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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38. Provided by R. Rothstein.
39. Provided by P. Schatz.
40. Provided by R. Schekman.
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Yeast Origin Recognition Complex Functions in Transcription Silencing and DNA Replication

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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

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yeast origins of replication. Binding of this complex requires the ACS, and the effect of mutations in the consensus sequence on *ARS1* function parallels the effect of the same mutations on ORC DNA binding. ORC binds to more than 10 yeast ARS elements, several of which are known origins of DNA replication (*13, 14*). Specific DNA binding by ORC requires adenosine triphosphate (ATP), suggesting that ORC binds ATP, a property of a number of initiator proteins (*15*). ORC also interacts with sequences outside of the ACS that are known to be important for ARS function (*14, 16, 17*). Further support for the hypothesis that ORC mediates the function of the ACS is provided by in situ deoxyribonuclease I (DNase I) footprinting experiments that identify a protected region of ARS1 remarkably similar to that observed with ORC in vitro (*18*).

To understand the role of ORC in vivo, we have begun to clone the genes encoding the subunits of this multiprotein complex. During this process, we found that the genes encoding the 50- and 72-kD subunits of ORC had been identified in two other laboratories (*19, 20*). The nature of the genetic screens and the properties of the mutations obtained confirm an in vivo role for ORC at the ACS and are consistent with ORC acting early in the DNA replication process. Moreover, the phenotype of the mutation isolated in the 72-kD subunit of ORC indicates that ORC is involved in transcriptional silencing at HMR-E (*19*). These findings provide strong in vivo support for the replicon model functioning at eukaryotic origins of replication (*21*) and suggest that DNA replication also plays an important role in the process of transcriptional control and determination of transcriptional states.

Transcriptional silencing and ORC. The presence of a match to the ACS at each of the mating type silencers, and their ability to function as ARS elements, suggested that ORC functioned at these sequences. To investigate this possibility, the binding of purified ORC to each of these four elements was tested with the use of a DNase I protection assay (*22*) (Fig. 1). Consistent with previous DNA binding studies (*13*), ORC protected the sequences that match the ACS at each of the four silencers in an ATP-dependent manner. At each silencer, characteristic hypersensitive sites of DNase I cleavage were observed that initiated 12 to 13 bp from the ACS. This pattern of DNase I protection and enhanced cleavage is nearly identical to that observed at nonsilencer sequences and indicates that ORC binding to these elements is not fundamentally different from ORC binding at other ARS elements.

At HML-E, HML-I, and HMR-E the only protection observed with purified ORC included the ACS. At HMR-I, however, we observed a second unexpected footprint that did not overlap a strong match to the ACS. Moreover, unlike all previous sites bound by ORC, this protection showed little dependence upon the addition of ATP to the binding reaction. Although there are two partial matches to the ACS in this region, similar sequences in other ARS elements and silencers were not recognized by ORC, suggesting that these sequences did not direct this unusual ATP-independent binding of ORC to DNA. This footprint was unlikely to be the result of contamination by another DNA binding activity. The ORC preparation used for these experiments was highly purified and the unusual binding activity cofractionated in a glycerol gradient with binding to the ACS and ORC protein (*14*). In combination with the protection observed at the ACS, the boundaries of the ORC footprint at HMR-I were similar to the boundaries of HMR-I defined by deletion mutagenesis (*23*). This finding also suggests the possibility that ORC can bind to sequences other than the ACS either through the same or alternative DNA binding domains present within the ORC polypeptides. These experiments demonstrate that ORC binds all four of the mating-type silencers and suggests that it plays an important role at *HML* and *HMR*.

The finding that a mutation in a gene encoding a subunit of ORC was defective for repression at *HMR* provided a link between ORC function and transcriptional silencing. To clone the genes encoding the various ORC subunits, peptides derived from each of the ORC subunits were sequenced (*24*). One candidate gene that might encode an ORC subunit, referred to as *ORC2*, was isolated by complementation of a temperature-sensitive mutation that showed silencing defects at the permissive temperature (*19*). Genetic experiments suggested that *ORC2* mediated the silencing function of the ACS at HMR-E, making it a good candidate to encode a subunit of ORC (*19*). Comparison of the predicted amino acid sequence of *ORC2* showed that all of the peptides derived from the 72-kD subunit of ORC were within the open reading frame of the *ORC2* gene, indicating that it encoded the second largest subunit of ORC (Fig. 2).

ORC2 mutations alter ORC function in vitro. To address the effect of *ORC2* mutations on ORC function in vitro, extracts were prepared from both *orc2-1* and *ORC2* strains (*25*). As expected, fractions derived from wild-type cells showed strong ORC DNase I protection over the A and B1 elements of ARS1 (Fig. 3, lane 2). In contrast, fractions derived from *orc2-1* cells showed a large reduction in ORC DNA binding activity. The A and B1 elements were no longer protected from DNase I cleavage. Only the characteristic enhanced

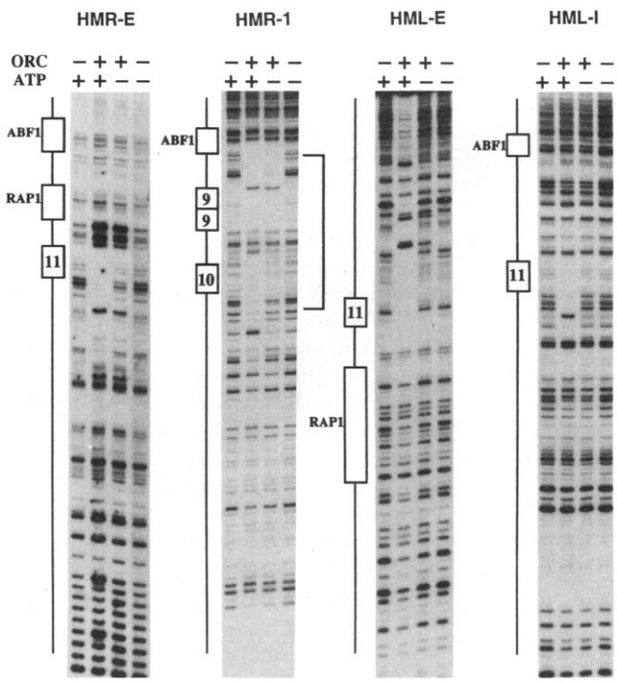

Fig. 1. ORC binds the four mating-type silencers. DNase I footprints were performed on each of the four mating-type silencers. The following DNA fragments were used for DNase I footprinting. In each case, the first position indicated is the labeled end of the fragment and the labeled strand of the ACS is indicated: HMR-E, position 500 to position 31 (A-rich strand); HMR-I, position 419 to position 171 (A-rich strand); HML-E, position 16 to position 159 (T-rich strand; this fragment also includes 180 base pairs of vector sequence beyond position 159); HML-I, position 318 to position −80 (A-rich strand). Numbering for HMR-E and HMR-I is according to (*43*), and numbering for HML-E and HML-I is according to (*23*). The addition of ORC (approximately 15 ng total) and ATP (10 μM final) to the footprinting reactions is indicated at the top of each panel. The location of the best matches to the ACS and binding sites for ABF1 and RAP1 at each of the silencers are indicated to the left of each footprint. In addition, the boundaries of HMR-I as determined by (*23*) are indicated to the right of the HMR-I footprint.

DNase I cleavages in the B domain of *ARS1* remained (lanes 3 and 4). Mutations that disrupt ORC DNA binding at *ARS1* prevented the residual DNA binding observed with the mutant fractions, indicating that this binding required the ACS (*14*). The DNA binding defects were not the result of a general inhibition of DNA binding because the mixing of mutant and wild-type fractions did not reduce binding of the wild-type protein (*14*). Incubation of the mutant cells at the nonpermissive temperature was not necessary to observe defects in ORC DNA binding, which may account for the defect observed in mating-type regulation at the permissive temperature (*19*).

To investigate the polypeptide composition of ORC derived from *orc2-1* and *ORC2* cells, immunoblots of these fractions were probed with polyclonal antibodies raised against ORC (*25*) (Fig. 4). Wild-type fractions contained the 120-, 72-, 62-, 56-, and 53-kD subunits of ORC in roughly equal quantity (the 50-kD subunit is not recognized by this serum). The mutant fractions, however, showed a distinctly different subunit composition. Whereas the amount of the 120- and 56-kD subunits was only slightly reduced relative to the wild-type fraction, the amount of the 72-, 62-, and 53-kD subunits was greatly reduced. In ultraviolet cross-linking experiments, the same three subunits are specifically cross-linked to DNA in an ACS- and ATP-dependent manner, which suggests an important role for one or more of these subunits in ORC DNA binding (*13*). Thus, the absence of these subunits is likely to be responsible for the defects in DNA binding observed in vitro and suggests that the *orc2-1* mutation results in a reduction of ORC stability. We cannot rule out, however, that a defect in Orc2p also results in reduced DNA binding of an intact ORC complex.

***orc2-1* cells are defective for entry into S-phase.** The availability of a temperature-sensitive mutation in the *ORC2* gene provided a valuable tool to address the role of ORC in vivo. In addition to the defects observed in silencing, *ORC2* mutant cells also exhibited a specific cell cycle arrest. At the nonpermissive temperature, *orc2*^{ts} mutant cells arrest with a single, large bud with the nucleus at the neck between the mother and the daughter cell (*19*) similar to that observed in other yeast DNA replication mutants (*9*). To further characterize the role of ORC in vivo, we asked at what point in the cell cycle the essential function of Orc2p is performed with the use of alpha factor and hydroxyurea (HU) as cell cycle landmarks (*26*). Our results were consistent with a requirement for Orc2p function between late G1 and the initiation of DNA synthesis (Table 1). Arrest with HU followed by release into the nonpermissive temperature resulted in 89 percent of the cells completing an additional cell cycle, indicating that the essential function for Orc2p was executed before the HU arrest point in the cell cycle. In contrast, blocking the cell cycle with alpha-factor followed by release at the nonpermissive temperature resulted in only 41 percent of the cells completing an additional cell cycle. This

```
        10         20         30         40         50
MLNGEDFVEH NDILSSPAKS RNVTPKRVDP HGERQLRRIH SSKKNLLERI

        60         70         80         90        100
SLVGNERKNT SPDPALKPKT PSKAPRKRGR PRKIQEELTD RIKKDEKDTI

       110        120        130        140        150
SSKKKRKLDK DTSGNVNEES KTSNNKQVME KTGIKEKRER EKIQVATTTY

       160        170        180        190        200
EDNVTPQTDD NFVSNSPEPP EPATPSKKSL TTNHDFTSPL KQIIMNNLKE

       210        220        230        240        250
YKDSTSPGKL TLSRNFTPTP VPKNKKLYQT SETKSASSFL DTFEGYFDQR

       260        270        280        290        300
KIVRTNAKSR HTMSMAPDVT REEFSLVSNF FNENFQKRPR QKLFEIQKKM

       310        320        330        340        350
FPQYWFELTQ GFSLLFYGVG SKRNFLEEFA IDYLSPKIAY SQLAYENELQ

       360        370        380        390        400
QNKPVNSIPC LILNGYNPSC NYRDVFKEIT DLLVPAELTR SETKYWGNHV

       410        420        430        440        450
ILQIQKMIDF YKNQPLDIKL ILVVHNLDGP SIRKNTFQTM LSFLSVIRQI

       460        470        480        490        500
AIVASTDHIY APLLWDNMKA QNYNFVFHDI SNFEPSTVES TFQDVMKMGK

       510        520        530        540        550
SDTSSGAEGA KYVLQSLTVM SKKMYKLLIE TQMQNMGNLS ANTGPKRGTQ

       560        570        580        590        600
RTGVELKLFN HLCAADFIAS NEIALRSMLR EFIEHKMANI TKNNSGMEII

       610        620
WVPYTYAELE KLLKTVLNTL
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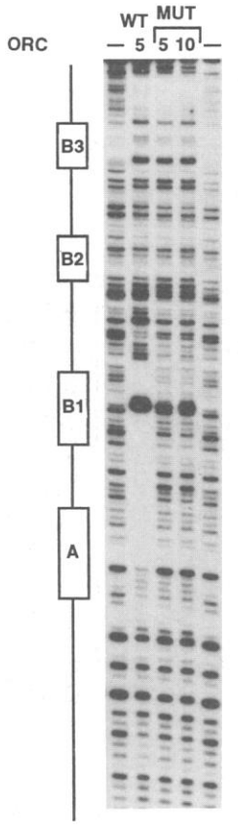
Fig. 2. Polypeptide sequence of the ORC2 protein. The sequence of the ORC2 protein is shown (*19*). Peptides derived from the 71-kD subunit of ORC are underlined. Boldfaced amino acids were correctly predicted by amino acid sequencing.



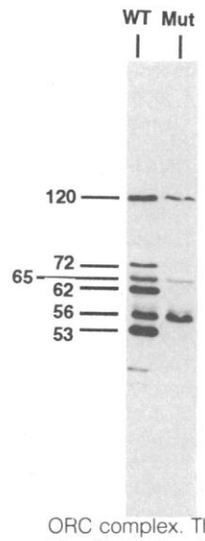


Fig. 3 (**left**). DNA binding by ORC derived from *orc2-1* cells. Partially purified ORC (see experimental procedures) derived from either JRY3688 (*ORC2*) or JRY3687 (*orc2-1*) yeast cells was used in DNase I footprinting of *ARS1* DNA. *ARS1* DNA was labeled with ^{32}P at position 926 and extended to position 734 [numbering according to (*44*)]. The number of micrograms of protein present in each ORC fraction used in the footprinting reaction is indicated above each lane. The elements of *ARS1* as defined in (*12*) are indicated to the left. **Fig. 4** (**right**). Polypeptide composition of ORC derived from *orc2-1* cells. 30 μg of partially purified ORC derived from either JRY3688 (*ORC2*) or JRY3687 (*orc2-1*) was separated on a 10 percent SDS-polyacrylamide gel and transferred to nitrocellulose. The resulting protein blot was incubated with polyclonal mouse sera raised against the entire ORC complex. This sera detects all but the 50-kD subunit of ORC. The 65-kD band is a breakdown product of the 120-kD subunit. Efforts to detect the 53-, 62- and 72-kD subunits in other fractions during the purification using either DNA binding assays or immunoblotting were unsuccessful. Antibody-antigen complexes were detected with horseradish peroxidase conjugated secondary antibodies followed by incubation with a chemiluminescent substrate.

phenotype suggests that the Orc2p function was performed at or near the G1-S phase boundary. The partial nature of the alpha factor phenotype could be the result of a number of factors including a nonuniform block with alpha factor or a requirement for new protein synthesis for the cell to express the temperature-sensitive phenotype.

To address the role of ORC in yeast DNA replication more directly, the DNA content of asynchronous cultures of either *orc2-1* or isogenic wild-type cells was measured by fluorescent cytometric analysis at various times after shifting from the permissive to the nonpermissive temperature (Fig. 5A) (*27*). In addition, a small number of cells (approximately 1000) from each time point were returned to the permissive temperature to determine the percentage of cells that remained viable at a given time point (Fig. 5B). Initially, the DNA content of both wild-type and mutant cells was equally divided between 1C and 2C with approximately 10 percent of the cells in S phase (between 1C and 2C; 0-minute time point). At early time points after the temperature shift (15 to 70 minutes), there was a dramatic loss of *orc2-1* cells in S phase, suggesting that entry into S phase had been halted. Consistent with this hypothesis, as the time course continued, the *orc2-1* mutant showed a rapid accumulation of cells with a 1C DNA content and a commensurate decrease in cells with a 2C DNA content (50 to 100 minutes). Between 100 and 120 minutes, a new population of *orc2-1* cells was observed that appeared to enter into a delayed S phase. By 150 minutes, the bulk of the mutant cells were in this population and after 180 minutes only a few cells remained with a 1C DNA content. We observe a strong correlation between entry into the new round of DNA synthesis and a loss of *orc2-1* cell viability (Fig. 5B). Similar experiments with isogenic ORC2 cells showed that these effects were specific to the *orc2-1* mutation (Fig. 5, A and B). These findings suggest that at the nonpermissive temperature the *orc2-1* cells were initially unable to enter S phase, but later entered into an abortive round of DNA replication. Entry into this type of replication appears to be a lethal event. Overall, the analysis of the *orc2-1* mutation provides strong in vivo evidence supporting the hypothesis that ORC acts early in S phase in general, and functions as the initiator protein at yeast origins of replication in particular.

Table 1. Functional ordering of the *orc2-1* mutation. JRY4170 cells (*orc2-1 MAT***a** *HMR***a** *ade2 his3 leu2 trp1 ura3*) were arrested with the indicated growth inhibitors and then released into media without inhibitors at the indicated temperature. Cells were then observed at regular intervals to determine the number of subsequent cell cycles performed at the given temperature. A dash indicates that less than 0.5 percent of the cells arrested at this stage. Control experiments in which cells were released into media at the permissive temperature indicate that these effects were not due to alpha factor or HU treatment alone.

Shift	Arrest in first cell cycle (%)	Arrest in second cell cycle (%)
HU ⇒ 37°C	11	89
HU ⇒ 24°C	5	–
α-F ⇒ 37°C	59	41
α-F ⇒ 24°C	4	–

Identification of the ORC6 gene. A second gene that represented a strong candidate to encode one of the subunits of ORC was the *AAP1* gene. This gene was cloned with the use of a novel screen for proteins that bound to the ACS in vivo (*20*). When compared to the predicted amino acid sequence of this gene, we found that all of the peptides derived from the 50-kD subunit of ORC were encoded by the open reading frame of the *AAP1* gene (*28*). For this reason, we now refer to *AAP1* as *ORC6*, because it encodes the smallest of the six ORC subunits. The identification of this gene as a subunit of ORC provides direct evidence that ORC is bound to the ACS in vivo.

A complete understanding of eukaryotic chromosomal replication requires the identification of the sequences that direct initiation of DNA replication and the proteins that mediate their function. In the yeast *Saccharomyces cerevisiae*, origins of DNA replication have been clearly delineated. However, the proteins that direct the early events at these sequences are not fully understood. In this study, we have taken the first steps towards an understanding of the in vivo role of ORC, which represents a strong candidate to act as an initiator protein at yeast origins of replication. The identification of the *ORC6* and *ORC2* genes both support the hypothesis that ORC binds to the ACS in vivo. Moreover, our findings using the *orc2-1* mutation indicate that ORC also functions early in the DNA replication process. The identification of a gene encoding a subunit of ORC in a screen for mutations that effect transcriptional silencing at the mating-type loci (*19*) indicates that ORC also plays a role in the regulation of gene expression in yeast cells.

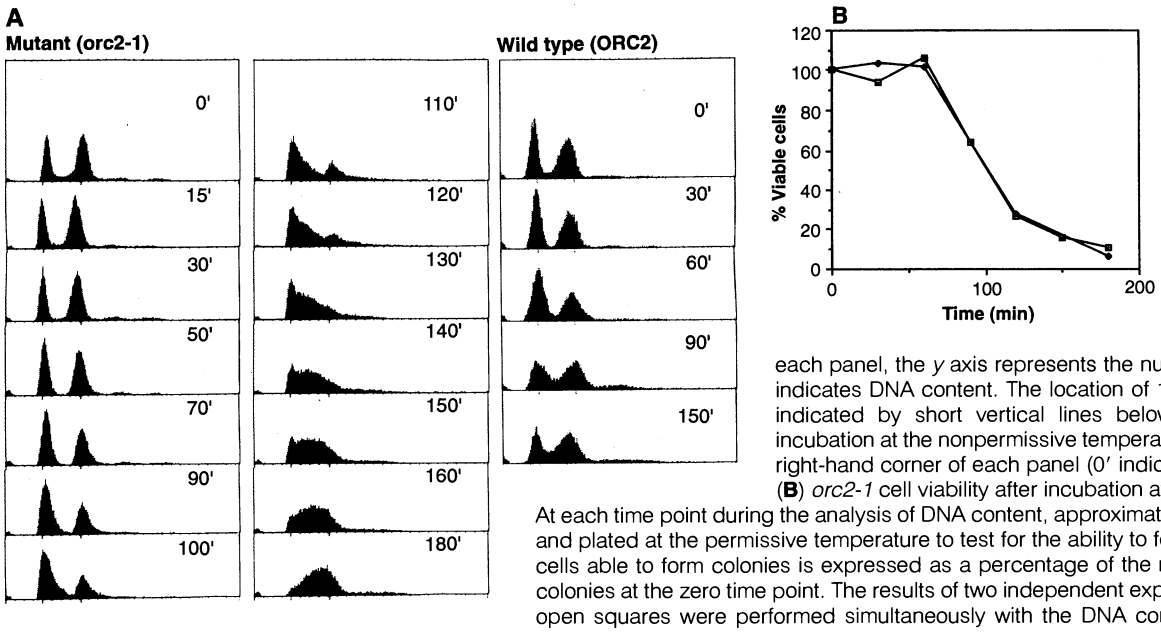


Fig. 5. DNA content of *orc2-1* cells. (**A**) JRY-3687 (*orc2-1*) or JRY-3688 (ORC2) cells grown at 24°C (0-minute time point) or at various times after shifting to the nonpermissive temperature (37°C) were fixed, stained with propidium iodide, and analyzed for DNA content with a Coulter Model Epics-C Flow Cytometer. For each panel, the *y* axis represents the number of cells and the *x* axis indicates DNA content. The location of 1C and 2C DNA content are indicated by short vertical lines below the *x* axis. The time of incubation at the nonpermissive temperature is indicated in the upper right-hand corner of each panel (0′ indicates culture grown at 24°C). (**B**) *orc2-1* cell viability after incubation at 37°C for the time indicated. At each time point during the analysis of DNA content, approximately 1000 cells were removed and plated at the permissive temperature to test for the ability to form colonies. The number of cells able to form colonies is expressed as a percentage of the number of cells able to form colonies at the zero time point. The results of two independent experiments are presented. The open squares were performed simultaneously with the DNA content analysis shown in (A). During the same time course, the number of *ORC2* cells (JRY3688) doubled in approximately 3 hours.

ORC is involved in yeast DNA replication. Whereas the in vitro characterization of ORC derived from *orc2* cells allows some insight into the defect caused by this mutation, the true value of a temperature-sensitive mutation is its ability to shed light on the in vivo role of the mutated protein. Initial characterization of the *orc2* mutations, including the arrest point during the cell cycle and the essential nature of the complementing gene, were consistent with this hypothesis (*19*). The reduced plasmid stability observed in *orc2-1* strains and the identification of an early S phase execution point of the ORC2 gene are also consistent with a replicative role for ORC in yeast cells. Analysis of the DNA content of *orc2-1* cells after incubation at the nonpermissive temperature provide compelling in vivo evidence that ORC is indeed required to play a positive role in the initiation of DNA replication.

Whereas these studies provide strong evidence in favor of a requirement for ORC in the DNA replication process, the precise function of ORC is not yet known. Because ORC specifically binds to the only sequence conserved at all yeast origins of DNA replication, at the very least ORC is likely to mark these sites in the chromosome. The involvement of six proteins in this complex, however, suggests that ORC also performs other functions at the origin. By analogy to known initiator proteins, such functions could include unwinding the DNA at the origin of replication or directing the assembly of other DNA replication proteins at the origin or both (*15*). The complex nature of eukaryotic chromosomal replication suggests a number of other possible functions for ORC, including the localization of origins of replication within the nucleus, assisting the chromatin assembly process, or maintaining a chromatin-free region at the origin of replication. ORC also represents a likely target for cell cycle regulators that control the G1 to S phase transition and the replication of the genome once per cell cycle. Further genetic analysis of the genes encoding the subunits of ORC as well as continued biochemical analysis of ORC should provide insights into both the functions of ORC during yeast DNA replication and the biochemical mechanisms through which they are exerted.

In addition to the defects in the entry into S phase, at later time points in the analysis of the DNA content of the *orc2-1* cells at the nonpermissive temperature a new population of cells is observed that has entered into a new round of DNA replication. Entry into this abortive S phase was linked to a loss in cell viability. The cells were also clearly delayed in the entry into this abortive DNA synthesis relative to entry into normal DNA replication, suggesting an adaptive process occurs before the cells are competent to continue DNA synthesis. Although it is possible this new round of DNA synthesis is due to leakiness in the *orc2-1* mutation, the close correlation between new DNA synthesis and cell death and the complete loss of S phase cells observed early in the time course suggest otherwise. Mutations in the *CDC46* gene show a similar FACS profile (*14, 29*) and also show genetic interactions with the *ORC6* gene (*20*). These findings, together with the likelihood that ORC is bound to the ACS throughout the cell cycle (*18*), suggest that ORC not only acts positively to initiate DNA replication but also acts to prevent inappropriate DNA synthesis in the cell, possibly in concert with CDC46 protein.

ORC and transcriptional silencing. There are several possible roles for ORC in the process of transcriptional silencing. One possibility is that the involvement of ORC is related to a critical role for DNA replication during the silencing process. If this is the case, it is not simply the passage of a replication fork through this region that is important for silencing but the actual initiation of replication at the silencer that is crucial for setting up the repressed state. It is possible that the origins associated with the silencers are important because they direct this region of the chromosome to replicate at a particular time during S phase. Alternatively, one or more of the events involved in the initiation of DNA replication may be modified during the establishment of the repressed state, possibly resulting in the alternative chromatin structure observed at the *HM* loci (*3*). The transcriptionally repressed regions observed adjacent to the telomeres in yeast, which utilize many of the same components as silencing at the mating-type loci (for example, SIR3 protein), do not necessarily include either ARS elements or origins of DNA replication (*30*). Thus, if replication is required for transcriptional silencing at the *HM* loci, a distinct mechanism must be used at telomeric sites to establish the repressed state.

An alternative explanation for the involvement of ORC in the silencing process is that ORC does not play a replicative role at the silencers but instead is recognized by other proteins (for example, SIR1-4 proteins) and merely acts to tether these silencing factors to the appropriate site in the chromosome. Such a model could still account for the requirement for passage through S phase to establish silencing if ORC is only accessible to these factors at a certain stage in the cell cycle or if disassembly of the chromatin is required to establish the silenced state. This mechanism is also consistent with the lack of origin function observed at HML-E and HML-I (*4*). If this model is correct, there must be other factors in addition to ORC DNA binding that distinguish the silencer loci from other ORC binding sites. Studies of the HMR-E silencer indicate that ABF1 and RAP1 represent these additional factors (*31, 32*), but binding of these factors near ORC is not sufficient to form a silencer in yeast. Both the silencer at HML-I and the well-studied *ARS1* origin of replication include a binding site for both ABF1 and ORC, but HML-I acts as a transcriptional silencer whereas *ARS1* does not.

Early studies of eukaryotic DNA replication found that there were far more origins of DNA replication than would be required to replicate the genome based upon estimated rates of fork movement (*1, 2*). The evidence suggesting a role for origins of DNA replication in the process of transcriptional silencing may begin to shed light on the reasons for the apparent excess of origins. It is possible that beyond the primary role of initiating the DNA replication process, these chromosomal elements also are involved in directing or assisting in the formation of transcriptional domains at eukaryotic chromosomes (*33, 34*). A close correlation between the timing of DNA replication and the level of expression of nearby genes has been observed, with active genes generally replicated early in S phase and inactive genes replicated later (*35–37*). Passage through S phase has also been implicated as an important step in changing the developmental program of individual cells during embryogenesis (*38, 39*). The identification of ORC as a player in the process of transcriptional silencing in yeast will allow a more detailed analysis of the connection between the function of origins and their role in the regulation of gene expression.

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22. DNase I footprinting was performed as described (*13*). Binding reactions with purified ORC contained only 10 μM ATP, no creatine phosphate, and no creatine phosphokinase. The vectors containing the fragments used were as follows: *ARS1* DNA, p*ARS1*/WT; HMR-E DNA, pJR315; HMR-I DNA, pSK/HMR-I; HML-E DNA, p119/HML-E; HML-I DNA, Δ637.
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24. To obtain sufficient protein for peptide sequencing, a revised purification procedure for ORC was devised, based on the procedure reported previously (*13*). Whole-cell extract was prepared from 400 g of frozen BJ926 cells using a bead beater (Biospec Products) until greater than 90 percent breakage was achieved. One twelfth volume of a saturated (at 4°C) solution of ammonium sulfate was added to the broken cells and stirred for 30 mins. This solution was then spun at 13,000*g* for 20 minutes. The resulting supernatant was spun in a 45 Ti rotor (Beckman) at 44,000 rpm for 1.5 hours. 0.27 g/ml of ammonium sulfate was added to the resulting supernatant, and the resulting precipitate was collected by spinning in the 45 Ti rotor at 40,000 rpm for 30 minutes. The resulting pellet was resuspended in buffer H/0.0 (*13*) and dialyzed versus H/0.15 M KCl (H with 0.15 M KCl added). Preparation of ORC from this extract was essentially as described (*13*) with the following changes. The double-stranded DNA cellulose column was omitted from the preparation and only a single glycerol gradient was performed. Sequencing of peptides derived from ORC subunits was performed using a modification of an in gel protocol described previously (*40, 41*). Purified ORC (~10 μg per subunit) was separated by SDS–polyacrylamide gel electrophoresis (PAGE) and stained with 0.1 percent Coomassie brilliant blue G (Aldrich). After destaining, the gel was soaked in water for 1 hour. The protein bands were excised, transferred to a microcentrifuge tube, and treated with 200 ng of *Achromobacter* protease I (Lysylendopeptidase: Wako). The resulting peptides were separated by reverse-phase chromatography and sequenced by automated Edman degradation (Applied Biosystems model 470).
25. To isolate and assay ORC from *ORC2* and *orc2-1* cells, four liters of JRY3687 (*orc2-1, MATα, hmrΔA::TRP1 ade2 his3 leu2 trp1 ura3*) or the isogenic wild-type strain JRY3688 (*ORC2 MATα, hmrΔA::TRP1 ade2 his3 leu2 trp1 ura3*) were grown to a density of 2×10^7 cells per milliliter. Extracts were prepared as described (*24*) and fractionated over the first two columns in the preparation of ORC. The peak fraction of ORC DNA binding activity eluted from the Q-Sepharose (Pharmacia) column of each preparation was used for subsequent analysis. Antibodies were raised against the entire ORC complex with the use of a single mouse. The resulting sera was able to recognize all but the 50-kD subunit of ORC. Proteins were transferred to nitrocellulose and antigen-antibody complexes were detected with horseradish peroxidase conjugated secondary antibodies and a chemiluminescent substrate.
26. Yeast cells were grown to a density of 1×10^7 to 4×10^7 cells per milliliter at 24°C, then diluted to a density of 2×10^6 to 4×10^6 cells per milliliter into YPD containing 6 μM alphafactor and incubated for 2 to 2.5 hours at 24°C (>90 percent unbudded cells). For the hydroxyurea arrest experiments, alpha factor was washed away and the cells were resuspended in YPD containing 100 mM hydroxyurea and incubated an additional 2.5 hours (>90 percent large budded cells). After incubation with the growth inhibitor, cells were briefly sonicated and plated on YPD plates preincubated at either 24°C or 37°C and observed at 0, 3, and 6 hours after plating.
27. Yeast cells were grown to a density of 1×10^7 to 4×10^7 cells per milliliter at 24°C and diluted into fresh YPD at either 37°C or 24°C and a density of 2×10^6 to 4×10^6 cells per milliliter. At various times after dilution, 3×10^6 cells were removed and processed as described (*42*).
28. The position of the five peptides derived from the 50-kD subunit of ORC in the *ORC6* gene are as follows: 51 QDEEVARCHICAYIA 65; 91 AKHLMNLFRQSL 102; 110 QFAWTP 105; 207 SITGTRNVDSDEYENHESDP 226; 424 RIEMDLA 430. The two cysteines in the first peptide sequence and the arginine in the second peptide could not be read.
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