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Separable Functions of *ORC5* in Replication Initiation and Silencing in *Saccharomyces cerevisiae*

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ABSTRACT

Origin recognition complex (ORC) is a six subunit complex that functions as the replication initiator and is required for silencing the *HML* and *HMR* loci in the yeast *Saccharomyces cerevisiae*. The roles of *ORC5* in replication initiation and silencing were investigated to determine whether the two roles were mechanistically coincident or separable. Some spontaneous revertants of *orc5-1* were functional for replication initiation, but not silencing. Other alleles of *ORC5* were obtained that were nonfunctional for replication initiation, but fully competent for silencing. The two types of alleles, when put in the same cell, complemented, establishing two separable functions for *ORC5*. These data implied that replication initiation at *HMR-E* was not required for silencing. The data were consistent with a model in which different ORC species functioned at different origins within the genome and that only one Orc5p subunit functioned at any given origin.

THE origin recognition complex (ORC) is a six polypeptide complex that binds to the ARS consensus sequence found at all known origins of replication in the yeast *Saccharomyces cerevisiae*. Temperature-sensitive (ts) mutations in *ORC* genes block replication initiation at origins of replication at the restrictive temperature and, in the case of *orc2-1*, cause cells to arrest in the cell cycle at the G_1/S boundary with unreplicated DNA (Bell *et al.* 1993; Fox *et al.* 1995; Liang *et al.* 1995; Loo *et al.* 1995). *In vivo*, mutations in *ORC2* and *ORC5*, which encode the second and fifth largest ORC subunit, respectively, are defective in replication initiation at several origins of replication, including the origin found at the *HMR-E* silencer (Fox *et al.* 1995). Mutations in *ORC* genes derepress the mating type genes at *HML* and *HMR*, implying a link between DNA replication and silencing (Foss *et al.* 1993; Loo *et al.* 1995).

Silencing is a form of gene regulation that involves the assembly of certain regions of the genome into an inactive chromatin structure, which blocks gene expression. In *S. cerevisiae*, silencing blocks expression of the mating type genes at *HML* and *HMR*, and blocks gene expression at telomeres (Rine and Herskowitz 1987; Gottschling *et al.* 1990). The silent domain of *HMR* is inaccessible to nucleases, RNA polymerases, and DNA methylases (Schnell and Rine 1986; Singh and Klar 1992; Loo and Rine 1994). In addition to ORC, silencing depends upon regulatory sites, known as silencers, which flank *HML* and *HMR*, the silencer binding proteins Rap1p and Abf1p and the four *SIR* genes (reviewed in Loo and Rine 1995). Moreover, mutations in the genes encoding either histone H3 or histone H4 cause derepression of both *HMR* and *HML* loci, indicating that silencing involves an inactive structure of chromatin (Kayne *et al.* 1988; Johnson *et al.* 1990; Park and Szostak 1990; Thompson *et al.* 1994).

Both *HML* and *HMR* are flanked by a pair of silencers, with the *E* silencer on the left and the *I* silencer on the right of both loci. *HMR-E*, the most thoroughly studied of the four silencers, contains three functional domains. Two of the domains correspond to binding sites for two well studied transcription factors: Abf1p and Rap1p. The third domain corresponds to a binding site for ORC. A synthetic silencer composed of only these three domains is fully capable of silencing expression of flanking genes (McNally and Rine 1991).

Both the *E* and *I* silencers at *HMR* are bona fide origins of replication, suggesting a link between DNA replication and silencing (Rivier and Rine 1992; D. Rivier and J. Rine, unpublished data; reviewed in Dillin and Rine 1995). However, several observations already hint that the relationship between DNA replication initiation and silencing is not simple. First, ORC is required outside of S phase for silencing, implying that its role in silencing is not restricted to ORC's role in replication initiation (Fox *et al.* 1995). Second, tethering Sir1p to synthetic silencers bypasses the need for ORC in silencing (Triolo and Sternglanz 1996; Fox *et al.* 1997). Third, expression of the *ORC2* gene of *Drosophila melanogaster* in a yeast *orc2-1* mutant complements the silencing defect but not the replication defect of *orc2-1* (Ehrenhofer-Murray *et al.* 1995). Finally, like *orc2-1*, the *orc5-1* mutation decreases initiation of replication at *ARS1*, a bona fide origin of replication (Liang *et al.* 1995). However, unlike *orc2-1*, which causes a simi-

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TABLE 1

Strains used in this study

Strain	Genotype
W303-1B	*MATα ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100* (R. Rothstein)[a]
JRY2726	*MAT***a** *his-4*
JRY4154	W303-1B *ssΔI orc5Δ* pRS316-*ORC5*
JRY4253	W303-1B *ssΔI orc5-1*
JRY4555	W303-1B *ssΔI orc5-R1*
JRY5297	JRY4253 *HIS3::ORC5::his3-11,15*
JRY5298	JRY4253 *HIS3::orc5-1::his3-11,15*
JRY5299	JRY4253 *HIS3::orc5-R1::his3-11,15*
JRY5300	JRY4253 *HIS3::orc5-R3::his3-11,15*
JRY5301	JRY4253 *HIS3::orc5-S1::his3-11,15*
JRY5302	JRY4253 *HIS3::orc5-S2::his3-11,15*
JRY5303	JRY4253 *HIS3::orc5-S3::his3-11,15*
JRY5304	JRY4253 *HIS3::orc5-S4::his3-11,15*
JRY5305	JRY4555 *HIS3::ORC5::his3-11,15*
JRY5306	JRY4555 *HIS3::orc5-1::his3-11,15*
JRY5307	JRY4555 *HIS3::orc5-R1 his3-11,15*
JRY5308	JRY4555 *HIS3::orc5-S1::his3-11,15*
JRY5309	JRY4555 *HIS3::orc5-S2::his3-11,15*
JRY5310	JRY4555 *HIS3::orc5-S3::his3-11,15*
JRY5311	JRY4555 *HIS3::orc5-S4::his3-11,15*

[a] Except for this strain, the strains listed in this table were from the laboratory collection or were constructed for experiments in this study.

lar reduction in replication initiation at the *HMR-E* origin, *orc5-1* causes only a modest reduction in replication initiation at *HMR-E* (Fox *et al.* 1995). Collectively, these data suggest that ORC may have separable roles in silencing and replication. We tested this hypothesis by determining whether the replication and silencing roles of *ORC5* were genetically separable.

MATERIALS AND METHODS

Strains and genetic methods: The genotypes of strains used in this work are in Table 1. 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). *ORC5* alleles were cloned into the multiple cloning site of pRS403, a *HIS3* integrating vector (Sikorski and Hieter 1989). The resulting plasmids were then cleaved within the *HIS3* gene with *Apa*I to direct integration into the *his3-11* locus. Integration was confirmed by DNA-blot hybridization of genomic DNA isolated from the transformants with a 1-kb fragment unique to *HIS3* as a probe (Hoffman and Winston 1987). An identical blot was also probed with a 1.2-kb fragment unique to *ORC5*.

Isolation of replication-competent, silencing-defective alleles of *ORC5*: Ten independent cultures of an *orc5-1* strain (JRY4253) were grown at 23° to an A_{600} = 1.0 and plated onto individual rich medium plates at 1×10^8 cells per plate. Three to seven temperature-resistant revertants were recovered from each plate following incubation at 37° for 2 days after double replica-plating. Four independent intragenic revertants were analyzed in the experiments reported here.

All four alleles were cloned by gap-repair using a plasmid that contained *ORC5*, cleaved at the *Sph*I and *Eco*NI sites internal to *ORC5*. *orc5-R3* was cloned using the same plasmid, but missing a *Sna*BI-*Sna*BI fragment that removed the 5′ end of the *ORC5* coding sequence. Plasmids were recovered and transformed into an *orc5-1* strain and tested for viability at 37° and for silencing at 23°. Plasmids able to restore viability, but not silencing were sequenced (Figure 3).

Site-directed mutagenesis of leucine 380: Site-directed mutagenesis of a uracil-substituted single-stranded *orc5-1* plasmid was used as a template for mutagenesis (Kunkel *et al.* 1987). Primer 5′ CAGCCTTCTNNATTTGCTATTGAAG 3′ was used to create mutations at codon 380. N refers to any of the four possible nucleotides. Aliquots from the mutagenesis reaction were used to transform an *orc5-1* strain (JRY4253). Transformants were grown at 23°, replica-plated and incubated overnight at 37°, and then replica-plated and placed for an additional night at 37°. Transformants were also screened for their ability to mate at 23°. Random transformants were also isolated, plasmid DNA extracted, purified and sequenced to identify all possible mutant alleles that could be generated by the degenerate primer.

Isolation of replication-defective, silencing-competent alleles of *ORC5*: Mutant *ORC5* alleles were created using the cloned *ORC5* gene and the primers 5′ CCAACTGTCTCG CATCGTATATTTCTGCTGATCC 3′ and 5′ CCACCTGACT TTAGGACTCAAATAGTCG 3′ in an error-prone PCR reaction with limited amounts of dATP. The relative position of these primers on the *ORC5* gene is depicted in Figure 3. The products of six independent PCR reactions were gel purified and used to transform an *orc5-1* strain along with a gapped plasmid containing *ORC5* missing its unique *Sph*I-*Eco*NI fragment (Muhlrad *et al.* 1992). Approximately 200 transformants per plate were replica-plated onto a lawn of *MAT***a** cells and a 37° YPD plate. Colonies that grew at 37° were replica-plated to a second plate that was incubated an additional day at 37°. Transformants unable to complement *orc5-1* at 37°, but capable of mating at 23°, were isolated and further characterized. Approximately 16,000 transformants were screened in this manner.

Noncomplementation of *orc5Δ*: *ORC5* alleles on a *CEN/TRP1* plasmid were transformed into an *orc5Δ* strain harboring a *CEN/URA3* plasmid containing *ORC5* (JRY4154). Transformants were selected on minimal medium (YM) lacking tryptophan and uracil (YM -trp -ura). Transformants were picked and patched onto YM -trp medium and grown for 2 days at 23°. After 2 days the patch was streaked onto YM -trp medium with or without 5′FOA and allowed to grow for 3 days at 23°. Strains unable to grow on 5′FOA-containing medium were judged to have a mutation in the *ORC5* gene carried on the *TRP1*-marked plasmid.

Quantitative mating assays: *MATα* cells with the mutation of interest were grown into log phase in rich medium supplemented with adenine and mixed with 1.2×10^7 cells of **a** mating-type tester strain (JRY2726) in 0.3 ml YPD. The cell suspensions were plated onto minimal plates and grown at 23°. Dilutions of the tested strain were also plated onto fully supplemented minimal plates to determine the number of viable cells. Mating efficiencies were calculated as the number of prototrophic diploid colonies formed per viable cell. Results were presented as the mean of three independent experiments.

Plasmid loss-rates: Transformants were grown into early log phase in liquid minimal medium selecting for the plasmid of interest. Aliquots were then innoculated into liquid rich medium supplemented with adenine 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 minimal medium either containing or lacking the supplement needed for growth in the absence of the plasmid, and dividing the number of colonies

TABLE 2

Plasmid loss-rates of *orc5* strains

Strain	Relevant genotype	*ARS1* relative loss-rate	*SS* relative loss-rate
JRY5297	*orc5-1/ORC5*	1.0	1.0
JRY5298	*orc5-1/orc5-1*	3.4	19
JRY5299	*orc5-1/orc5-R1*	1.2	6.8
JRY5300	*orc5-1/orc5-R3*	1.0	4.1
JRY5301	*orc5-1/orc5-S1*	3.4	27
JRY5302	*orc5-1/orc5-S2*	2.3	29
JRY5303	*orc5-1/orc5-S3*	1.6	24
JRY5304	*orc5-1/orc5-S4*	5.3	16
JRY5305	*orc5-R1/ORC5*	1.0	1.0
JRY5306	*orc5-R1/orc5-1*	1.6	1.8
JRY5307	*orc5-R1/orc5-R1*	1.2	4.0
JRY5308	*orc5-R1/orc5-S1*	0.6	9.6

Relative loss-rate of plasmids replicated by *ARS1* or the synthetic silencer. Relative loss-rate was calculated for strains indicated in Figure 4 (JRY5297-JRY5304) and normalized to the loss-rate of each plasmid in a haploid *orc5-1* strain containing a wild-type *ORC5* allele at *HIS3* (JRY5297). Relative loss-rate was also calculated for strains indicated in Figure 9 (JRY5305-JRY5308) and normalized to the loss-rate of each plasmid in a haploid *orc5-R1* strain containing a wild-type *ORC5* allele at *HIS3* (JRY5305).

TABLE 3

Mating efficiencies of *ORC5* alleles

Strain	Relevant genotype	Mating efficiency	SD
JRY5297	*orc5-1/ORC5*	3.1×10^{-2}	1.2×10^{-2}
JRY5298	*orc5-1/orc5-1*	3.0×10^{-4}	6.2×10^{-5}
JRY5299	*orc5-1/orc5-R1*	2.9×10^{-5}	1.4×10^{-5}
JRY5300	*orc5-1/orc5-R3*	1.3×10^{-5}	6.5×10^{-6}
JRY5301	*orc5-1/orc5-S1*	2.2×10^{-2}	2.4×10^{-3}
JRY5302	*orc5-1/orc5-S2*	2.6×10^{-2}	8.2×10^{-4}
JRY5303	*orc5-1/orc5-S3*	2.5×10^{-2}	9.4×10^{-4}
JRY5304	*orc5-1/orc5-S4*	2.3×10^{-2}	4.7×10^{-4}
JRY5305	*orc5-R1/ORC5*	2.4×10^{-2}	4.5×10^{-3}
JRY5306	*orc5-R1/orc5-1*	2.9×10^{-4}	1.2×10^{-4}
JRY5307	*orc5-R1/orc5-R1*	4.6×10^{-6}	7.8×10^{-7}
JRY5308	*orc5-R1/orc5-S1*	1.9×10^{-2}	3.6×10^{-3}

Quantitative mating assays were performed on the strains containing an *orc5-1* allele at the *ORC5* locus and an additional *orc5* allele integrated at the *HIS3* locus, described in Table 1 (JRY5297–JRY5304). Quantiative mating efficiencies were also calculated for strains containing an *orc5-R1* allele and an additional *orc5* allele integrated at *HIS3* (JRY5305–JRY5308). Data were mean values from three independent experiments carried out in parallel at 23°.

on selective medium by the total number of colonies on nonselective medium. After ~13 doublings at 23°, 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 (McNally and Rine 1991). Results presented in Table 2 and Table 3 were the results of three independent experiments carried out in parallel.

Two-dimensional origin analysis: The analysis of replication intermediates was performed essentially as described. (Brewer and Fangman 1987; Rivier and Rine 1992).

RESULTS

Reversion analysis of *orc5-1*: One approach to testing whether the silencing and replication functions of ORC were separable was to determine whether both phenotypes co-revert. Ten independent cultures of an *orc5-1* strain (JRY4253) were plated at 37° to select for temperature-resistant revertants (Figure 1A). Temperature-resistant isolates from each culture were crossed to a wild-type strain of the opposite mating type to determine which revertants were intragenic and which were extragenic. The six revertants in this study were judged to be intragenic by the absence of any temperature-sensitive recombinants among 20 tetrads from each cross.

Of the six intragenic revertants of *orc5-1*, two restored both the ability to grow at 37° as well as silencing of *HML* and *HMR*, and hence were not studied further. The remaining four mutations, designated *orc5-R1*, *orc5-R2*, *orc5-R3*, and *orc5-R4*, restored growth at 37° but did not restore silencing as judged by the non-mating phenotype that these revertants shared with the original *orc5-1* mutation (Figure 2). These alleles are referred to collectively as *orc5-R* to reflect their replication competence.

Characterization of the *orc5-R* alleles: To identify the change in *orc5-1* responsible for these partial revertants, the mutant alleles were cloned onto a plasmid by gap repair, and the recovered alleles were sequenced (Orr-Weaver *et al.* 1983). The original *orc5-1* allele was a change of a leucine to a proline at position 331 (L331P). All four revertants were due to changes in nearby amino acids. *orc5-R3* resulted from a change of serine 335 to alanine (S335A). Remarkably, the remaining three alleles all resulted in the identical substitution of a leucine at position 380 to serine (L380S) even though each revertant was independent (Figure 3). In the absence of L331P, neither S335A nor L380S mutations had any discernable phenotype with respect to either replication or silencing (data not shown).

The isolation of three independent yet identical changes at position 380 was surprising in that the Leu380 codon, TTA, could mutate to codons for a variety of different amino acids by a single base change. However, nearly all such changes would result in the substitution of a leucine hydrophobic side group for a different hydrophobic side group. The leucine to serine change is the only qualitatively different amino acid substitution that can be achieved at this codon with a single nucleotide substitution. To determine whether other changes at this position in the Orc5p could also restore viability to an *orc5-1* strain, site-directed mutagenesis with a degenerate oligonucleotide was used to

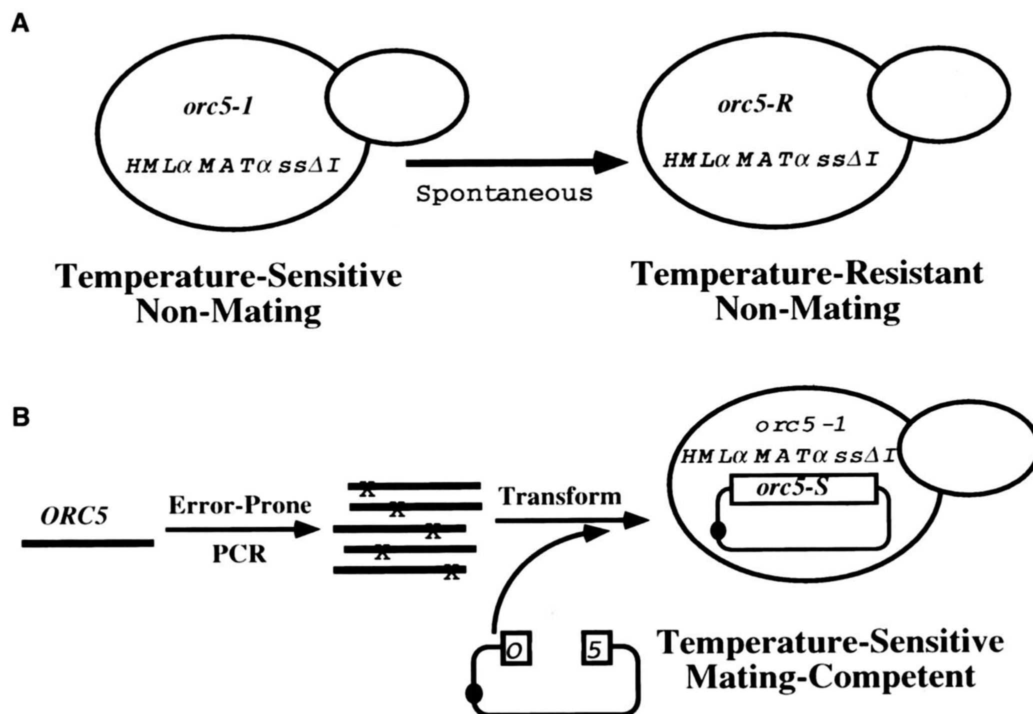

FIGURE 1.—A schematic representation of the genetic screens used to identify *orc5-R* and *orc5-S* alleles. The *orc5-R* alleles were found as spontaneous temperature-resistant revertants of an *orc5-1* strain (JRY4253). Revertants were isolated based on their ability to grow at 37° and were subsequently assayed for their ability to silence *HMR* flanked by the synthetic silencer and without the I silencer (*ss*Δ*I*). To identify *orc5-S* alleles, *ORC5* was mutagenized by error-prone PCR and the products, along with a gapped plasmid whose ends were homologous to *ORC5,* were used to transform the same *orc5-1* strain (Figure 1B). Transformants unable to grow at 37° but able to mate at 23° were chosen for further study.

create a variety of amino acid substitutions at position 380 on an *orc5-1* template. Change of leucine 380 to amino acids with either a hydrophobic side group, alanine, valine, or isoleucine; a positively charged group, lysine, glutamine or arginine; a negatively charged group, glutamic acid or aspartic acid; a polar group, threonine; or changes to glycine or a stop codon resulted in no change in phenotype; *orc5-1* mutant cells containing any of these mutations in *cis* to the *orc5-1* mutation on a plasmid remained temperature-sensitive for growth and defective in silencing. In contrast, a leucine 380 proline substitution suppressed both the temperature sensitivity and the silencing defect (data not shown). Thus only the leucine to serine substitution at position 380 could create a replication-competent but silencing-defective revertant of *orc5-1.*

Replication initiation in orc5-R mutants: The previous section established that changes in the *orc5-1* encoded protein could restore viability without restoring silencing, presumably by restoring replication initiation. To provide a more quantitative evaluation of the properties of the *orc5-R* alleles, we evaluated replication initiation at the *ARS1* origin and at the synthetic silencer by measuring the loss-rate for plasmids with either *ARS1* or the synthetic silencer as their only origin (Figure 4). *orc5-1* caused an elevated plasmid loss-rate for plasmids replicated by *ARS1* (3.4-fold) and for plasmids replicated by the synthetic silencer (19-fold) in cells grown at the permissive temperature. Cells containing either the *orc5-R1* or *orc5-R3* allele partially suppressed the loss-rate of plasmids replicated either by *ARS1* or by the synthetic silencer (Figure 4 and Table 2).

In addition to plasmid loss-rate analysis, we performed two-dimensional (2-D) origin mapping experi-

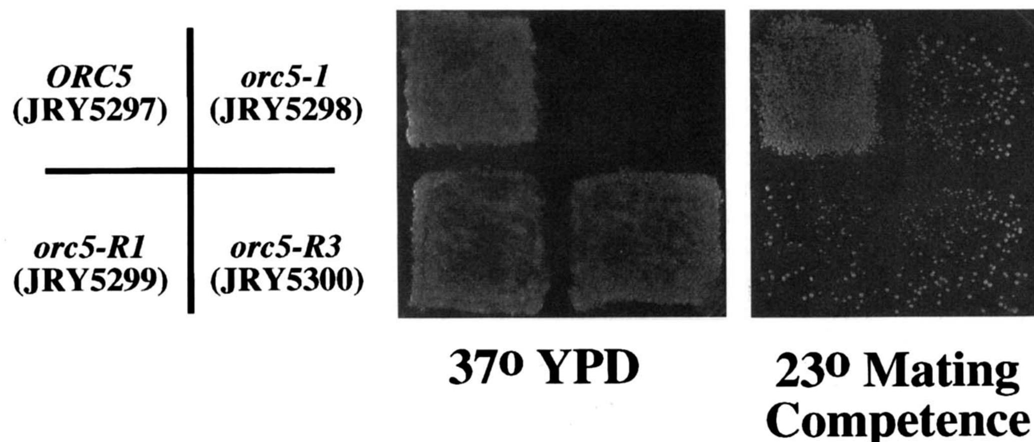

FIGURE 2.—*orc5-R* alleles restored viability without restoring silencing to *orc5-1.* Strain legend is shown on the left. Growth after two days at 37° on rich medium is shown as patches in the middle. Matings are shown as growth of diploids on the right; absence of growth reflected a loss of silencing at *HMR.* All strains were isogenic *orc5-1* haploids with an additional *ORC5, orc5-1,* or *orc5-R* allele integrated at the *HIS3* locus, as indicated.

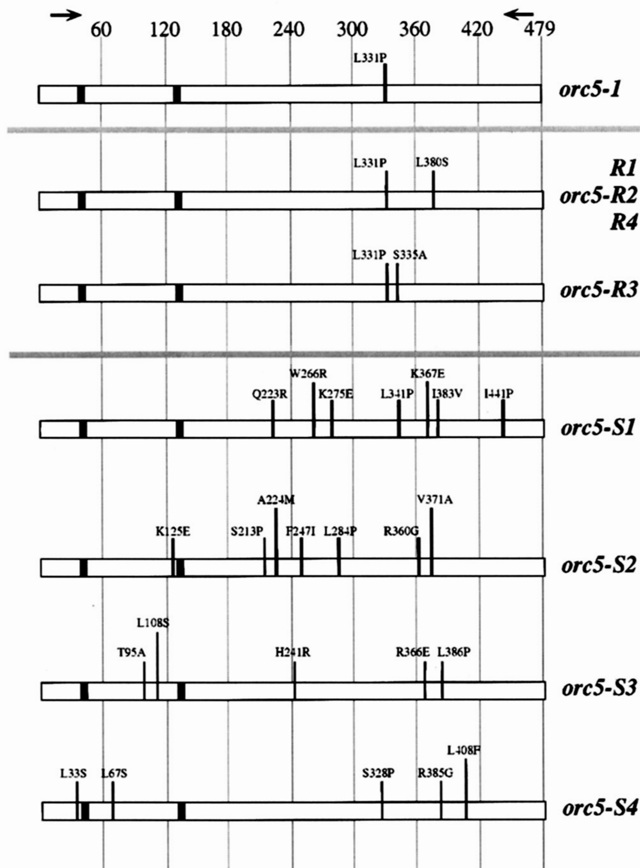

FIGURE 3.—Schematic representation of *ORC5* alleles. Orc5p is a 479-amino acid protein with a nucleotide binding domain located at the N-terminus. The A and P loops of this domain are represented as black boxes. Amino acid positions are indicated at the top of the figure. The position of each mutation and its corresponding change are shown above each site. The position of primers used for PCR mutagenesis are shown as arrows at the top of the figure.

ments (FANGMAN and BREWER 1991) on the *ARS1* origin in cells containing different alleles of *ORC5* (Figure 5A). In cells with a wild-type *ORC5* allele, replication initiation at *ARS1* occurred in a substantial fraction of cell divisions, whereas initiation was reduced but not eliminated in cells containing the *orc5-1* allele grown at the permissive temperature (Figure 5, A1 and A2, respectively). The presence of the *orc5-R1* allele restored replication initiation at *ARS1* to near wild-type levels (Figure 5A3).

In cells with a wild-type *ORC5* allele, replication initiation at the synthetic silencer occurred in a large fraction of cell divisions (Figure 5B1). In contrast, replication initiation at the synthetic silencer in an *orc5-1* cell was reduced, but not eliminated (Figure 5B2, compare the ratio of the signal from the fork forms to the replication bubble forms). The *orc5-R1* allele restored replication initiation to near wild-type levels (Figure 5B3). Thus by both quantitative and qualitative measures, the *orc5-R* alleles restored near wild-type replication function of ORC.

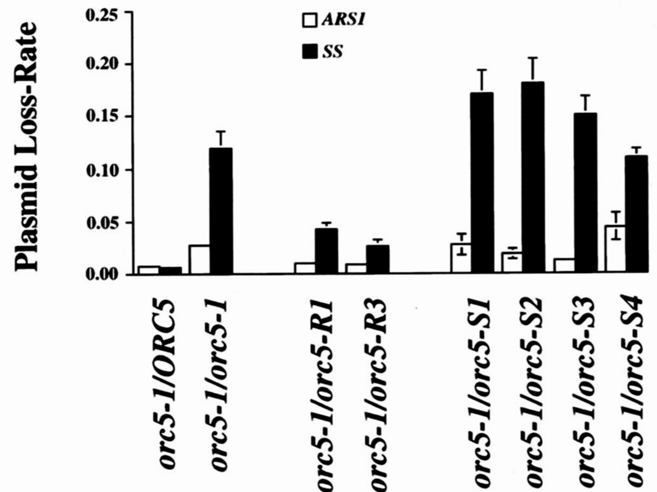

FIGURE 4.—Quantitative plasmid loss-rates caused by *ORC5* alleles. All strains were isogenic *orc5-1* haploids with either *ORC5, orc5-1, orc5-R,* or *orc5-S* alleles integrated at the *HIS3* locus. Plasmids containing the synthetic silencer (pJR950) or *ARS1* (pJR1469) as the sole origins of replication were used to determine the plasmid loss-rate of these strains. The average loss-rates obtained from three independent experiments are shown with corresponding error bars. The small error bars of some points are not depicted due to the scale of the graph.

Silencing in orc5-R mutants: The data of the previous section established that replication initiation was restored in *orc5-R* strains. We analyzed silencing in these strains by the mating efficiency of *MATα* strains, which is determined by the extent of silencing of *HMR***a**. Loss of silencing of *HMR***a** results in loss of mating ability of α strains. Qualitative patch mating analysis revealed that a *MATα ORC5* strain could mate efficiently, reflecting a silenced *HMR*, whereas the *orc5-1, orc5-R1* and *orc5-R3* strains were unable to mate efficiently, due to expression of *HMR* (Figure 2). Quantitatively, *orc5-1* strains were much worse at silencing *HMR* than was an isogenic wild-type strain, and *orc5-R1* and *orc5-R3* strains were even more defective in silencing (Table 3). No noticeable silencing defect was observed in cells containing both a wild-type *ORC5* gene and either *orc5-R1* or *orc5-R3* (data not shown). Thus, the silencing defect caused by *orc5-R1* and *orc5-R3* was recessive. In summary, strains with *orc5-R* alleles were able to suppress the lethality of an *orc5-1* strain, initiate replication at the *ARS1* and *HMR-E* origins, yet failed to silence *HMR*. Taken together, these data indicated that these mutant forms of Orc5p could assemble into a replication-competent but silencing-defective ORC.

Isolation of replication-defective, silencing-competent *ORC5* alleles: The properties of the *orc5-R* alleles suggested that silencing and replication might be two qualitatively separate functions of *ORC5*. Alternatively, silencing might require a higher level of *ORC5* function than does replication initiation, implying a more quantitative explanation. The quantitative explanation for

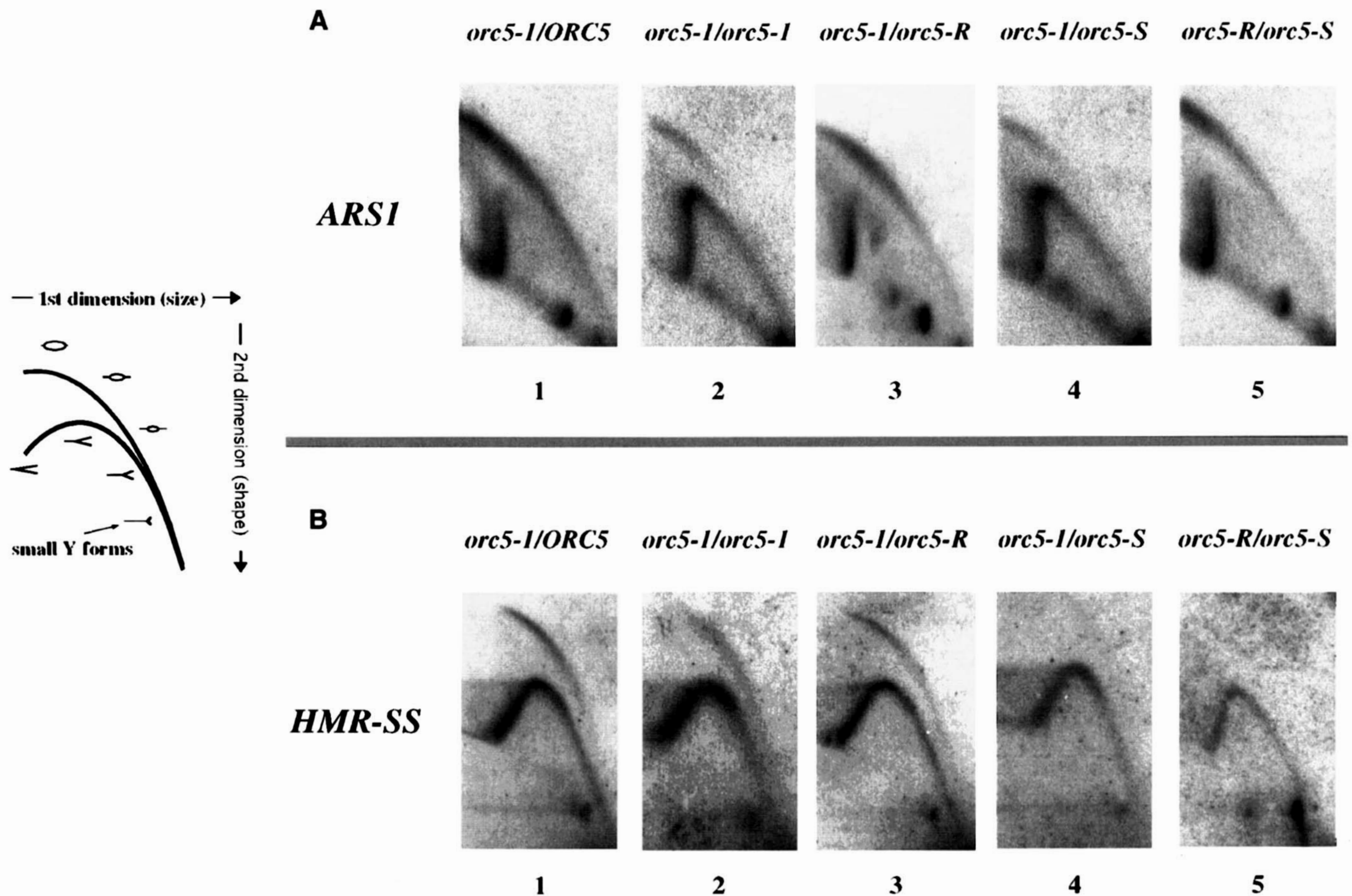

FIGURE 5.—Two-dimensional origin analysis of *orc5* strains. (A) Origin function of *ARS1*. In a set of isogenic *orc5-1* haploid strains containing either *ORC5* (JRY5297), *orc5-1* (JRY5298), *orc5-R1* (JRY5299) or *orc5-S1* (JRY5301) alleles integrated at *HIS3* (A1–A4, respectively). The strain used in A5 (JRY5307) was isogenic to JRY5301, except that it contained *orc5-R1* at the *ORC5* locus. (B) Origin function of the *HMR* synthetic silencer in the same strains used in A.

the single defect of the *orc5-R* mutants would be disproven if replication-defective but silencing-competent alleles of *ORC5* could be recovered.

The *ORC5* gene was mutagenized *in vitro* to screen for replication-defective and silencing-competent alleles. Such alleles should be unable to complement the temperature-sensitive defect of *orc5-1,* but should be able to complement its silencing defect. Sixteen plasmid-borne alleles of *ORC5* were recovered with this phenotype from 16,000 transformants (Figure 1B). Of the 16 *orc5* mutants, four were unable to complement the lethality of a null allele of *ORC5* at any temperature and were chosen for further study (Figure 6). These alleles were named *orc5-S1, orc5-S2, orc5-S3,* and *orc5-S4,* to reflect their silencing competence. The remaining 12 alleles could complement a null allele at 23°, but not at 37° and hence were additional temperature sensitive alleles of *ORC5.*

Each *orc5-S* allele contained several mutations, none of which were shared with another *orc5-S* allele (Figure 3). To determine if more than one single point mutation was required for the *orc5-S* phenotype, we constructed two new *ORC5* alleles. One allele contained three of the five mutations found in *orc5-S4,* L67S, S328P, and R385G. The other allele contained the remaining two mutations of *orc5-S4,* the L33S and L408F mutations. Neither allele complemented the silencing or replication defects of *orc5-1,* suggesting that at least for one *orc5-S* allele, *orc5-S4,* more than one mutation was required for the *orc5-S* phenotype (data not shown).

Replication initiation in *orc5-S* alleles: The phenotype of the *orc5-S* alleles suggested that they encoded proteins that were defective in replication initiation. Alternatively, *ORC5* might have another essential function in addition to replication initiation, with these alleles being defective in this other essential function. Because *orc5-S* alleles by themselves were lethal, we evaluated the replication capacity of these alleles in strains containing an *orc5-1* allele, which is compromised for replication initiation. For these experiments the *orc5-S* alleles were integrated at the *HIS3* locus of an *orc5-1* strain.

We measured replication initiation qualitatively at *ARS1* and the synthetic silencer by 2-D gel analysis and quantitatively by plasmid loss-rate determinations. From previous data, *orc5-1* had an elevated loss-rate for plasmids replicated by *ARS1* (3.4-fold) and the synthetic silencer (19-fold) when compared to an isogenic wild-

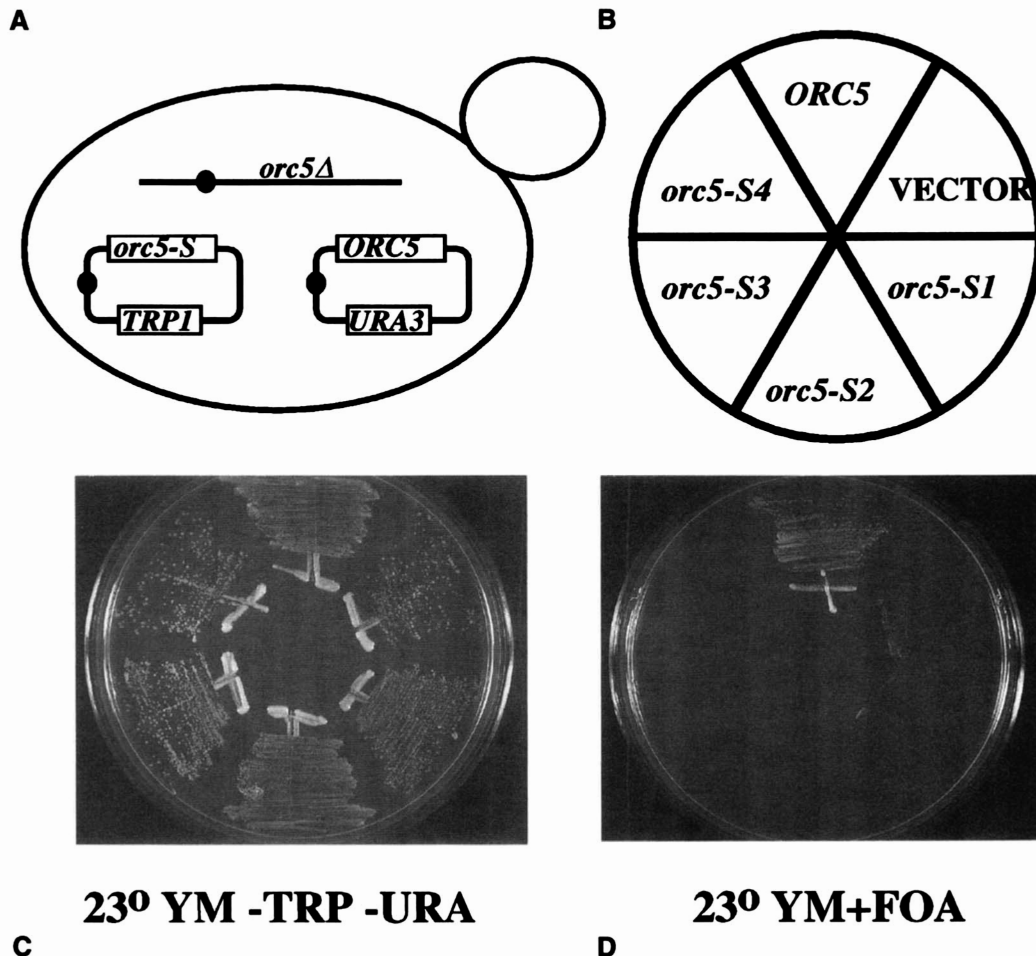

FIGURE 6.—*orc5-S* alleles did not provide the essential function of *ORC5*. (A) An *orc5*Δ strain with *ORC5* on a *CEN/URA3* plasmid (JRY4154) was transformed with a *CEN/TRP1* plasmid containing either *ORC5*, an *orc5-S* allele or no insert at all (VECTOR). (B) Legend showing which *ORC5* allele was transformed into JRY4154 on the *TRP1* plasmid. (C) Growth of transformants on minimal medium selecting for both the *TRP1*- and *URA3*-marked plasmids. (D) Growth of transformants on medium selecting against the *URA3*-marked plasmid. Lack of growth indicated that the mutant *orc5* alleles were unable to complement the *orc5*Δ.

type strain. Strains containing *orc5-S1*, *orc5-S2*, or *orc5-S4* lost the *ARS1* plasmid at an equal or slightly higher rate than the *orc5-1* strain. Cells with an *orc5-S3* allele were slightly better at replicating the *ARS1* plasmid than *orc5-1* cells (Figure 4 and Table 2). All four *orc5-S* alleles were at least as defective as *orc5-1* in propagating plasmids replicated by the synthetic silencer (Figure 4 and Table 2). Taken together, the data indicated that all four *orc5-S* alleles were defective in plasmid replication.

We analyzed the *ARS1* origin and the synthetic silencer origin by 2-D gel analysis as an additional measure of replication initiation in these strains. Replication initiation at *ARS1* in an *orc5-S1* strain and in an isogenic *orc5-1* strain were indistinguishable (compare Figure 5, A2 with A4). In contrast, replication initiation at the synthetic silencer was qualitatively poorer in a strain with *orc5-S1* combined with *orc5-1* than in a strain with *orc5-1* alone (compare Figure 5, B2 and B4). In summary, *orc5-S* alleles were unable to support life on their own and, by two independent assays of replication initiation, were compromised for replication initiation.

Transcriptional silencing in *orc5-S* alleles: The isolation of *orc5-S* alleles was based upon a qualitative mating assay. By this assay silencing of *HMR* in cells with an *orc5-S* allele was as complete as in wild-type cells, regardless of the presence of an *orc5-1* allele (Figure 7). To provide a more quantitative measure of the silencing capability of *orc5-S* alleles, quantitative mating efficiency of *MATα* strains containing *orc5-S* alleles were determined (Table 3). These data also revealed that silencing was restored to near wild-type levels in strains containing *orc5-S* alleles. In the absence of any one of the four *SIR* genes, *orc5-S* alleles were unable to silence *HMR* (data not shown). Thus *orc5-S* restored normal SIR-dependent silencing to wild-type levels.

Intragenic complementation at *ORC5*: The phenotypes of the *orc5-R* and *orc5-S* alleles were due to recessive mutations that disrupted *ORC5*'s silencing and replication functions, respectively. To determine if two independent functions were mutated in these alleles, we tested whether these two classes of recessive mutations could complement each other. The *orc5-S* alleles were integrated at the *HIS3* locus in a strain that contained *orc5-R1*. This strain contained the synthetic silencer that had a single ORC binding site at *HMR*. All strains grew at 37° due to the presence of the *orc5-R1* allele. Those strains that contained either an *ORC5* or an *orc5-S* allele silenced *HMR*, whereas strains with either *orc5-1* or an additional copy of *orc5-R1* did not (Figure 8). Quantitative mating analysis revealed that the *orc5-R1*/*orc5-S1* strain was able to silence *HMR-E* as well as a strain containing an *ORC5* allele (Table 2). Thus, the *orc5-S*

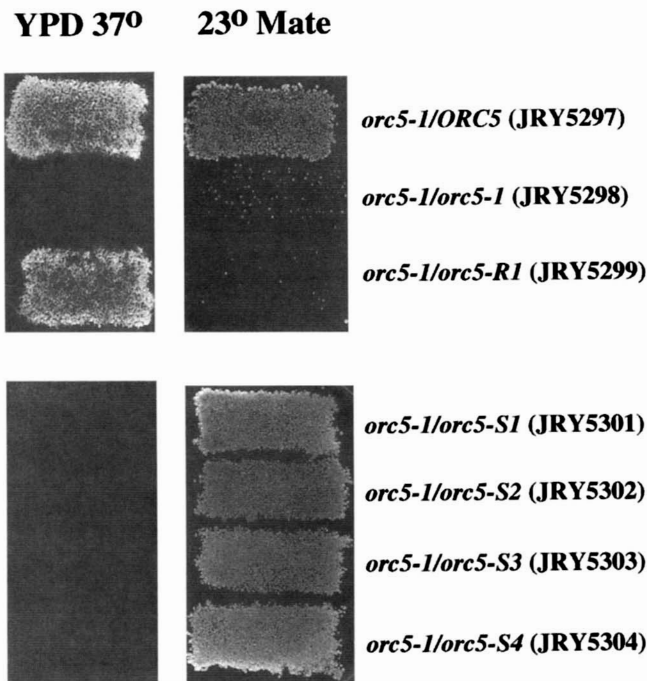

FIGURE 7.—*orc5-S* alleles restored silencing without restoring replication initiation to *orc5-1*. Growth (left) reflected the ability of these alleles to provide the essential function of *ORC5*. Matings (right) for *MATα* strains containing the synthetic silencer flanking *HMR*. Thus, lack of mating is indicative of failure to silence the **a** genes at *HMR*. All strains were isogenic *orc5-1* haploids with either *ORC5, orc5-1, orc5-R1,* or *orc5-S* alleles integrated at the *HIS3* locus.

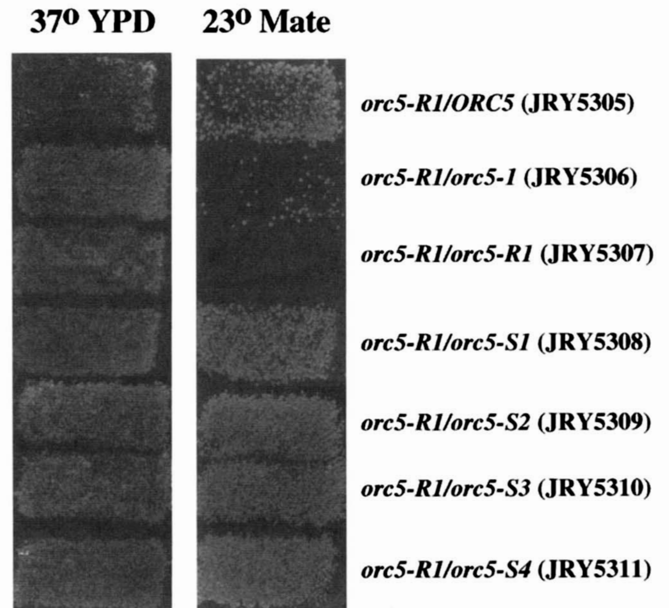

FIGURE 8.—Intragenic complementation of *ORC5* alleles. *ORC5, orc5-1, orc5-R1,* or *orc5-S* alleles were integrated at the *HIS3* locus in an *orc5-R1* (JRY4555) strain and tested for their ability to grow at 37° and silence at 23°. All strains grew at 37° (left) due to the presence of the *orc5-R1* allele at the *ORC5* locus, whereas only those strains harboring either *ORC5* or the *orc5-S* alleles were able to silence the *HMR* locus (right).

and *orc5-R* alleles could complement, even though both classes of mutations were in the same gene.

In this experiment the cells contained two forms of *ORC5* and only a single ORC binding site at the *HMR* synthetic silencer. The structure of the synthetic silencer offered the opportunity to test models of how *orc5-S* and *orc5-R* alleles could complement. One possibility was that the ORC complex contained two (or more) Orc5 subunits. Alternatively, ORC contained only a single Orc5 subunit. If ORC contains two or more Orc5 subunits, then a silencer binding an ORC would be expected to both act as an origin and promote silencing. In contrast, if ORC has only a single Orc5 subunit, then cells with an Orc5-S subunit bound the synthetic silencer, *HMR* would be silent with no replication initiation from the silencer. In contrast, in cells with an Orc5-R subunit bound the silencer, *HMR* would not be silent yet should initiate replication at the silencer. We analyzed replication initiation at the synthetic silencer in cells containing both an *orc5-S* and *orc5-R* allele. Initiation at this origin was noticeably reduced in this strain relative to a strain that contained an *orc5-R1* allele (compare Figure 5, B3 to B5). Plasmid loss-rate data revealed similar results; the loss of plasmids replicated by the synthetic silencer in an *orc5-R/orc5-S* strain was 9.6-fold higher than an *orc5-R/ORC5* strain (Figure 9 and Table 2). Thus, in the *orc5-R/orc5-S* strain, silencing of *HMR-E* was equivalent to a wild-type strain, but replication initiation at this origin was greatly reduced. These data were inconsistent with a model in which both forms of Orc5p acted at the synthetic silencer and suggested that ORC complexes with the Orc5-S subunit acted preferentially at the synthetic silencer.

The previous experiment indicated that the Orc5-S subunit acted at the synthetic silencer, but the Orc5-R subunit did not. We next asked whether the Orc5-S subunit could act at *ARS1*, preventing initiation at this locus. Two-dimensional gel analysis of the *ARS1* origin revealed that the *orc5-R/orc5-S* strain was as proficient in replication initiation at *ARS1* as an *orc5-1/orc5-R1* strain (compare Figure 5, A3 to A5). Additionally, plasmids replicated by *ARS1* were maintained in the *orc5-R/orc5-S* strain at least as well as in a wild-type cell (Figure 9 and Table 2). Taken together, these data suggested that the Orc5-S subunit acted at the synthetic silencer whereas the Orc5-R subunit acted at the *ARS1* origin.

DISCUSSION

This work addressed whether or not ORC's role in replication initiation and silencing were separable. We found alleles of *ORC5* that were replication-competent but silencing-defective and other alleles that were replication-defective but silencing-competent. These two classes of alleles of the same gene could complement. Thus *ORC5*, and by inference ORC itself, possessed two

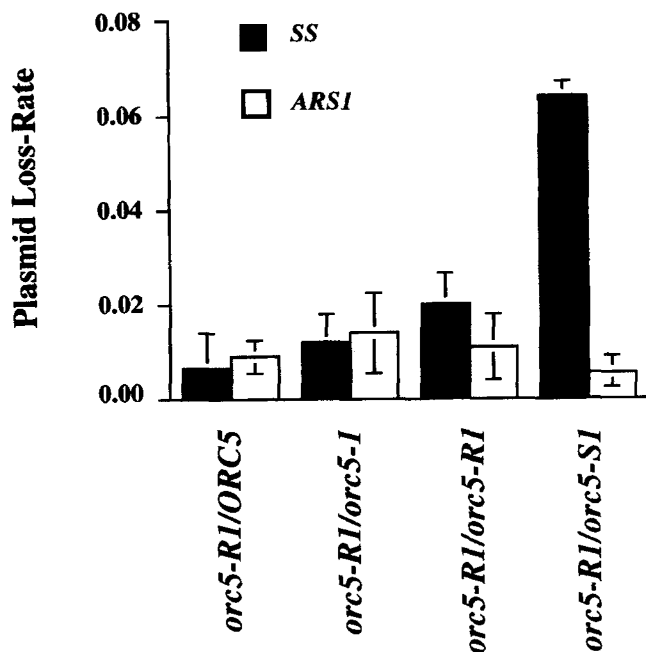

FIGURE 9.—Plasmid loss-rates were determined for *orc5-R1* haploid strains containing either *ORC5* (JRY5305), *orc5-1* (JRY5306), *orc5-R1* (JRY5307), or *orc5-S1* (JRY5308) at the *HIS3* locus. Plasmids used contained either the synthetic silencer (pJR950) or *ARS1* (pJR1469) as the sole origin of replication.

independent functions, one function essential for replication initiation, and a second function important for silencing. Interestingly, the degree of complementation was to near wild-type levels for both functions, suggesting that enough replication-competent ORC complexes were formed to support replication of the entire genome and enough silencing-competent complexes were formed to support repression of the silent loci.

In principle, the two functions of *ORC5* might be encoded in discrete portions of the *ORC5* gene corresponding to discrete domains of the protein. If true, one might expect the two classes of mutations to cluster in discrete regions of the *ORC5* gene. The two *orc5-R* alleles both required two mutations, the original *orc5-1* mutation and either a L380S or a S335A change, to restore the replication but not the silencing function of *ORC5*. The specificity of the changes at Leu380 suggested that there were few changes in the *orc5-1* gene that could restore replication without restoring silencing. It should also be noted that the *orc5-R* alleles were isolated as pseudorevertants of *orc5-1,* and we thus have no information on what amino acid substitution would be required to selectively ablate the silencing role of *ORC5*. Like the *orc5-R* alleles, the *orc5-S* alleles probably require more than one single mutation to disrupt the replication but not the silencing functions of *ORC5*. Thus, if two domains do exist, a more detailed analysis of the *ORC5* gene or protein is needed to reveal these domains.

Earlier studies had identified an S-phase dependence for silencing of *HML* and *HMR* (MILLER and NASMYTH 1984). Because ORC is required for silencing, DNA replication initiation at the silencers was an attractive candidate for the S-phase event required for the establishment of the silent state. However, the *orc5-S* alleles could promote efficient silencing of *HMR* in cells that failed to initiate replication at *HMR*-E. Thus replication initiation at a silencer was not required for silencing, in agreement with an independent line of experiments that led to the same conclusion (FOX *et al.* 1997).

Stoichiometry of ORC: The stoichiometry of subunits in the ORC complex is unknown (BELL and STILLMAN 1992). Similarly, the number of ORCs that bind an ARS sequence has not been defined. Thus we do not know whether ORC binds origins as a monomer, dimer or as a multimer. The intragenic complementation of the two different classes of *ORC5* alleles provided an opportunity to test if more than one Orc5 subunit could act at an origin. In cells containing both Orc5-S and Orc5-R subunits, only one type of Orc5 subunit functioned at an origin. The Orc5-S subunit functioned at the *HMR-E* origin and the Orc5-R subunit functioned at the *ARS1* origin. The simplest interpretation of these results was that only one Orc5 subunit, and by inference, one ORC, functioned at an origin. Of course, we cannot exclude the more complex model in which ORC functions as a multimer, but multimers with different forms of Orc5 are nonfunctional. If the homo-multimer model were correct, then the remarkably complete complementation of the replication and silencing defects would imply that wild-type cells have more ORC complexes than are required.

Why do the *ORC5* alleles have an origin bias? ORC is bound to origins throughout the genome, but only a subset of these origins function as silencers. Why does ORC function as a replication initiator at origins and as a silencer protein only at silencers? The different *orc5* alleles found in this study may shed some light on this question. Although the Orc5-R subunit could function at the *HMR* origin in the absence of any other type of Orc5 subunit, it lost this ability in the presence of the Orc5-S subunit (Figure 5). This result indicated that the Orc5-S subunit acted better at the *HMR* origin than did the Orc5-R subunit. One possible explanation for this result could be that the Orc5-S subunit interacted better with the proteins bound at the silencer than did the Orc5-R subunit. The silencer and *ARS1* origin both contain an Abf1p binding site, but the silencer contains a Rap1p binding site that *ARS1* lacks. Possibly, the Orc5-S subunit interacted better with Rap1p at the silencer origin, thus enabling it to outcompete the Orc5-R subunit at this origin.

At the *ARS1* origin in this heteroallelic strain, the Orc5-R subunit could out-compete the Orc5-S subunit. This result could be explained in a similar manner if the Orc5-R subunit interacted better than the Orc5-S subunit with proteins that bind the B1 and B2 elements

of the *ARS1* origin, which are lacking in the silencer origin. We imagine that the wild-type Orc5 subunit can interact with both Rap1p and with the other proteins that bind at *ARS1*, and specificity of ORC function is achieved through these interactions. The *ORC5* alleles described here have presumably lost the ability to interact with a subset of their potential partners.

The time of origin activation of the *ARS1* and *HMR* silencer origins might also explain the origin bias displayed by the different *ORC5* alleles. The *ARS1* origin initiates in early S-phase, whereas the silencer origin functions in late S-phase (FANGMAN and BREWER 1991). Perhaps the *orc5-S* alleles have lost the ability to interact with factors that cause replication early in S-phase, and the *orc5-R* alleles have lost the ability to interact with factors that cause replication in late S-phase.

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