutants displayed cell cycle defects characteristic of mutants defective in DNA replication and raised the possibility that Orc2p had an essential role in replication. Evidence by Bell *et al.* (*16*) indicated that the cells arrested prior to initiating DNA replication.

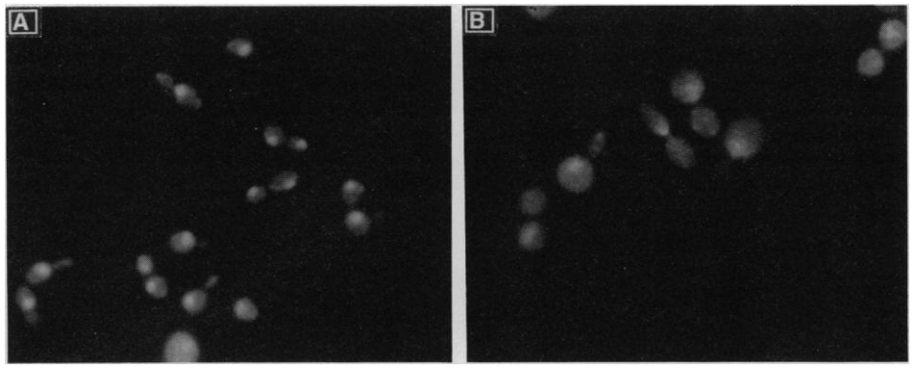

Fig. 2. The *orc2-1* cells (JRY3683) were grown to log phase at the permissive temperature (23°C), and the culture was divided. Half was kept at the permissive temperature and was grown for an additional 5 hours; the other half was shifted to the nonpermissive temperature (37°C) and grown for an additional 5 hours. Both cultures were then fixed and stained with DAPI (4′, 6-diamidino-2-phenylindole) to allow visualization of the nucleus. At the permissive temperature (**A**), cells at all phases of the cell cycle were observed. Cells later in the cell cycle, as evidenced by the presence of large buds, frequently exhibited nuclei in both the mother and the daughter cell. In contrast, in the culture shifted to the restrictive temperature, approximately 90 percent of the cells arrested as large budded cells (**B**). Nuclei were only present in the mother cell and not in the daughter cells. In addition, the cells were larger than those grown at the permissive temperature, indicating that protein synthesis and cell wall synthesis continued in the absence of *ORC2* function. Similar results were obtained with two additional *orc2-1* strains (JRY3685 and JRY3687, data not shown).

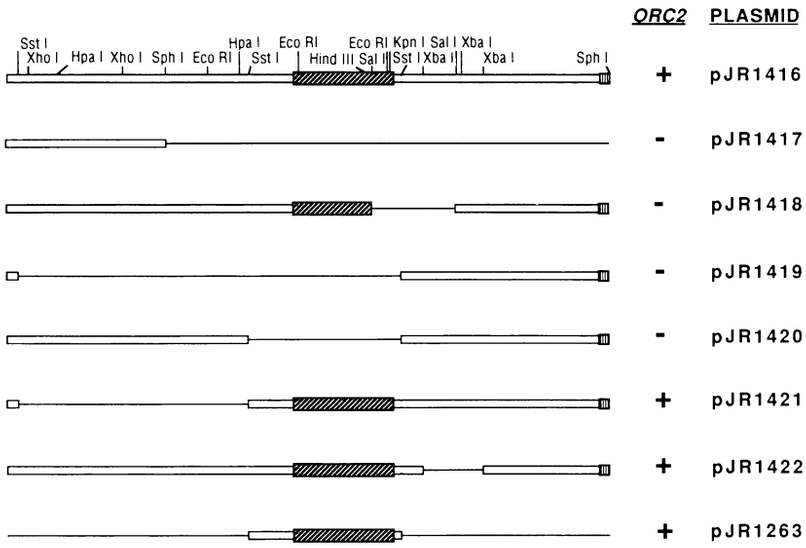

Fig. 3. The original *ORC2*-complementing clone (top row) is drawn to scale as are the six subclones used to map further the *ORC2* gene (middle six rows), and the smallest *orc2*-complementing subclone revealed by this analysis (bottom row) (*31*). The *ORC2* coding region is represented by diagonal stripes. The bars indicate DNA present in the subclones, and the thin lines represent DNA deleted in the subclones. The small vertically striped bars on the right sides of the clones represent nonessential vector sequence. The bottom insert was in pRS316, and all others in a YCp50-based (*LEU2*) vector. A "+" or "−" designates whether the plasmid did or did not complement the temperature sensitivity of an *orc2-1* strain.

The *ORC2* gene was cloned by complementation of the *orc2-1* temperature sensitivity (*22*). One complementing clone (pJR1416) was chosen for further analysis. Subclones missing various fragments from the insert were transformed into an *orc2-1* strain to test whether the deletion affected the clone's ability to complement *orc2-1*'s temperature sensitivity. The key observations were that the deletion of a 2.8-kb Sst I–Sst I fragment destroyed complementation activity, whereas the deletions of flanking sequences (Xba I, and the larger Sst I fragment) had no effect. The 2.8-kb fragment was subcloned (pJR1263), and shown to have complementing activity (Fig. 3).

To determine whether the gene on the clone was indeed allelic to the *ORC2* mutation, we subcloned a fragment of the original clone into a yeast integrating vector. This plasmid (pJR1423) was cleaved within the insert to direct homologous integration and transformed into a wild-type strain (W303-1A). As a result, the site of integration was marked by the plasmid's *URA3* gene. The resulting strain (JRY4134) was crossed to an *orc2-1* strain (JRY3685). In each of 59 tetrads, *URA3* segregated opposite to the temperature sensitivity caused by *orc2-1*, indicating that *ORC2* had indeed been cloned.

ORC2 was disrupted by *URA3* (*23*) and integrated into a diploid homozygous for *ura3* and *ORC2* (creating JRY3444). Of the 41 tetrads dissected, 40 tetrads had two live and two dead segregants, and one tetrad had only one live segregant. The colonies that grew were Ura-, without exception. By inference, the dead segregants contained the *URA3* gene, and thus the *ORC2* disruption, indicating that *ORC2* function was essential for cell viability at all temperatures. We examined the dead segregants under a microscope to gain some insight into the true null phenotype. Most of the spores germinated into cells that were elongated or otherwise deformed and had not divided. In no case did the cell divide more than two times. Thus in many spores, the absence of *ORC2* blocked cell division but not growth.

Role of *ORC2* in plasmid replication. If *ORC2* were involved in DNA replication, then a mutation in *ORC2* would be expect-

ed to impair the replication and inheritance of plasmids whose replication depends on a chromosomal origin of replication. Colony color sectoring assays offer a sensitive and simple measure of plasmid stability. In one such assay, a plasmid containing a centromere, a suppressor tRNA, and an origin of replication was transformed into strains containing a nonsense mutation in the *ADE2* gene. If the plasmid is replicated and segregated properly, transformants with the plasmid grow into uniformly white colonies. However, if a plasmid is lost in a cell division during the growth of the colony, the colony exhibits a red sector in an otherwise white colony (*24*).

In a test for the role of *ORC2* in plasmid stability, an isogenic pair of strains, one wild type (W303-1B) and one *orc2-1* (JRY4125), were transformed with a plasmid containing a centromere, a suppressor tRNA (*SUP11-1*), *URA3*, and ARS1, a chromosomal origin of replication (YRP14/CEN4/ARS1/ARS1) (*24*), selecting for uracil prototrophy. Transformants were grown on selective medium at 23°C, the permissive temperature for *orc2-1*. The colonies were picked from the selective plate, serially diluted, plated onto solid rich medium and grown to colonies at 23°C. The wild-type transformants grew into colonies most of which were white with a few exhibiting red sectors. The small number of red colonies were from cells in the selectively grown colony that had lost the plasmid. In contrast, most the colonies from the *orc2-1* mutant were red, reflecting a high degree of plasmid loss among the cells in the selectively grown colony. Moreover, in the *orc2-1* strain, red sectors were present in most of the white colonies with some white colonies displaying multiple red sectors (Fig. 4).

It is possible to quantitate the number of cell cycles in which a plasmid is lost from the number of colonies that are half red and half white. Only those colonies that lose the plasmid in the first cell division have half sectors. In the case of the wild-type strain, 0.9 percent (10 of 1168) of the colonies were half red and half white, indicating that the plasmid was lost in 0.9 percent of cell cycles. In contrast, the frequency of half red and half white colonies in the *orc2-1* strain grown at the permissive temperature was 11 percent (58 of 512), indicating that the same plasmid was lost approximately 12 times as often in the strain with partially defective Orc2p. These data indicated a profound defect in plasmid stability specific to the *orc2-1* strain and, in combination with the cell cycle phenotype of *orc2-1*, suggested that *orc2-1* strains were defective in DNA replication. These results were consistent with the flow cytometry studies of *orc2-1* strains (*16*).

Sequence of ORC2. The sequence of the 2.8-kb Sst I–Sst I *orc2*-complementing fragment was determined. The only open reading frame of significant length was deduced to be *ORC2*, and predicted a 620-residue protein of approximately 68-kD (Fig. 5). The Sst I fragment included 806-bp of upstream sequence and 140-bp of downstream sequence. Because of the connection between *ORC2* and replication, we considered it likely that *ORC2* might encode a subunit of the origin recognition complex described by Bell and Stillman (*15*). Comparison of the predicted Orc2p sequence with the sequence of two peptides derived from purified Orc2p revealed a perfect match (*16*). This identity established that the gene identified in this study encoded a subunit of ORC, and was the reason for naming the gene *ORC2*.

The deduced Orc2p protein was 15 percent basic residues and 16 percent serine or threonines. Fully 50 percent of the NH_2-terminal residues (residues 15 to 280) were lysine, arginine, proline, serine, or threonine. The KeyBank motif program revealed several matches to peptide motifs within Orc2p. Orc2p contained many potential phosphorylation sites (3 for cyclic adenosine monophosphate– and cyclic guanosine monophosphate–dependent protein kinase, 14 for casein kinase II, and 12 for protein kinase C), and an excellent match to the nuclear targeting sequence (residues 103 to 107). There were no sequences in the promoter region, such as Mlu I sites, suggestive of S phase–specific transcription.

Orc2p was not similar enough to any protein in the databases to identify a specific biochemical activity. Nonetheless,

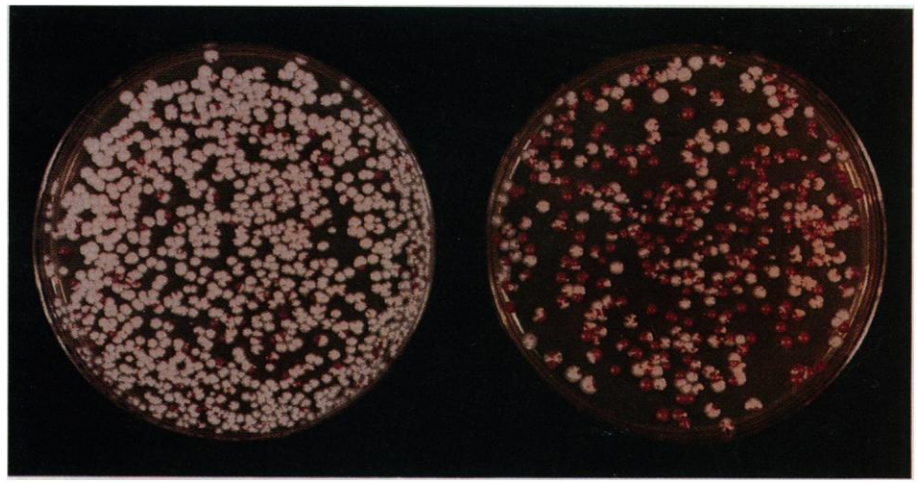

Fig. 4. *ORC2* is required to stabilize plasmids. An isogenic pair of wild-type (W303-1B) and *orc2-1* (JRY4125) strains transformed with a plasmid containing CEN4, ARS1, *URA3*, and *SUP11-1* were grown selectively and then plated onto the nonselective plates shown. The wild-type strain is on the left and the *orc2-1* strain is on the right. Colonies bearing the plasmid are white, and colonies which have lost the plasmid are red. Colonies in which the plasmid is lost during the growth of the colony are white with red sectors (*24, 25*). The stability of the plasmid is severely compromised in the *orc2-1* strain, as is seen by the high frequency of red and sectored colonies compared to the wild type.

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1    MLNGEDFVEHNDILSSPAKSRNVTPKRVDPHGERQLRRIHSSKKNLLERISLVGNERKNTSPDPALK
68   PKTPSKAPRKRGRPRKIQEELTDRIKKDEKDTISSKKKRKLDKDTSGNVNEESKTSNNKQVMEKTGI
135  KEKREREKIQVATTTYEDNVTPQTDDNFVSNSPEPPEPATPSKKSLTTNHDFTSPLKQIIMNNLKEY
202  KDSTSPGKLTLSRNFTPTPVPKNKKLYQTSETKSASSFLDTFEGYFDQRKIVRTNAKSRHTMSMAPD
269  VTREEFSLVSNFFNENFQKRPRQKLFEIQKKMFPQYWFELTQGFSLLFYGVGSKRNFLEEFAIDYLS
336  PKIAYSQLAYENELQQNKPVNSIPCLILNGYNPSCNYRDVFKEITDLLVPAELTRSETKYWGNHVIL
403  QIQKMIDFYKNQPLDIKLILVVHNLDGPSIRKNTFQTMLSFLSVIRQIAIVASTDHIYAPLLWDNMK
470  AQNYNFVFHDISNFEPSTVESTFQDVMKMGKSDTSSGAEGAKYVLQSLTVNSKKMYKLLIETQMQNM
537  GNLSANTGPKRGTQRTGVELKLFNHLCAADFIASNEIALRSMLREFIEHKMANITKNNSGMEIIWVP
604  YTYAELEKLLKTVLNTL
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Fig. 5. The deduced amino acid sequence of *ORC2*. The genomic sequence has been deposited in Genbank (accession number L23924). Single-letter abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

one partial similarity was intriguing. Comparison of the entire 620-residue protein to the PIR29 database revealed that human DNA topoisomerase I showed the closest identity (8.69 standard deviations above the mean). The topoisomerase was 21 percent identical to Orc2p over the entire length of Orc2p if gaps were allowed in the alignment. The region of greatest similarity between Orc2p and human topoisomerase I was near the NH_2-terminus of both proteins, far from the catalytic domain of the topoisomerase. The region near the catalytic domain of topoisomerase I proteins has diverged among topoisomerase I proteins from other species except for the region surrounding the invariant active-site tyrosine. This region includes a consensus sequence consisting of a serine and lysine residue near the tyrosine (SKXXY) (*25*). The Orc2p protein also contained an SKXXY sequence near its COOH-terminus. However, mutation of this putative active-site tyrosine to phenylalanine had no detectable effect on the ability of *ORC2* to complement the temperature-sensitivity or mating defect of an *orc2-1* strain.

In vivo roles of ORC2. Genetic screens aimed at identifying genes involved in silencing have not found the genes encoding the proteins that bind to silencers. In large part, this difficulty has been due to functional redundancy in the silencer, a common theme in complex regulatory sites. In our study, a strain containing a genetically challenged *HMR-E* silencer was used to identify mutations affecting silencing that mapped to a gene not found in previous hunts for mutants. A prescreen for temperature-sensitive viability allowed a subset of the mutants to be chosen for further study. The properties of one of these genes, *ORC2*, is described below.

ORC2 defective mutants were recovered by their ability to cause derepression of *HMR* in two different strains. Strains with a RAP1 site mutation in the *HMR-E* silencer are only partially derepressed for *HMR* because of some degree of functional redundancy in *HMR-E* (*8*). Mutations in *ORC2*, combined with the mutation in the RAP1 binding site of *HMR-E*, resulted in complete derepression of *HMR*. In addition, the *orc2-1* mutation caused derepression of *HMR* flanked by a synthetic silencer lacking the functional redundancy found in a wild-type silencer. These data indicated that *ORC2* was an important contributor to silencing.

The connection of *ORC2* with the ORC complex came from the discovery that the sequence of the *ORC2* gene described here contained an exact match to the sequence of two peptides from the Orc2p. Moreover, the *orc2-1* mutation resulted in altered properties of the ORC complex in vitro (*16*), and hence was the strongest indication that the role of the Orc2p in silencing was through the role of the ORC complex at the ARS consensus sequence.

The properties of cells containing the *orc2-1* mutation offered strong support for a role for *ORC2* in DNA replication. On shifting an exponentially growing population of *orc2-1* cells from the permissive to the restrictive temperature, the population arrested with most of the cells containing large buds. This terminal phenotype is a hallmark of yeast *cdc* mutants involved in replication. Thus *ORC2* function was required for passage through the cell cycle. The other striking effect of *orc2-1* was on the mitotic stability of plasmids. When a plasmid-based colony sectoring assay was used, it was apparent that even at the permissive temperature, *orc2-1* mutants had a profound defect in plasmid mitotic stability. This assay did not distinguish an effect on replication from an effect on segregation. However, the results complement the flow cytometric analysis on *orc2-1* at the restrictive temperature described by Bell *et al.* (*16*) and fortify the connection between ORC and DNA replication.

One feature of the *ORC2* sequence was the presence of multiple matches to motifs recognized by a variety of protein kinases. The cell-cycle phenotype of *orc2-1* mutants and the presumptive role of ORC in replication raise the possibility that a subset of these sites may undergo phosphorylation in a cell cycle–regulated manner.

The connection between replication and silencing. As described above, there are several lines of evidence indicating that

Table 2. Strain list.

Strain	Genotype*	Reference †
DBY1034	*MAT***a** *his4-539 lys2-801 ura3-52 SUC2*	*(37)*
W303-1A	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*	*(38)*
W303-1B	*MATα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*	*(38)*
PSY152	*MAT***a** *his3Δ200 leu2-3,112 lys2-801 ura3-52*	*(39)*
JRY4130	*MATα his4 ura3 sec18*	*(40)*
JRY438	*MATα* Gal$^+$ *his4-519 leu2-3,112 SUC2 ura3-52*	
JRY543	*MAT***a**/*MATα ade2-101/ade2-101 his3Δ200/his3Δ200 lys2-801/lys2-801 met2/MET2 TYR1/tyr1 ura3-52/ura3-52*	
JRY2640	*mat***a***1 ade2 leu2-3,112 lys2-801 ura3*	
JRY2698	*MATα HMRα ade2-101 his3 leu2 trp1 ura3-52*	
JRY2699	*MATα HMRα ade2-101 his3 leu2 trp1 ura3-52 sir4ΔN::HIS3*	
JRY2700	*MATα HMRα ade2-101 his3 leu2 trp1 ura3-52* + pJR924	
JRY2903	*MATα HMRα ade2-101 his3 leu2 orc2-1 trp1 ura3-52*	
JRY2904	*MATα HMRα ade2-101 his3 leu2 orc2-1 trp1 ura3-52* + pJR924	
JRY3444	*MAT***a**/*MATα ade2-101/ade2-101 his3Δ200/his3Δ200 lys2-801/lys2-801met2/MET2 TYR1/tyr1 ura3-52/ura3-52 orc2::Tn10LUK/ORC2*	
JRY3683	*mat***a***1 {HMRα} ade2 his3 leu2 orc2-1ura3*	
JRY3685	*MATα HMR***a***-e-rap1-10 ade2 leu2 trp1 orc2-1 ura3*	
JRY3687	*MATα hmrΔA::TRP1 ade2 his3 leu2 trp1 ura3 orc2-1*	
JRY3690	*MATα HMR***a***-e-rap1-10 ade2 his3-11,15 leu2 orc2-1 trp1 ura3*	
JRY4125	*MATα ade2-1 can1-100 his3-11,15 leu2-3,112 orc2-1 trp1-1 ura3-1*	
JRY4132	*MATα HMR***a***-e-rap1-10 ade2 his3 ura3*	
JRY4133	*MATα HMR***a***-e-rap1-10 ade2 leu2 orc2-1trp1 ura3*	
JRY4134	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 ORC2::*pJR1423	
JRY4135	*mat***a***1 ade2 leu2-3,112 lys2-801 ura3 ste14*	
JRY4136	*mat***a***1 ade2 leu2-3,112 lys2-801 orc2-2 ura3 ste14*	
JRY4137	*mat***a***1 ade2 leu2-3,112 lys2-801 orc2-3 ura3 ste14*	
JRY4138	*mat***a***1 ade2 leu2-3,112 lys2-801 orc2-4 ura3 ste14*	

*Unless otherwise noted, all strains were *HMLα* and *HMR***a**. *HMR***a***-e-rap1-10* refers to the allele of *HMR-E*, originally described as *PAS1-1*, that contains a mutation in the RAP1 binding site (*21*). Strain 5401-1*a* is *MATα HMR***a***-e-rapl-10 ade2 trpl ura3* (*21*). †Unless referenced, all strains were constructed in the laboratory of J. Rine.

DNA replication and transcriptional silencing share some mechanistic connection. The results described here substantially strengthen the connection between replication and transcriptional silencing by establishing that a protein exerting its role through the ARS consensus sequence of *HMR-E* was likely to be functionally involved in DNA replication. Although one could imagine that *ORC2* itself may have more than one role in the cell, we have recently identified an allele of *ORC5* that has a silencing defect (*26*), indicating that the four independent alleles of *ORC2* with silencing defects was not due to a secondary role of a specialized domain of Orc2p. These results eliminated models involving two different proteins acting through the ARS consensus sequence of *HMR-E*. Thus it seems reasonable to propose that silencing itself requires the participation of proteins intimately connected to DNA replication.

There are three classes of models for a connection between replication and silencing. (i) Silencing requires the assembly of a complex at the silencer that involves the ORC complex for assembly, but does not require initiation of replication nor passage of a replication fork through *HMR*. (ii) Silencing requires assembly of a complex including ORC at *HMR-E* and initiation of replication, but not passage of a fork through *HMR*. (iii) Silencing requires assembly of a complex at *HMR-E* including ORC, initiation of replication, and the passage of a fork through *HMR-E*. Support for the first model comes from the existence of an ARS consensus sequence at *HML-E* bound by ORC (*16*), but with no detectable origin of replication (*27*). A limitation to these data is the difficulty of detecting low frequency firing at the origin, which might be sufficient for a role for replication in silencing. Support for the third model comes from the bidirectional spread of the silenced state on both sides of the origin for several kilobases (*28*). Bidirectional spreading of silencing is reminiscent of the bidirectional replication fork observed beginning at *HMR-E*. However, a replication fork itself cannot be sufficient to establish silencing since *HMR* is replicated even in the absence of the *HMR-E* silencer. Thus, the fork initiated at *HMR-E* would need to have a different property from forks initiated elsewhere if the third model proves correct.

Several major issues regarding the origin of replication at *HMR-E* and silencing remain unresolved. First, does the *orc2-1* mutation affect the frequency of origin firing at *HMR-E*? Models in which the initiation of replication is part of the link between replication and silencing demand that mutations in *ORC2* that affect silencing also decrease the frequency of origin firing. Second, is *ORC2* required for the establishment or the maintenance of the repressed state? Finally, there must be some feature of the *HMR-E* origin that endows it with the capability of silencing adjacent DNA. A molecular understanding of this ability will require elucidation of the connection between the SIR proteins and the proteins that bind the silencer.

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20. Two genetic screens were devised to identify ts mutations in essential genes involved in silencing. The screen that led to isolation of *orc2-1* started with JRY2698 (*HMLα, MATα, HMRα, ade2, his3, leu2, trp1, ura3-52*), which had α mating–type cassettes at all three chromosomal mating-type loci and was transformed with a plasmid (pJR924) containing the a mating-type cassette at *HMR* (JRY2700). The plasmid-borne *HMR*a locus had two synthetic silencers substituted for the *E* silencer, and also had a deletion of the *I* element. The use of two silencers rather than one minimized the risk of being distracted by site mutations in the silencer. Colonies (162,000) of ethyl methyl sulfonate (EMS)–mutagenized colonies were grown on supplemented minimal media (without uracil) at 25°C and screened for derepression of the plasmid-borne a cassette at *HMR*. Mutagenized colonies were replica-plated onto lawns of the mating tester strain DBY1034 (*MAT*a, *his4-539, lys2-801, ura3-52*) on minimal media either with or without uracil supplementation. Replicas were incubated for 1 hour at 25°C, and then overnight at 30°C. Only plasmid-containing JRY2700 cells were able to mate with the tester strain to yield diploids capable of growing on the unsupplemented plates because the only functional *URA3* gene was on the plasmid. Cells bearing mutations causing derepression of the plasmid-borne a cassette could be distinguished from the other classes of mutations by exploiting a feature of yeast plasmids. Approximately 10 percent of the cells in these colonies lacked the plasmid and thus could, in principle, mate with the tester strain and form Ura$^-$ diploids capable of growth on the plates supplemented with uracil. If a colony had a mutation in the mating response pathway, the cells would be unable to mate even in the absence of the plasmid, and thus would be unable to form diploids capable of growth on medium supplemented with uracil. Twenty-eight strains were identified that were temperature sensitive for growth and that mated with the tester strain only on plates supplemented with uracil. Plasmid-free isolates of each strain were again transformed with the plasmid bearing the synthetic silencer at the *HMR*a locus (pJR924) and with the plasmid bearing the wild-type *HMR*a locus (pJR919) (*17*). Three strains were able to mate when carrying the wild-type *HMR* locus (pJR919) but not when carrying the synthetic silencer-containing *HMR* locus (pJR924). In order to determine whether the ts growth phenotype and the mating phenotype were due to the same mutation, spontaneous revertants of the ts phenotype were selected. A spontaneous revertant of the ts growth of one strain, JRY2904, mated as well as the wild-type JRY2700, suggesting that the mating phenotype and ts growth were due to the same mutation which was named *orc2-1*.
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22. The *ORC2* gene was cloned by complementation of the temperature sensitivity of *orc2-1*. An *orc2-1* strain (JRY3683) was transformed with a CEN *LEU2*-based *S. cerevisiae* genomic library (*32*). Approximately 1000 to 1500 transformants formed colonies at 23°C. Replica prints of these colonies were incubated at 37°C to screen for the ability to grow at elevated temperatures. Plasmids were isolated from temperature-resistant strains and retested. Those plasmids that complemented the defect a second time were analyzed by restriction digestion. One plasmid from the CEN-*LEU2* library (pJR1416) was chosen for further analysis.
23. *ORC2* was disrupted with the *Tn10* LUK transposon (*33*), which inserted within the *ORC2* coding sequence on the plasmid (pJR1146) carrying the Sst I *orc2-1* complementing fragment. Plasmid pJR1147 had the *Tn10*LUK insertion within the *ORC2* coding region. The *ORC2*-containing Sst I fragment, disrupted by the transposon, was removed from pJR1147 by partial digestion with Sst I. The fragment was transformed into the wild-type diploid JRY543. The integration of this disruption allele at the *ORC2* locus was confirmed by DNA blot hybridization analysis (*34*), and the diploid was named JRY3444.
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30. A mutation was introduced into the RAP1 binding site at *HMR-E* adjacent to the *HMR*α locus by oligonucleotide-directed mutagenesis (*35*), and the change was confirmed by sequencing. The RAP1 site mutation was identical to the *PAS1-1* mutation of *HMR-E* characterized previously that blocks RAP1 protein binding in vitro (*21*), and is described here as *HMRα-e-rap1-10*. The plasmid

consisting of the *HMRα-e-rap1-10* Hind III fragment in pRS316 was named pJR1425. The wild-type *HMRα* version of the same plasmid was named pJR1426. Approximately 100,000 mutagenized cells from 12 independent cultures of the *HMLα mat*a*1 HMR*a *ste14* strain with the *HMRα* plasmid (pJR1425) were grown into colonies at 23°C and replica-plated to a *MAT*a *ura3* mating-type tester lawn (PSY152) culture to identify mutants exhibiting the α mating phenotype. The mating plates were incubated at 30°C in order to identify mutants defective enough to be derepressed at *HMR* yet not so defective as to be inviable. Of 900 haploid mating proficient colonies that were picked, 50 mutants were temperature sensitive for growth at 37°C to some degree. These mutants were subjected to further study and the remainder were discarded. All 50 mutants were recessive to wild type. Only the subset of mutants relevant to *ORC2* are presented with this research article.

31. The *ORC2* gene was defined by the *orc2-1* mutation. An *orc2*-complementing plasmid (pJR1416) was obtained by complementation of the temperature sensitivity of *orc2-1*. In order to map the approximate position of the *orc2*-complementing gene in the plasmid, six derivatives of pJR1416 were made and tested for complementation (Fig. 2). The Sal I–Sal I fragment was removed from the insert to yield pJR1418. Three adjacent Xba I–Xba I fragments were removed to yield pJR1422. Sph I cleaved once in the insert and once just inside the vector. Deleting this Sph I–Sph I fragment produced pJR1417. Cleavage by Sst I released two fragments from the insert. Deletion of both fragments created pJR1419. Isolates in which only the larger Sst I fragment (pJR1421) or only the smaller Sst I fragment (pJR1420) was deleted were also recovered. The 2.8-kb Sst I–Sst I *orc2*-complementing fragment was cloned into the Sst I site of the CEN *URA3* vector pRS316 (*36*) to yield pJR1263. Two plasmids were made that allowed the chromosomal integration of part or all of *ORC2*. The first, pJR1423, contained an Xho I–Kpn I insert (from pJR1416) that extended from a few kilobases upstream of the *ORC2* start codon to about 60-bp upstream of the stop codon inserted into Xho I–Kpn I–cut pRS306 (*36*), a yeast-integrating vector marked by *URA3*. The second plasmid, pJR1424, contained the Sst I *orc2*-complementing fragment inserted into the Sst I site of pRS306.
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Yeast Origin Recognition Complex Functions in Transcription Silencing and DNA Replication

Stephen P. Bell, Ryuji Kobayashi, Bruce Stillman

The genes encoding two of the subunits of the *Saccharomyces cerevisiae* origin recognition complex (ORC) have been isolated. Characterization of a temperature-sensitive mutation in the gene encoding the 72-kD subunit of ORC (*ORC2*) indicates that this protein complex functions early in the DNA replication process. Moreover, ORC derived from *orc2*^{ts} cells is defective for DNA binding. Others have shown a defect in *orc2*^{ts} cells in transcriptional silencing at the silent mating-type loci. Consistent with this finding, ORC specifically binds to each of the four mating-type silencers identified in yeast. These findings support the hypothesis that ORC acts as an initiator protein at yeast origins of DNA replication and suggest that ORC also functions in the determination of transcriptional domains.

Eukaryotic DNA replication requires the coordinate control of numerous origins of DNA replication spread over the length of each chromosome. Comparison of the estimated number of origins in a eukaryotic cell to the rate of the DNA replication fork movement and the length of S phase indicates that there are as many as ten times more replication origins than would be required to replicate the cellular DNA during S phase (*1*, *2*). These findings suggest that there may be additional functions for these sequences beyond that of directing the duplication of chromosomal DNA.

Transcriptional silencing at the yeast silent mating-type loci represents an example of a process that may be influenced by origin function. Four cis-acting elements (HMR-E, HMR-I, HML-E, and HML-I), as well as a number of trans-acting factors including the four SIR proteins (SIR1-SIR4), NAT1, ARD1, RAP1 proteins, and histone H4 are involved in this process (*3*). Each of the four cis-acting elements involved in the maintenance of transcriptional repression functions as an autonomously replicating sequence (ARS) in plasmids, and each contains a match to the ARS consensus sequence, indicating that each has the potential to function as an origin of replication in the chromosome. Only in the case of HMR-E, however, has it been established that the sequence acts as an origin of replication in its native chromosomal position (*4*, *5*). Several lines of evidence support a role for origin function in the repression of these sites. Studies of the establishment of the repressed state indicate that passage through S phase is a necessary step in this process (*6*, *7*). Mutations in HMR-E that prevent origin function in vivo also abolish silencing (*5*), and mutations in *CDC7*, a yeast gene required for the initiation of DNA replication, suppress silencing defects (*8*). To understand how origins of DNA replication may influence silencing and other gene regulatory events in eukaryotic cells requires an understanding of the basic components controlling these chromosomal elements.

The elements involved in the early events of eukaryotic DNA replication have begun to emerge in the yeast *Saccharomyces cerevisiae*. A critical first step was the identification of ARS elements derived from yeast chromosomes, a subset of which were subsequently shown to act as chromosomal origins of DNA replication (reviewed in *9*). Sequence comparison of a number of ARS elements resulted in the identification of the ARS consensus sequence (ACS) (*10*). This sequence is essential for the function of yeast origins of DNA replication (*5*, *10*, *11*). Three additional elements required for efficient ARS1 function have been identified (*12*). When mutated individually, these elements, referred to as B1, B2, and B3, result in a slight reduction of ARS1 activity. When two or three of the B elements are simultaneously mutated, however, *ARS1* function is severely compromised (*12*).

Proteins that recognize two elements of *ARS1* have been identified. The yeast transcription factor ABF1 binds to and mediates the function of the B3 element (*9*, *12*). The critical function of the B3 element appears to involve the localization of a transcriptional activation domain to the origin (*12*). More recently we have identified a multiprotein complex that specifically recognizes the highly conserved ACS (*13*). This activity, referred to as the origin recognition complex (ORC), has several properties that make it likely to be an initiator protein at

Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 11724.