Roles of *ABF1*, *NPL3*, and *YCL54* in Silencing in *Saccharomyces cerevisiae*

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ABSTRACT

A sensitized genetic screen was carried out to identify essential genes involved in silencing in *Saccharomyces cerevisiae*. This screen identified temperature-sensitive alleles of *ORC2* and *ORC5*, as described elsewhere, and *ABF1*, *NPL3*, and *YCL54*, as described here. Alleles of *ABF1* that caused silencing defects provided the genetic proof of Abf1p's role in silencing. The roles of Npl3p and Ycl54p are less clear. These proteins did not act exclusively through any one of the three protein binding sites of the *HMR-E* silencer. Unlike the *orc2*, *orc5*, and *abf1* mutations that were isolated in the same (or a similar) screen for silencing mutants, neither temperature-sensitive mutation in *NPL3* or *YCL54* caused overt replication defects.

REGIONS of eukaryotic chromosomes are organized into functional domains that greatly influence gene expression. Some regions are enriched for expressed genes whereas others form transcriptionally inert heterochromatin. The repression of expressed genes when they are transposed next to heterochromatin is known as position effect (reviewed in WILSON *et al.* 1990). Heterochromatin in species such as *Drosophila melanogaster* includes DNA at centromeres, telomeres, and at some positions in the midst of euchromatin. In *Saccharomyces cerevisiae*, a form of position effect called silencing occurs at the *HML* and *HMR* mating-type loci. The mating-type genes at these loci are not transcribed even though they are identical in sequence to the mating-type genes located at the expressed *MAT* locus. Some of the genes required for silencing at *HML* and *HMR* are also required for repression of genes introduced next to yeast telomeres (APARICIO *et al.* 1991), indicating that mating-type and telomere silencing probably share features of chromosome organization and metabolism.

Silencing causes DNA to become refractory to a variety of molecular and biological processes. The most obvious result of silencing is the transcriptional repression of the mating-type genes at *HML* and *HMR*. Moreover, a number of unrelated genes, when inserted at *HML* and *HMR*, are also repressed by the same mechanism, demonstrating that silencing is a general process that does not distinguish among different genes or promoters (reviewed in LOO and RINE 1995). In addition, transcription is only one of many protein-DNA interactions affected by silencing. For example, the *S. cerevisiae* HO endonuclease (STRATHERN *et al.* 1982; KLAR *et al.* 1984), the *E. coli dam* methylase (GOTTSCHLING 1992), as well as a host of bacterial restriction endonucleases (LOO and RINE 1994) are among those proteins that are unable to act on their DNA substrates when they are silenced.

A number of genes required for silencing have been identified by genetic screens for mutations affecting the mating ability of yeast strains. Screens for mutations that derepress *HML* and *HMR* have identified the *SIR1*, *SIR2*, *SIR3*, and *SIR4* genes (HABER and GEORGE 1979; KLAR *et al.* 1979; RINE *et al.* 1979; RINE and HERSKOWITZ 1987). Loss of *SIR2*, *SIR3*, *or SIR4* function leads to complete derepression of the silent mating-type loci as well as loss of telomere silencing (APARICIO *et al.* 1991). Mutations in *SIR1*, which have a less severe derepression phenotype, have revealed the heritable nature of silenced DNA and the distinction between the establishment and maintenance processes (PILLUS and RINE 1989). By and large, the sequences of the *SIR* genes have not been informative with regard to mechanism. Silencing does, however, involve chromatin because mutations in either histone H3 or H4 cause silencing defects (KAYNE et al. 1988; JOHNSON *et al.* 1990; MEGEE *et al.* 1990; PARK and SZOSTAK 1990; THOMPSON et al. 1994). In addition, the product of the *SIR2* gene is likely to be involved in the regulation of histone N-terminal acetylation (BRAUNSTEIN *et al.* 1993). Taken together, these observations indicate that the mechanism of silencing involves the formation of a specialized chromatin structure.

DNA sequence elements called silencers flank the silent mating-type loci and are required for silencing. *HML* and *HMR* are each flanked by *E* and *I* silencers (ABRAHAM *et al.* 1984; FELDMAN *et al.* 1984). Each silencer is a compound site consisting of up to three recognized protein-binding sequences. *HMR-E*, the most well characterized of the four silencers, comprises an ARS consensus sequence that binds the origin recog-

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TABLE 1

Strains used in this std1

Strain	Genotype (source)
W303-1A	*MAT***a** *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100* (R. ROTHSTEIN)
W303-1B	*MATα ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100* (R. ROTHSTEIN)
YAB102	W303-1B; *hmr-Δe331-324* (BRAND *et al.* (1987)
JRY2252	*MAT***a** *his3Δ200 leu2-3,112 lys2-801 ura3-52*
JRY2726	*MAT***a** *his4*
JRY2728	*MATα his4*
JRY3009	W303-1A; *MATα*
JRY3765	*MAT***a***abf1-102 ade2 his3-11,15 leu2-3,112 lys2-801 ura3*
JRY3774	*MATα hmr-Δe331-324 abf1-102 ade2 his3-11,15 leu2-3,112 trp1-1 ura3*
JRY3789	JRY3009; *ABF1::URA3*
JRY3790	JRY3009; *ABF1::URA3*
JRY3795	*MATα HMR-SS ΔI abf1-102 ade2 his3-11,15 leu2-3,112 lys2-801 ura3*
JRY3935	JRY3009; *HMR-SS ΔI*
JRY3990	*MATα HMR-SS ΔI npl3-95 ade2 his3-11,15 leu2-3,112 trp1-1 ura3*
JRY4047	W303-1A/JRY3935; *NPL3/npl3Δ::URA3*
JRY4048	W303-1A/JRY3935; *NPL3/npl3Δ::URA3*
JRY4116	JRY3009; *HMR-SS ΔI npl3Δ::URA3*
JRY4125	JRY3009; *orc2-1*
JRY4135	*mat***a**1 *ste14 ade2 leu2-3,112 lys2-801 ura 3*
JRY4186	W303-1A; *mat***a** *Δp hmrΔ::URA3*
JRY4249	JRY3009; *orc5-1*
JRY4253	JRY3009; *HMR-SS ΔI orc5-1*
JRY4423	*mat***a***Δp hmrΔ::URA3 abf1-102 ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3*
JRY4426	*mat***a** *Δp hmrΔ::URA3 abf1-102 ade2-1 his3-11,15 leu2-3,112 ura3*
JRY4429	*mat***a** *Δp hmrΔ::URA3 npl3-95 ade2-1 his3-11,15 leu2-3,112 lys2-801 trp1-1 ura3*
JRY4433	*mat***a** *Δp hmrΔ::URA3 npl3-95 ade2-1 his3-11,15 leu2-3,112 lys2-801 trp1-1 ura3*
JRY4431	*MATα hmr-Δe331-324 npl3-95 ade2 his3-11,15 leu2-3,112 ura3*
JRY4662	*MATα hmr-Δe331-324 ycl54-1 ade2 his3-11,15 leu2-3,112 trp1-1 ura3*
JRY4710	*MAT***a** *ycl54-1 his3-11,15 leu2-3,112 lys2Δtrp1-1 ura3*
JRY4765	*mat***a***Δp hmrΔ::URA3 ycl54-1 ade2-1 his3-11,15 leu2-3, 112 trp1-1 ura3*
JRY4766	*mat***a***Δp hmrΔ::URA3 ycl54-1 ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3*
JRY4828	*MATα HMR-SS ΔI ycl54-1 ade2-1 his3-11,15 leu2-3,112 lys2Δ trp1-1 ura3*
JRY4829	*MATα HMR-SS ΔI ycl54-1 his3-11,15 leu2-3,112 lys2Δ trp1-1 ura3*
JRY4830	*MATα HMR-SS ΔI ycl54-1 ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3*
JRY4831	*MATα HMR-SS ΔI ycl54-1 his3-11,15 leu2-3,112 trp1-1 ura3*
JRY4889	*JRY3009; HMR-SSabf1⁻ ΔI*
JRY4904	*MATα HMR-SS ΔI abf1-102 ade2 his3-11,15 leu2-3,112 lys2-801 ura3*
JRY4905	*MATα HMR-SS ΔI abf1-102 ade2 his3-11,15 leu2-3,112 lys2-801 ura3*
JRY4906	*MATα HMR-SS ΔI abf1-102 ade2 his3-11,15 leu2-3,112 lys2-801 ura3*
JRY4907	*MATα HMR-SS ΔI abf1-102 ade2 his3-11,15 leu2-3,112 lys2-801 ura 3*
JRY4908	*MATα HMR-SSabf1⁻ ΔI abf1-102 ade2 his3-11,15 leu2-3,112 ura3*
JRY4909	*MATα HMR-SSabf1⁻ ΔI abf1-102 ade2 his3-11,15 leu2-3,112 lys2-801 trp1-1 ura3*
JRY4910	*MATα HMR-SSabf1⁻ ΔI abf1-102 ade2 his3-11,15 leu2,3,112 trp1-1 ura3*
JRY4911	*MATα HMR-SSabf1⁻ ΔI abf1-102 ade2 his3-11,15 leu2-3,112 lys2-801 ura3*
JRY4963	*MATα HMR-SSabf1⁻ ΔI npl3-95 ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3*
JRY4964	*MATα HMR-SSabf1⁻ ΔI npl3-95 ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3*
JRY4965	*MATα HMR-SSabf1⁻ ΔI npl3-95 ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3*
JRY4966	*MATα HMR-SSabf1⁻ ΔI npl3-95 ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3*
JRY4967	*MATα HMR-SSabf1⁻ ΔI ycl54-1 ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3*
JRY4968	*MATα HMR-SSabf1⁻ ΔI ycl54-1 ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3*
JRY4969	*MATα HMR-SSabf1⁻ ΔI ycl54-1 ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3*
JRY4970	*MATα HMR-SSabf1⁻ ΔI ycl54-1 ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3*
JRY4996	*mat***a**1 *abf1-101 ste14 ade2 leu2-3,112 lys2-801 ura3*
JRY4997	*mat***a**1 *abf1-102 ste14 ade2 leu2-3,112 lys2-801 ura3*
JRY4998	*mat***a**1 *abf1-103 ste14 ade2 leu2-3,112 lys2-801 ura3*
JRY4999	*mat***a**1 *abf1-104 ste14 ade2 leu2-3,112 lys2-801 ura3*
JRY5000	*mat***a**1 *npl3-95 ste14 ade2 leu2-3,112 lys2-801 ura3*
JRY5001	*MAT***a** *npl3-95 ade2 leu2-3,112 lys2-801 ura3*
JRY5002	*MAT***a** *ycl54-1 leu2-3,112 lys2-801 trp1-1 ura3*
JRY5003	*MATα hmr-Δe331-324 ycl54-1 ade2 his3-11,15 leu2-3,112 trp1-1 ura3*

TABLE 1

Continued

Strain	Genotype (source)
JRY5004	*MAT***a** *hmr-Δe331-324 ycl54-1 ade2 his3-11,15 leu2-3,112 lys2-801 trp1-1 ura3*
JRY5005	JRY3009; *YCL54*::*LEU2*
JRY5019	*MATα HMR-SS ΔI abf1-102 orc5-1 ade2-1 his3-11,15 leu2-3,112 lys2 trp1-1 ura3*
JRY5020	*MATα HMR-SS ΔI abf1-102 orc5-1 his3-11,15 leu2-3,112 lys2 trp1-1 ura3*
JRY5021	*MATα HMR-SS ΔI abf1-102 orc5-1 ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3*
JRY5022	*MATα HMR-SS ΔI abf1-102 orc5-1 his3-11,15 leu2-3,112 lys2 ura3*
JRY5023	*MATα HMR-SS ΔI npl3-95 orc5-1 ade2 his3-11,15 leu2-3,112 ura3*
JRY5024	*MATα HMR-SS ΔI npl3-95 orc5-1 ade2 his3-11,15 leu2-3,112 trp1-1 ura3*
JRY5025	*MATα HMR-SS ΔI npl3-95 orc5-1 ade2 his3-11,15 leu2-3,112 trp1-1 ura3*
JRY5026	*MATα HMR-SS ΔI npl3-95 orc5-1 ade2 his3-11,15 leu2-3,112 trp1-1 ura3*
JRY5027	*MATα HMR-SS ΔI ycl54-1 orc5-1 his3-11,15 leu2-3,112 trp1-1 ura3*
JRY5028	*MATα HMR-SS ΔI ycl54-1 orc5-1 his3-11,15 leu2-3,112 trp1-1 ura3*
JRY5029	*MATα HMR-SS ΔI ycl54-1 orc5-1 his3-11,15 leu2-3,112 trp1-1 ura3*
JRY5030	*MATα HMR-SS ΔI ycl54-1 orc5-1 ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3*
JRY5039	*MATα hmr-Δe331-324 ycl54-1 ade2 his3-11,15 leu2-3,112 lys2Δ trp1-1 ura3*
JRY5040	*MATα hmr-Δe331-324 ycl54-1 ade2 his3-11,15 leu2-3,112 lys2Δ trp1-1 ura3*
JRY5041	*MATα hmr-Δe331-324 ycl54-1 ade2 his3-11,15 leu2-3,112 trp1-1 ura3*
JRY5042	*MATα hmr-Δe331-324 ycl54-1 ade2 his3-11,15 leu2-3,112 trp1-1 ura3*
JRY5043	*MATα hmr-Δe331-324 ycl54-1 ade2 his3-11,15 leu2-3,112 trp1-1 ura3*
JRY5044	*MATα hmr-Δe331-324 abf1-102 ade2 his3-11,15 leu2-3,112 lys2Δ trp1-1 ura3*
JRY5045	*MATα hmr-Δe331-324 abf1-102 ade2 his3-11,15 leu2-3,112 lys2Δ trp1-1 ura3*
JRY5046	*MATα hmr-Δe331-324 abf1-102 ade2 his3-11,15 leu2-3,112 lys2Δ trp1-1 ura3*
JRY5047	*MATα hmr-Δe331-324 abf1-102 ade2 his3-11,15 leu2-3,112 lys2Δ trp1-1 ura3*
JRY5048	*MATα hmr-Δe331-324 abf1-102 ade2 his3-11,15 leu2-3,112 lys2Δ trp1-1 ura3*
JRY5049	*MATα HMR-SS ΔI npl3-95 ade2 his3-11,15 leu2-3,112 lys2Δ trp1-1 ura3*
JRY5050	*MATα HMR-SS ΔI npl3-95 ade2 his3-11,15 leu2-3,112 lys2Δ trp1-1 ura3*
JRY5051	*MATα HMR-SS ΔI npl3-95 ade2 his3-11,15 leu2-3,112 lys2Δ trp1-1 ura3*
JRY5052	*MATα HMR-SS ΔI npl3-95 ade2 his3-11,15 leu2-3,112 lys2Δ trp1-1 ura3*
JRY5053	*MATα HMR-SS ΔI npl3-95 ade2 his3-11,15 leu2-3,112 lys2Δ trp1-1 ura3*
JRY5054	*MATα hmr-Δe331-324 npl3-95 ade2 his3-11,15 leu2-3,112 lys2Δ trp1-1 ura3*
JRY5055	*MATα hmr-Δe331-324 npl3-95 ade2 his3-11,15 leu2-3,112 lys2Δ trp1-1 ura3*
JRY5056	*MATα hmr-Δe331-324 npl3-95 ade2 his3-11,15 leu2-3,112 trp1-1 ura3*
JRY5057	*MATα hmr-Δe331-324 npl3-95 ade2 his3-11,15 leu2-3,112 trp1-1 ura3*
JRY5058	*MATα hmr-Δe331-324 npl3-95 ade2 his3-11,15 leu2-3,112 trp1-1 ura3*
JRY5063	JRY3795; spontaneous temperature-resistant, silencing-competent revertant
JRY5064	JRY3935/JRY4186
JRY5065	JRY3935/JRY4429
JRY5066	JRY3935/JRY4433

Unless otherwise indicated, all strains were either from the laboratory collection or produced during the course of this work.

nition complex (ORC) (BELL *et al.* 1993), as well as a Rap1p binding site and an Abf1p binding site (BRAND *et al.* 1985; MCNALLY and RINE 1991).

There are two noteworthy features of the silencers. First, the presence of an ARS consensus sequence at each silencer suggests a role for replication initiation in silencing. Indeed, correlations between these two processes have been observed (for example, MILLER and NASMYTH 1984; BRAND *et al.* 1987; MCNALLY and RINE 1991; RIVIER and RINE 1992). Second, the silencers appear to be over-engineered, such that mutation of any one element within the *HMR-E* silencer leads to very little derepression. Mutation of any two elements, however, leads to complete loss of silencing (BRAND *et al.* 1985). This redundancy among the silencer elements is likely to be the reason for why mutations in genes encoding silencer-binding proteins were not identified in early screens for silencing mutants. By using weakened silencer alleles, the screen described in this report revealed mutations in genes encoding ORC subunits (FOSS *et al.* 1993; LOO *et al.* 1995), establishing a role for the yeast replication initiator in silencing. This work describes mutations in *ABF1, NPL3,* and *YCL54* that affect silencing and evaluates their silencing defects and other phenotypes associated with mutations in these genes.

MATERIALS AND METHODS

General: The genotypes of strains used in this work are presented in Table 1. Standard yeast media, genetic, and recombinant DNA methods were as described (ROSE *et al.* 1989; SAMBROOK *et al.* 1989). Yeast transformations used the lithium

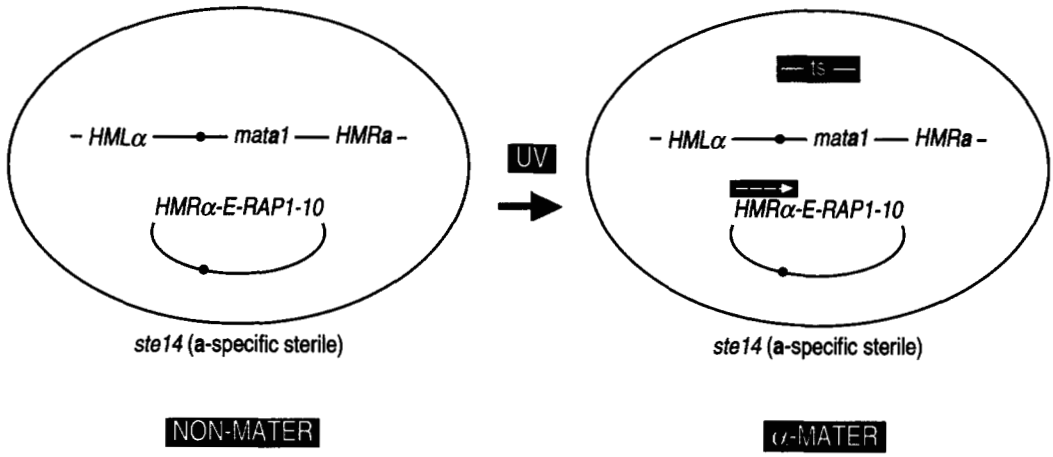

FIGURE 1.—A schematic representation of the genetic screen used to isolate the *abf1*, *npl3*, and *ycl54* mutations. Modified from FOSS *et al.* (1993). The key point is that derepression of *HMRα-E-RAP1-10* in this strain converts a nonmating cell into a cell that displays the α-mating phenotype.

acetate procedure (ITO *et al.* 1983). The substitution of synthetic silencer alleles at *HMR* were confirmed by DNA-blot hybridization using genomic DNA isolated by glass bead lysis of cells in detergent followed by organic extractions (HOFFMAN and WINSTON 1987).

Total RNA was isolated by hot phenol extractions. In this method, each sample of 2–4 × 10^8 cells was harvested from a log-phase culture grown in rich medium, washed once in water, and frozen in a dry ice-ethanol bath. Cells were thawed by adding 0.7 ml of 0.3 M NaCl, 20 mM Tris-Cl pH 8.0, 10 mM EDTA. Then 0.6 ml of 65° phenol was added and the suspension vortexed for 30 sec before incubating 4 min at 65°. The aqueous phase was recovered by centrifugation and the 65° phenol extraction was repeated, followed by two phenol-chloroform extractions at 4°. Total RNA was precipitated by the addition of 2.5 volumes of ethanol.

For RNA-blot hybridizations, 30–40 μg of total RNA was separated by formaldehyde-agarose gel electrophoresis (SAMBROOK *et al.* 1989). DNA and RNA blots were onto Zeta-Probe membrane (Bio-Rad), and hybridizations were done according to the membrane manufacturer's instructions. Probe DNA was labeled using Amersham's Multiprime kit. Blots were exposed to Kodak XAR film at −80° in the presence of DuPont Cronex Lightning-Plus intensifying screens. RNA blots were quantified using a Molecular Dynamics PhosphoImager and ImageQuant software.

Mutant screen: Mutagenesis of the starting strain (JRY4135 with pJR1425) was carried out on 19 independent cultures grown in liquid YM. Cells from each culture were plated onto casamino acid plates at a density of several hundred surviving colonies per plate. The plates were exposed to UV light for ~30 sec (55–60% killing) and immediately wrapped in foil to reduce photorepair of mutagenesis-induced lesions. The plates were incubated at 23° for 3 days, and the ~130,000 surviving colonies were replica plated onto a lawn of *MAT***a** cells (JRY2252) on minimal medium supplemented with leucine and lysine. Among the background of nonmating colonies, colonies that contained α-mating cells that formed prototrophic diploids at 30° were picked for analysis. The plasmid bearing the compromised *HMRα-E-RAP1–10* allele (pJR1425) has been described (FOSS and RINE 1993).

Plasmid and strain construction: Allelism of *ABF1* to the temperature-sensitive mutations that were complemented by the cloned *ABF1* gene was determined by crossing each of the temperature-sensitive strains to strains with the *URA3* gene integrated adjacent to *ABF1*. The integrating plasmid used to place the *URA3* gene at the *ABF1* locus was constructed by ligating a 3-kb *Cla*I-*Pvu*II fragment containing *ABF1* from pRS315/ABF1 (a gift of J. DIFFLEY and B. STILLMAN) into YIp5 (BOTSTEIN *et al.* 1979) that was digested with *Cla*I and *Nru*I, generating pJR1455. Integration of pJR1455 into a wild-type strain (JRY3009) was directed by digesting the plasmid with *Kpn*I. Candidates for the correct integration of pJR1455 were screened by DNA-blot hybridization. Two isolates with the *URA3* gene correctly integrated next to the *ABF1* locus were named JRY3789 and JRY3790.

Allelism of the *npl3-95* mutation to the authentic *NPL3* locus was suspected because the temperature-sensitive mutation isolated here, as well as *NPL3* and *STE14*, were all linked to the *ADE8* gene (BOSSIE *et al.* 1992; S. MICHAELIS, personal communication). In crosses between a *npl3-95 ste14* strain and a wild-type strain, *NPL3* mapped to within 11 cM of *STE14* (47PD:14T:0NPD). Allelism was confirmed by crosses to a strain bearing a deletion of *NPL3* that was marked by a transplacement of *URA3* (see below).

A deletion of the *NPL3* open reading frame and its replacement by the *URA3* gene was constructed in a diploid strain homozygous for *ura3-1* (W303-1A/JRY3935) using plasmid pSB149 (BOSSIE *et al.* 1992). This plasmid was digested with *Xho*I and *Eco*RI, releasing a linear fragment containing the *npl3Δ::URA3* allele before transformation into the diploid. Candidate integrants were screened by DNA-blot hybridization, and two strains with the integrated deletion allele (JRY4047 and JRY4048) were sporulated and dissected. None of the *MATα HMR-SS Δ1 npl3Δ::URA3* strains exhibited silencing defects (38 tetrads examined).

Three plasmids (pJR1677–1679) containing overlapping inserts that complemented the temperature-sensitivity and silencing defect of a *npl3-95* strain (JRY4431) were isolated from a genomic DNA library (SPENCER *et al.* 1990). A 5.2-kb *Spe*I-*Pvu*II fragment from pJR1679 was ligated into pRS316 (SIKORSKI and HIETER 1989) previously digested with *Spe*I and *Sma*I, generating pJR1680. pJR1680 was subject to exonuclease III deletions, and the three largest noncomplementing clones were sequenced using primers that hybridized to vector sequences. All of the deletions extended into the *NPL3* open reading frame. Neither a 2.5-kb *Spe*I-*Bgl*II fragment to the left of *NPL3* (in pJR1718) nor a 2.5-kb *Bgl*II-*Bgl*II fragment to the right of *NPL3* (in pJR1717) complemented the temperature sensitivity or silencing defect of a *npl3-95* strain; both of these fragments were isolated from pJR1680 and truncated the *NPL3* gene at internal *Bgl*II sites.

TABLE 2

AFB1, NPL3,* and *YCL54* were required for silencing at *HMR

Relevant genotype	Mating efficiency	Strains used
MATα HMR-E	1.0	W303-1B
MATα hmr-Δe331-324	$1.9 \times 10^{-1} \pm 9.8 \times 10^{-2}$	YAB102
MATα hmr-Δe331-324 abf1-102	$3.1 \times 10^{-3} \pm 1.2 \times 10^{-3}$	JRY3774, 5044-48
MATα hmr-Δe331-324 npl3-95	$9.0 \times 10^{-4} \pm 6.4 \times 10^{-4}$	JRY4431, 5054-58
MAYα hmr-Δe331-324 ycl54-1	$2.3 \times 10^{-2} \pm 3.0 \times 10^{-2}$	JRY4662, 5039-43
MATα HMR-SS ΔI	$1.7 \times 10^{-1} \pm 4.5 \times 10^{-2}$	JRY3935
MATα HMR-SS ΔI abf1-102	$2.0 \times 10^{-3} \pm 2.4 \times 10^{-2}$	JRY4904-07
MATα HMR-SS ΔI npl3-95	$1.7 \times 10^{-4} \pm 7.5 \times 10^{-5}$	JRY3990, 5049-53
MATα HMR-SS ΔI npl3Δ::URA3	$2.0 \times 10^{-1} \pm 4.6 \times 10^{-2}$	JRY4116
MATα HMR-SS ΔI ycl54-1	$2.5 \times 10^{-2} \pm 3.0 \times 10^{-2}$	JRY4828-31

Mating efficiencies are shown for *MATα* strains bearing compromising *HMR-E* silencer alleles and mutations in *ABF1, NPL3,* or *YCL54. hmr-Δe331-324* consists of the natural *HMR-E* silencer that is lacking its Rap 1p binding site (BRAND *et al.* 1987). *HMR-SS ΔI* consists of the synthetic *HMR-E* silencer and lacked *HMR-I.* All mating efficiencies were normalized to a wild-type strain (W303-1B).

Allelism of the *ycl54-1* complementing DNA clones to the temperature-sensitive mutation was determined by integrating DNA sequences from the complementing *LEU2*-containing clone into the genome of a wild-type cell and then testing allelism of the integrated *LEU2* gene with the temperature-sensitive phenotype of *ycl54-1.* The integrating plasmid was constructed by ligating a 5.6-kb *Hin*dIII fragment from a complementing clone (pJR1682) into the *LEU2*-containing vector pRS305 (SIKORSKI and HIETER 1989) that was digested with *Hin*dIII, resulting in pJR1688. Integration of pJR1688 into the genome of JRY3009 was directed by linearizing the plasmid at a unique *Sph*I site located within the insert, creating JRY5005.

Linkage of the silencing and temperature-sensitive phenotypes: In the crosses to determine if the *npl3-95* mutation caused both silencing and temperature-sensitive growth, 60 tetrads were examined from crosses in which the *MAT, NPL3,* and *HMR* loci were all heterozygous (for example, *MAT***a**/*MATα NPL3/npl3-95 HMR***a**/*hmr-Δe331-324*); the predicted number of *MATα npl3-95 hmr-Δe331-324* (or *HMR-SS ΔI*) segregants was 36 and the observed number of nonmating *MATΔ npl3-95* segregants was 35. Similarly, for *ycl54-1,* 55 tetrads were examined from crosses in which the *MAT, YCL54,* and *HMR* loci were all heterozygous (for example, *MAT***a**/*MATα YCL54/ycl54-1 HMR***a**/*hmr-Δe331-324*); the predicted number of *MATα ycl54-1 hmr-Δe331-324* (or *HMR-SS ΔI*) segregants was 33 and the observed number of nonmating *MATΔ ycl54-1* segregants was 32. These calculations assumed ~40% recombination between *MAT* and *HMR* (MORTIMER *et al.* 1992).

Quantitative mating assays: Some quantitative mating efficiencies were determined for nonisogenic strains. To account for variation due to differences in genetic background, mating efficiencies were determined for at least four strains with the desired genotype.

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

Cell cycle arrest and staining: Cells used to photograph terminal phenotypes at the restrictive growth temperature were grown to mid log phase in liquid YPD at 23°. The cultures were then divided and either left at 23° or placed at 37° for 5–6 hr. Cells were harvested by low speed centrifugation, washed once with water, and fixed with 70% ethanol in a dry ice bath for 10 min. After two more washes with water, the fixed cell pellet was suspended in 100 μl of 1 μg/ml 4′6-diamidino-2-phenylindole (DAPI), immediately washed twice with water, and photographed.

Plasmid loss rates: Transformants were grown into early stationary phase in liquid YM before inoculating into liquid YPD that was further supplemented with adenine, histidine, leucine, lysine, tryptophan, and uracil at standard concentrations (ROSE *et al.* 1989) to ensure a maximum number of doublings. The initial fraction of cells that contained the plasmid (F_i) was determined by plating dilutions of the new culture onto solid YM either containing or lacking the supplement needed to support growth in the absence of the plasmid. 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

A sensitized screen for silencing mutants: In previous screens for mutations affecting silencing, the recovery of mutations in genes encoding proteins that bind to the mating-type silencers was prevented by the functional redundancy among silencer elements. That is, mutation in any single binding site of the silencer has little effect on silencing, but two or more binding site mutations result in much larger silencing defects (BRAND *et al.* 1987). Thus the precise roles of the silencer binding proteins in silencing was not known, and with the exception of Rap1p, there was no information on whether the proteins that bind the silencers *in vitro* actually functioned in silencing. To overcome this redundancy and to identify genetically new proteins involved in silencing, a sensitized screen was used that

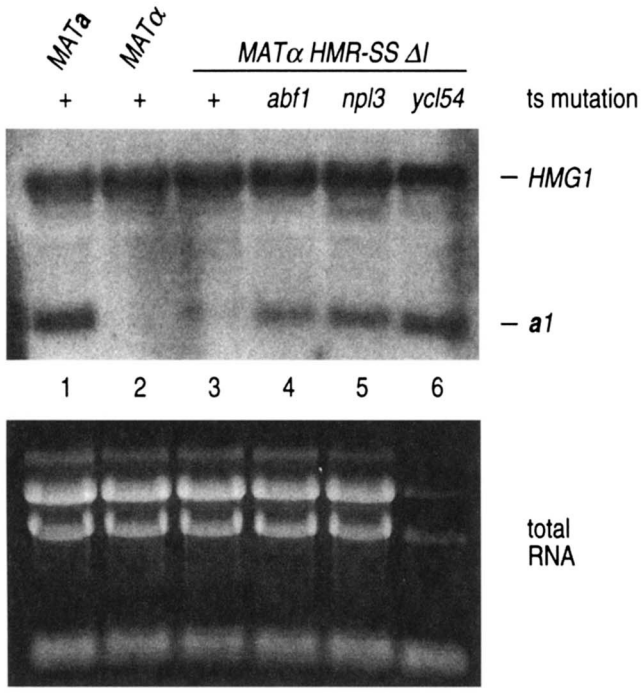

FIGURE 2.—Transcription of **a**1 mRNA from *HMR***a** correlated with the mating defect of *abf1-102*, *npl3-95*, and *ycl54-1* strains. The strains used were, from left to right, *MAT***a** ts^+ (JRY2334), *MATα* ts^+ (JRY3009), *MATα* ts^+ *HMR-SS ΔI* (JRY3935), *MATα abf1-102 HMR-SS ΔI* (JRY3795), *MATα npl3-95 HMR-SS ΔI* (JRY3990), and *MATα ycl54-1 HMR-SS ΔI* (JRY4830). Ribosomal RNA was underrepresented in the total RNA from *ycl54-1* mutant strains (bottom); this reduction in rRNA was also observed in a separate experiment (not shown) where only ethidium stained total RNA was examined.

allowed mutations to be isolated in genes that were otherwise not crucial for silencing (Figure 1). This screen used an *HMR-E* allele, *HMR-E-RAP1-10*, that is mutated at its Rap1p binding site and is unable to bind Rap1p (FOSS and RINE 1993). The uppercase designation of the *RAP1-10* allele reflects its *cis*-dominant phenotype and distinguishes this mutation from recessive loss-of-function mutations in the *RAP1* gene. The *HMR-E-RAP1-10* allele results in only slight derepression of *HMR*. However, in the absence of a functional Rap1p binding site, a mutation in either one of the two remaining silencer elements (the ARS consensus sequence or the Abf1p binding site) results in almost total derepression of *HMR* (BRAND *et al.* 1987). Thus mutations that reduce the DNA binding or silencing function of proteins that recognize either the ARS consensus sequence or the Abf1p binding site at *HMR-E-RAP1-10* should allow complete derepression of *HMR* and result in a mating phenotype.

In principle, derepression of the chromosomal *HMR***a***-E-RAP1-10* allele in a *MATα* strain would result in a detectable loss of α-mating ability due to simultaneous expression of both **a** and α genes (STRATHERN *et al.* 1981). However mutation of any of a large number of other genes can cause the nonmating phenotype, including *SIR* genes that derepress both *HMLα* and *HMR***a***-E-RAP1-10*, as well as genes that, when mutant, result in general sterility. To avoid isolating mutations in previously characterized genes, the screen was designed to detect specifically derepression of a plasmid-borne *HMRa-E-RAP1-10* allele. Expression of α genes in a strain lacking functional mating-type genes at the *MAT* locus (*mat***a**1) is sufficient to cause α-mating among a background of non-α-maters (KASSIR and SIMCHEN 1976). Furthermore, sterile mutations or mutations in *SIR* genes that derepress both of the chromosomal *HMLα* and *HMR***a** loci, both present in the parent strain, would not be recovered in this screen because they would cause the nonmating phenotype. A final refinement of the screen was made by introducing a *ste14* mutation into the strain. *STE14* is required for the processing of **a** factor and hence is an **a**-specific sterile gene (HRYCYNA *et al.* 1991). Mutations that weakly derepress *HMRα-E-RAP1-10* in a *mat***a**1 strain would have created strains capable of mating as both an **a** strain and as an α strain, resulting in cultures of polyploid cells (RINE and HERSKOWITZ 1987). The *ste14* mutation eliminated this complication by blocking the **a**-mating phenotype of the potentially bimating cultures.

To identify essential genes that might also be involved in silencing, analysis of mutants focused on those that caused both a temperature-sensitive growth defect at 37° and mating phenotypes at 23 or 30°. Genes that encode components of the origin recognition complex and *ABF1* are known to be essential (DIFFLEY and STILLMAN 1989; HALFTER *et al.* 1989a; RHODE *et al.* 1989; FOSS *et al.* 1993; LOO *et al.* 1995). Therefore, new mutations affecting silencing at the permissive temperature and viability at the restrictive temperature were tested for ORC and *ABF1* function during early stages of their characterization.

Ten recessive temperature-sensitive mutations were recovered in the screen for silencing mutants. These mutations were in five different genes. Among these were three alleles of *ORC2* (*orc2-2*, *orc2-3*, and *orc2-4*) and one allele of *ORC5* (*orc5-1*). *ORC2* and *ORC5* encode the second and fifth largest subunits of the origin recognition complex, and these mutations are described elsewhere (FOSS *et al.* 1993; LOO *et al.* 1995). The six remaining silencing-defective mutations each affected one of three different genes.

ABF1: Four temperature-sensitive alleles of *ABF1* that derepressed *HMRα-E-RAP1-10* were recovered among the 10 mutants. Because circumstantial evidence implicated Abf1p as having a role in silencing (SHORE *et al.* 1987; BUCHMAN *et al.* 1988; KIMMERLY *et al.* 1988), strains from the screen that bore mutations that appeared to derepress the plasmid-borne *HMRα-E-RAP1-10* allele were tested for their ability to be complemented by a plasmid bearing the *ABF1* gene. The cloned *ABF1* gene (in plasmid pRS315/ABF1) complemented the growth defect of four independently isolated mutant strains

(JRY4996–4999). As shown below, these four strains contained mutations in *ABF1*, designated *abf1-101, abf1-102, abf1-103,* and *abf1-104.*

Reversion analysis and crosses were performed with these *abf1* candidate strains to determine whether the temperature-sensitivity and the mating phenotypes were due to single nuclear mutations and whether the mutations were allelic to *ABF1.* In crosses between each of the presumptive *abf1* strains and a wild-type strain (W303-1A), two temperature-sensitive and two nontemperature-sensitive segregants were recovered in almost all tetrads, indicating that each mutant strain contained a single mutation (≥40 tetrads were analyzed for each mutant strain; rare tetrads that yielded non-2:2 segregation of the temperature-sensitivity were presumed to be due to gene conversion events). Allelism of each of the putative *abf1* alleles was confirmed by crossing temperature-sensitive strains to strains with the *URA3* gene integrated at the *ABF1* locus (JRY3789 or JRY3790). In the four crosses, all tetrads that gave 2:2 segregation of both temperature-sensitivity and uracil prototrophy also showed opposite segregation of both phenotypes, indicating that the mutations were allelic to *ABF1* (a minimum of 23 tetrads were analyzed from each cross and linkage between the mutation causing temperature-sensitivity and *ABF1* was therefore <2.2 cM).

Linkage of the temperature-sensitivity and mating phenotypes was determined in two ways. First, for each of the *abf1* strains, six YPD plates containing ~10^8 cells each were placed at 37° to select for temperature-resistant revertants. Several independent revertants of *abf1-102, abf1-103,* and *abf1-104* were recovered in this manner. Of these revertants, four out of six revertants of the *abf1-102* strain coreverted the temperature-sensitive and mating phenotypes, two out of five corevertants were recovered from the *abf1-103* strain, and three out of five corevertants were recovered from the *abf1-104* strain, indicating that the growth and mating phenotypes were likely due to the same mutation. Next, to test linkage of the mating and growth phenotypes further and to determine if the alleles of *ABF1* would cause a silencing defect at the chromosomal *HMR***a** locus, strains bearing each of the four *abf1* alleles were crossed to two strains bearing compromised *HMR-E* silencers. One strain had an allele of the *HMR-E* silencer that lacked a Rap1p binding site (*hmr-Δe331-324* in YAB102) and hence is a chromosomal analog of the plasmid-borne *HMR-E-RAP1-10* allele used in the mutant screen. The other strain possessed a synthetic *HMR-E* silencer and lacked the *HMR-I* silencer (*HMR-SS ΔI* in JRY3935). Both of these silencers are sensitive to loss of any one of the remaining *HMR-E* elements (BRAND *et al.* 1987; MCNALLY and RINE 1991) as well as to *orc* mutations (FOSS *et al.* 1993; FOX *et al.* 1995; LOO *et al.* 1995). Segregants that were *MATα abf1* and bore either *hmr-Δe331-324* or *HMR-SS ΔI* were able to mate as α*s* only weakly, indicating derepression of the **a**1 gene at *HMR* (Table 2; Figure 2). Segregants with either an *abf1* mutation alone or the silencer mutations alone were invariably able to mate as α*s* efficiently.

NPL3: Another temperature-sensitive strain obtained from the screen for silencing defective mutants contained a mutation in a gene known both as *NOP3* (RUSSELL and TOLLERVEY 1992) and *NPL3* (BOSSIE *et al.* 1992), as shown below. The allele isolated in this study is referred to as *npl3-95.*

As with the *abf1* mutants, temperature-resistant revertants of a *npl3-95* strain were selected at 37° and their mating phenotypes determined. Two temperature-resistant isolates of the original *npl3-95* strain (JRY5000) were obtained and both had restored the nonmating phenotype of the starting strain (*mat***a**1 *HMR-E-RAP1-10*) used in the genetic screen, indicating that the temperature-sensitivity and mating phenotypes were probably due to mutation of a single gene. To determine if the *npl3-95* allele would reduce silencing at the chromosomal *HMR***a** locus, a *npl3-95* strain was crossed to strains that bore either *hmr-Δe331-324* or *HMR-SS ΔI.* On dissection of the resulting diploids (JRY5001/YAB102 and JRY5001/JRY3935), temperature-sensitivity segregated 2:2, providing independent evidence that the phenotype was due to mutation of a single nuclear gene. Moreover, the number of nonmating segregants (all were temperature sensitive) was consistent with the temperature-sensitive mutation causing derepression of *hmr-Δe331-324* and *HMR-SS ΔI* (from 60 tetrads examined, the predicted number of *MATα npl3-95 hmr-Δe331-324* or *HMR-SS ΔI* segregants was 36, and the observed number of nonmating *MATα npl3-95* segregants was 35; see MATERIALS AND METHODS and Table 2; Figure 2).

To clone the gene affected by the temperature-sensitive mutation, DNA clones that complemented both the temperature-sensitivity and mating defect of a *MATα hmr-Δe331-324 npl3-95* strain (JRY4431) were isolated from a genomic library. Plasmids that contained three overlapping inserts were isolated (pJR1677–1679), and restriction analysis and subcloning experiments revealed a complementing fragment that was common to all three plasmids (see MATERIALS AND METHODS). A search of sequence databases revealed that the complementing gene was the previously sequenced gene named *NOP3* (RUSSELL and TOLLERVEY 1992) or *NPL3* (BOSSIE *et al.* 1992).

In some strains, *NPL3* is an essential gene (RUSSELL and TOLLERVEY 1992), whereas in other strains a deletion of *NPL3* causes temperature-sensitive growth (BOSSIE *et al.* 1992). To determine which was the case in our strains and to determine the silencing phenotype of the null mutant if it were viable, a complete deletion of the *NPL3* open reading frame was made in a diploid (W303–1A/JRY3935). Upon dissection of the heterozygous diploid, haploid strains carrying the deletion were found to be viable but temperature sensitive for growth.

The restrictive temperature for strains bearing the deletion of *NPL3* was lower than for strains bearing the *npl3-95* mutation (data not shown). Curiously, *MATα npl3Δ HMR-SS ΔI* strains showed no silencing defect (Table 2); however, the *npl3-95* mutation may be semidominant because several diploid strains heterozygous for the *npl3-95* mutation (for example, JRY5066) grew more slowly at 37° than a wild-type *NPL3* diploid strain (JRY5064) or a diploid strain heterozygous for a *NPL3* deletion (JRY5065), whereas the growth rates of JRY5064 and JRY5065 were indistinguishable.

YCL54: Another temperature-sensitive strain obtained from the screen for silencing defective mutants contained a mutation in a gene defined by open reading frame YCL54 on chromosome *III* (OLIVER *et al.* 1992). Until the primary biochemical function of the YCL54 protein is determined, the gene and mutation described in this report will be referred to as *YCL54* and *ycl54-1*, respectively.

The genetic characterization of *ycl54-1* paralleled that of the *abf1* and *npl3* mutants. Temperature-resistant revertants of a *ycl54-1* strain (JRY4662) were selected at 37° and their mating phenotypes determined. Eighteen temperature-resistant isolates of the *ycl54-1* strain were obtained, 14 of which had restored the nonmating phenotype of the starting strain, indicating that the temperature sensitivity and mating phenotypes were probably due to mutation of a single gene. Of the remaining three revertants, three had partial restoration of the mating phenotype and one continued to show derepression of *hmr-Δe331-324*. To determine whether a single mutation caused both the temperature-sensitive growth and mating phenotypes and to determine if the *ycl54-1* allele would reduce silencing at the chromosomal *HMR***a** locus, a *ycl54-1* strain was crossed to strains that bore either *hmr-Δe331-324* or *HMR-SS ΔI*. On dissection of the resulting diploids (JRY5002/YAB102 and JRY4710/JRY3935), temperature sensitivity segregated 2:2, providing independent evidence that the phenotype was due to mutation of a single nuclear gene. Moreover, the number of nonmating segregants (all were temperature sensitive) was consistent with the temperature-sensitive mutation causing derepression of *hmr-Δe331-324* and *HMR-SS ΔI* (from 55 tetrads examined, the predicted number of *MATα ycl54-1 hmr-Δe331-324* or *HMR-SS ΔI* segregants was 33 and the observed number of nonmating *MATα ycl54-1* segregants was 32; see MATERIALS AND METHODS and Table 2; Figure 2).

Genomic DNA clones were isolated that complemented both the temperature-sensitivity and mating defect of a *MATα hmr-Δe331-324 ycl54-1* strain (JRY5003). Six plasmids that contained overlapping inserts were isolated from two centromere-based plasmid libraries (pJR1681–1686). To determine if the cloned DNA fragments were linked to the lesion causing temperature-sensitive growth, a DNA fragment from one of the complementing clones was integrated into the genome, along with the *LEU2* gene (on pJR1688). A diploid (JRY5004/JRY5005) heterozygous for the *ycl54-1* mutation and the integrated fragment was dissected and the segregants scored for temperature sensitivity and leucine prototrophy. Of 47 tetrads dissected, the ratio of parental ditypes to tetratypes to nonparental ditypes was 43:4:0, indicating that the cloned fragment was ~4 cM from *ycl54-1*. Restriction analysis of the *ycl54-1* complementing clones revealed a 5.8-kb *Nhe*I-*Spe*I fragment that complemented *ycl54-1* (pJR1687). Mutation of a *Sph*I site within the fragment destroyed the ability of the fragment to complement *ycl54-1*. The sequence that flanked this *Sph*I site indicated that the complementing gene had a previously described open reading frame (YCL54) of unknown function on chromosome *III* (OLIVER *et al.* 1992). This gene exhibited significant homology to the *ftsJ* gene of *E. coli* (Figure 3) (OGURA *et al.* 1991; see DISCUSSION).

Silencing of *HML*: Silencing at *HML* was not reduced greatly in *abf1*, *npl3*, and *ycl54* mutants since *MAT***a** strains bearing these mutations mated well. However, the sensitivity of the mating assay can be increased by monitoring expression of *HMLα* in the absence of functional *MAT***a** genes, in a manner analogous to that used in the genetic screen that isolated these mutations. To determine whether the *abf1*, *npl3*, and *ycl54* mutations reduced silencing of *HMLα*, strains that were *HMLα mat***a***Δp hmrΔ::URA3* and either *abf1-102*, *npl3-95*, or *ycl54-1* were constructed. The *mat***a***Δp* allele lacked its promoter and hence was transcriptionally inactive (LOO and RINE 1994), and *HMR***a** was deleted to prevent interference with the mating assay by another potential source of **a** gene expression. The *abf1-102*, *npl3-95*, and *ycl54-1* mutations caused these strains to mate weakly as *α* cells at 23°, whereas the parental strain did not (Figure 4). Thus *ABF1*, *NPL3*, and *YCL54* were all required for silencing at *HML*, as well as at *HMR*, although the effect of *ycl54-1* on *HMLα* silencing was small.

Arrest phenotypes of *abf1*, *npl3*, and *ycl54* mutants: The *HMR-E* silencer functions as a chromosomal origin of DNA replication (RIVIER and RINE 1992), and its role as an origin may be related to its role as a transcriptional silencer. Because the genetic screen that identified the *abf1*, *npl3*, and *ycl54* mutations also identified mutations in genes encoding two subunits of the origin recognition complex (FOSS *et al.* 1993; LOO *et al.* 1995), and because *ABF1* has a role in ARS function (RHODE *et al.* 1992), it was possible that all mutations recovered from the screen would affect origin function or the cell cycle. To test this hypothesis, the arrest phenotypes of strains carrying the *abf1*, *npl3*, and *ycl54* mutations were examined microscopically, and the effects of these mutations on plasmid maintenance were determined.

None of the *abf1*, *npl3*, and *ycl54* mutations caused cell cycle specific arrest when strains bearing these mutations were placed at the restrictive temperature (Fig-

```
YCL54     2 GKTQKKNSKGRL.....DRYYYLAKEKGYRARSSFKIIQINEKYGHFLEK 46
            ||      |   |      | |   |  || | |  ||   |
ftsJ      3 GKKRSASSSRWLQEHFSDKYVQQAQKKGLRSRAWFKLDEIQQS DKLFKP 51

YCL54    47 SKVVIDLCAAPGSWCQVASKLCPVNSLIIGVDIVPMKPMPNVITFQSDIT 96
              | || |||| | |             ||  |  || |   |   | |
ftsJ     52 GMTVVDLGAAPGGWSQYVVTQIGGKGRIIACDLLPMDPIVGVDFLQGDFR 101

YCL54    97 TEDCRSKLRGYMKTWKADTVLHDGAPNVGLGWVQDAFTQSQLTLQALKLA 146
             |      |        |   |  | |||       |       |   ||
ftsJ    102 DELVMKALLERVGDSKVQVVMSDMAPNMSGTPAVDIPRAMYLVELALEMC 151

YCL54   147 VENLVVNGTFVTKIFRSKDYNKLIWVFQQLFEKVEATKPPASRNVSAEIF 196
              |   | || | |             || ||   ||  ||  | |
ftsJ    152 RDVLAPGGSFVVKVFQGEGFDEYLREIRSLFTKVKVRKPDSSRARSREVY 201

YCL54   197 VVCKGFKA 204
              | | |
ftsJ    202 IVATGRKP 209
```

FIGURE 3.—Sequence similarity between *Saccharomyces cerevisiae YCL54* and *E. coli ftsJ.* The first 209 amino acids of *YCL54* are shown aligned against the entire *ftsJ* protein. In these regions, the two proteins are 31% identical. The remainder of *YCL54* (amino acids 210–724) is not shown and was not homologous to proteins in the SwissProt database (Release 26). Sequence alignment was determined with Bestfit (Genetics Computer Group Sequence Analysis Software Version 7.3).

ure 5). At 37°, all four of the *abf1* mutations caused cells to arrest at various positions in the cell cycle. For those cells that had a bud, the *abf1* mutations caused the buds to become elongated, a phenotype previously reported for other temperature-sensitive *abf1* alleles (RHODE *et al.* 1992). In addition there was no block to the division and segregation of nuclei in cells that had formed elongated buds. The *npl3-95* mutation caused arrest at all positions in the cell cycle as judged by budding morphology; however, at the nonpermissive temperature, *npl3-95* cells became enlarged and lacked discrete DAPI-staining nuclei. Although *ycl54-1* cells ceased to divide at the restrictive temperature, no changes in budding or nuclear morphology were noted.

Another characteristic of mutations in *ORC2* and *ORC5* that were obtained in the genetic screen described in this study is that they caused replication defects (BELL *et al.* 1993; FOSS *et al.* 1993; FOX *et al.* 1995; LOO *et al.* 1995). To determine if mutations in *ABF1, NPL3,* and *YCL54* might also cause similar defects, strains bearing mutations in each of these genes, and strains containing either *orc2-1* or *orc5-1,* were transformed with a plasmid that relied on a synthetic *HMR-E* silencer as its sole origin of replication. In its capacity as an ARS, the synthetic silencer is sensitive to mutations in genes encoding ORC subunits; no stable transformants of these plasmids can be obtained in *orc2-1* strains, and *orc5-1* strains carrying these plasmids fail to maintain them efficiently (Table 3) (FOX *et al.* 1995). None of the *npl3* or *ycl54* mutants had a measurable effect on the stability of plasmids replicated by the synthetic silencer (Table 3), suggesting that their roles in silencing were not coupled to a role in replication. In contrast, the *abf1-102* mutation caused an approximately threefold increase in plasmid loss rate.

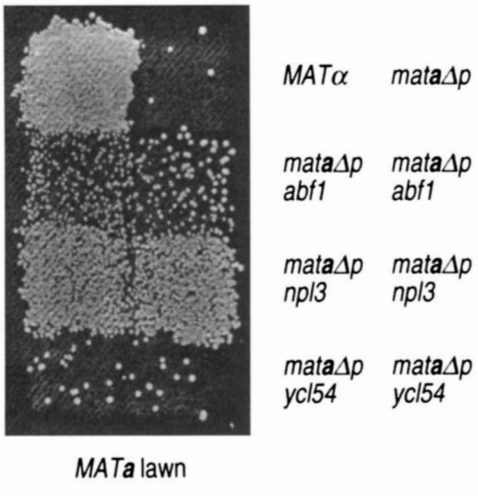

FIGURE 4.—*ABF1, NPL3,* and *YCL54* were required for silencing at *HML.* Matings are shown for strains lacking functional **a** genes at *MAT* or at *HMR,* thus the ability to mate as an a strain indicated derepression of *HMLα.* Of these three temperature-sensitive mutations, *ycl54-1* caused the least derepression of *HMLα.* Because the mutant strains were not isogenic, two isolates of each strain are shown.

The roles of *ABF1, NPL3,* and *YCL54* in silencer function: From the design of the genetic screen used to isolate the *abf1, npl3,* and *ycl54* mutants, none of these genes can function exclusively through the Rap1p binding site of the *HMR-E* silencer because these mutations increase derepression of *HMR* when combined with *HMR-E* silencers lacking their Rap1p binding sites (*HMRα-E-RAP1-10* or *hmr-Δe331-324,* Table 2). Similar double-mutant analysis to determine if *ABF1, NPL3,* and *YCL54* function through either or both of the two remaining silencer elements of the natural *HMR-E* silencer was precluded by the presence of multiple ARS consensus sequence matches and Abf1p binding sites at *HMR.* To overcome one of these complications, an *HMR* locus that possessed a synthetic *HMR-E* silencer and a deletion of *HMR-I* (*HMR-SS ΔI*) (MCNALLY and RINE 1991) was used to sensitize the mating assay. A *MATα* strain with *HMR-SS ΔI* mated with an efficiency of ~0.2, and mutation of the sole remaining Abf1p binding site at this locus (*HMR-SSabf1⁻ ΔI*) such that it is no longer capable of binding Abf1p causes another 10-fold reduction in mating ability (MCNALLY and RINE 1991; Table 4). If *ABF1, NPL3,* and *YCL54* function solely through the Abf1p binding site, then double mutants combining *HMR-SSabf1⁻ ΔI* with the temperature-sensitive mutants should mate no worse than a strain

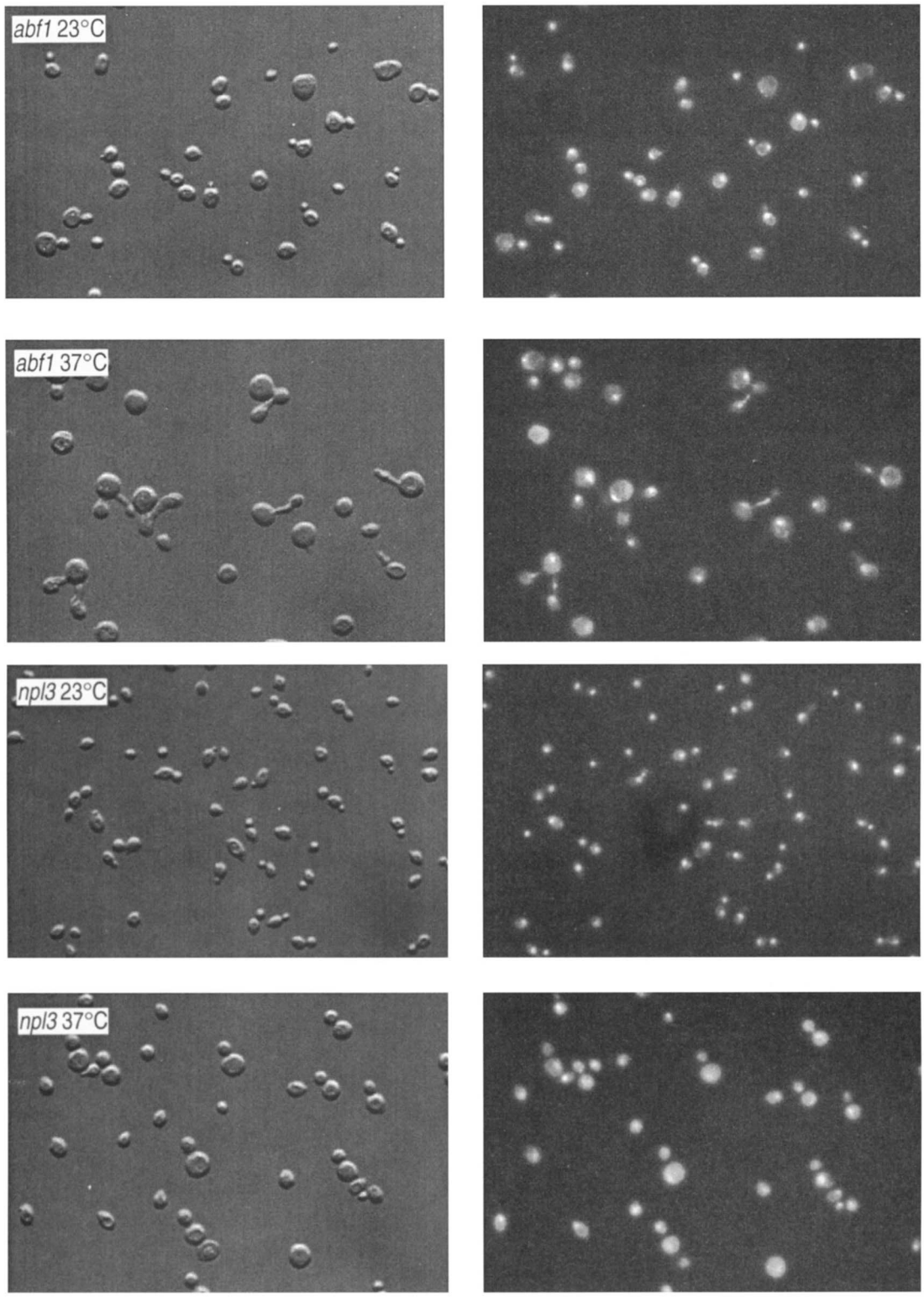

FIGURE 5. (A–C) Morphology of cells bearing mutations in *ABF1*, *NPL3*, or *YCL54* grown at the restrictive temperature. *abf1-102*, *npl3-95*, and *ycl54-1* strains (JRY4701, JRY4431, and JRY4662, respectively) were grown to mid-log phase in rich medium at 23°, after which the cultures were shifted to 37° for 6 hr. Cells were stained with DAPI and photographed using Nomarski optics (left) or under fluorescent illumination (right) to detect DAPI staining of nuclei.

that is *MATα* and *HMR-SSabf1*$^{-}$ ΔI alone. By this analysis, *ABF1* functioned exclusively through its cognate binding site at the synthetic silencer because the mating defect was no worse in the presence of the *abf1* mutation. In contrast, neither *NPL3* nor *YCL54* functioned in silencing solely though the Abf1p binding site because both mutations decreased the mating efficiency of a strain with a mutant Abf1p binding site (Table 4).

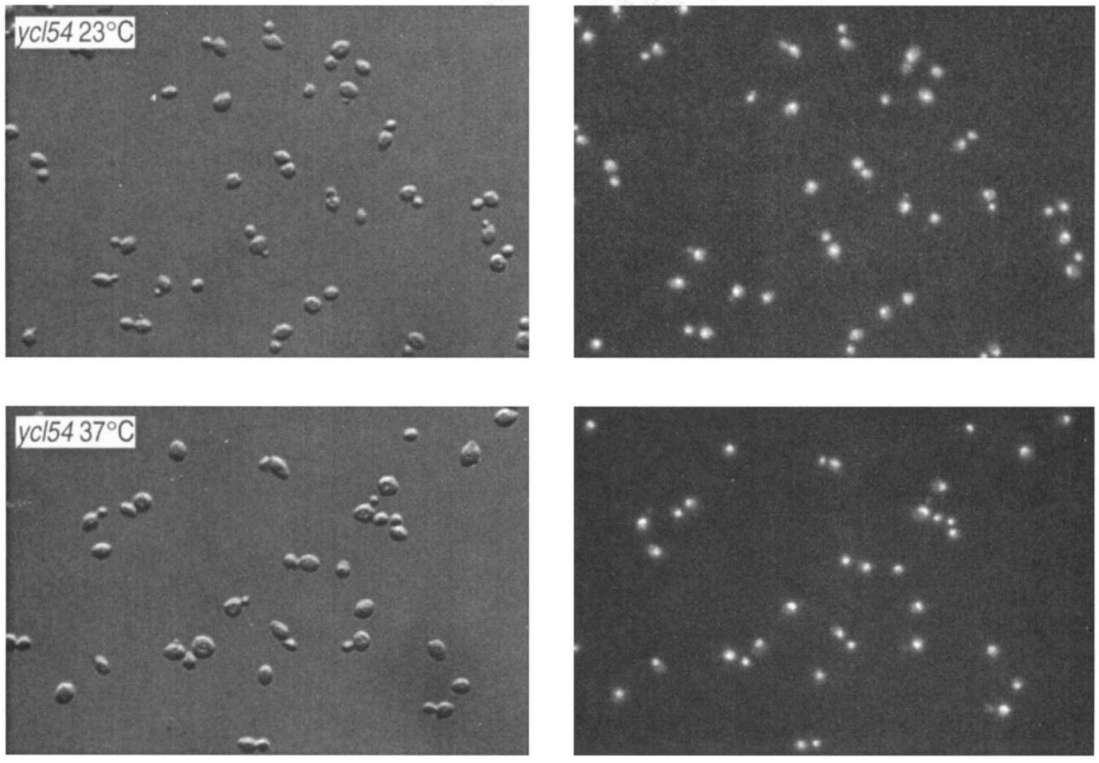

FIGURE 5.—*Continued*

Double-mutant analysis designed to test whether *ABF1, NPL3,* and *YCL54* act through the ARS consensus sequence of the *HMR-E* silencer was also complicated by the compound nature of the *HMR* silencers. The natural *HMR-E* silencer is positioned within a cluster of close matches to the ARS consensus sequence (LOO and RINE 1995). These elements, as well as an additional near match at the *HMR-I* silencer, may contribute to silencing. Thus, an *HMR-E* allele that lacks one ARS consensus sequence may still rely on proteins that regulate the function of the ARS consensus sequence for silencing. Unfortunately, the synthetic silencer allele that is mutated at its ARS consensus sequence is completely nonfunctional as a silencer. Hence it was not possible to carry out double-mutant analysis between that silencer and the temperature-sensitive mutations in *ABF1, NPL3,* and *YCL54.* Instead, the effects of these temperature-sensitive mutations on silencing in a strain that had an *orc5-1* mutation were determined. If the *abf1, npl3,* and *ycl54* mutations no longer had an effect (or as large an effect) on silencing in the presence of *orc5-1,* then these mutations may act through ORC, and presumably the ARS consensus sequence. No synthetic lethality or weakened growth phenotypes were noted for strains bearing *orc5-1* and another temperature-sensitive mutation in either *ABF1, NPL3,* or *YCL54* (data not shown). However, each of the *abf1, npl3,* and *ycl54* mutations exacerbated the silencing defect of a *MATα orc5-1 HMR-SS ΔI* strain (Figure 6).

TABLE 3

Plasmid loss rates of *ORC, ABF1, NPL3,* and *YCL54* mutants

Relevant genotype	Loss rate	Strains used
wild-type	0.004 ± 0.004	W303-1B
orc2-1	No transformants	JRY4125
orc5-1	0.256 ± 0.008	JRY4249
abf1-102	0.029 ± 0.010	JRY3795
abf1-102 (revertant)	0.009 ± 0.005	JRY5063
npl3-95	0.010 ± 0.006	JRY4431
ycl54-1	0.010 ± 0.008	JRY4662

Plasmids were replicated by the synthetic silencer (pJR954; MCNALLY and RINE 1991). The *abf1-102* (revertant) is a spontaneous temperature-resistant, silencing-competent revertant of a *MATα HMR-SS ΔI abf1-102* strain (JRY3765).

DISCUSSION

***ABF1*:** This report describes the isolation of new mutations affecting silencing, including four temperature-sensitive alleles of *ABF1.* Abf1p's ability to bind some silencers *in vitro* provides biochemical evidence for a role in silencing (BUCHMAN *et al.* 1988; DIFFLEY and STILLMAN 1988). However, without mutations in *ABF1* with silencing defects, it was possible that another protein bound the silencers *in vivo.* Thus the isolation of loss-of-function mutations in *ABF1* that reduced silencing provided the genetic proof of its involvement in silencing.

Abf1p shares many characteristics with another silencer-binding protein, Rap1p (reviewed in SHORE 1994). Both Abf1p and Rap1p are essential, and both

TABLE 4

Mating efficiencies of double mutants between *abf1-102*, *npl3-95*, or *ycl54-1* and compromised synthetic silencers

Relevant genotype	Mating efficiency	Strains used
Matα HMR-E	1.0	W303-1B
MATα HMR-SS ΔI	$1.7 \times 10^{-1} \pm 4.5 \times 10^{-2}$	JRY3935
MATα HMR-SSabf1⁻ ΔI	$1.8 \times 10^{-2} \pm 1.4 \times 10^{-2}$	JRY4889
MATα HMR-SSabf1⁻¹ ΔI abf1-102	$1.9 \times 10^{-2} \pm 2.4 \times 10^{-2}$	JRY4908-11
MATα HMR-SSabf1⁻ ΔI npl3-95	$2.2 \times 10^{-5} \pm 2.4 \times 10^{-6}$	JRY4963-66
MATα HMR-SSabf1⁻ ΔI ycl54-1	$4.2 \times 10^{-4} \pm 4.6 \times 10^{-4}$	JRY4967-70

HMR-SS ΔI is an allele of *HMR* flanked on the left by the synthetic *HMR-E* silencer and on the right by a deletion of *HMR-I*. *HMR-SSabf1⁻ ΔI* lacked the Abf1p binding site at the synthetic silencer as well.

bind to the UAS elements of a number of different genes where they serve as transcriptional activators (Huet *et al.* 1985; Halfter *et al.* 1989; Chambers *et al.* 1990; Della Seta *et al.* 1990a,b; Kurtz and Shore 1991). Abf1p and Rap1p binding sites are found in or near a number of ARS elements (reviewed in Zakian 1989; Campbell and Newlon 1991), and in the case of Abf1p, loss-of-function mutations cause a modest reduction in ARS efficiency (Table 3; Rhode *al.* 1992). Finally, both Rap1p and Abf1p share regions of limited sequence homology (Diffley and Stillman 1989).

How do proteins such as Rap1p and Abf1p fulfill their disparate activation and silencing roles? In the case of Rap1p, there is strong evidence that its activation and silencing functions are provided by separate domains: Rap1p possesses a C-terminal silencing domain that is dispensable for its activation function (Sussel and Shore 1991), and certain Rap1p fragments that are unable to activate transcription will silence when tethered to *HMR-E* (Buck and Shore 1995). Even though Rap1p and Abf1p share some sequence homology, their similarity does not include the portion of Rap1p that seems to be devoted to silencing. Extensive mutagenesis of *ABF1* has not yet been carried out. Thus, unlike for Rap1p, there is no strong evidence for or against Abf1p having separable functions in activation and in silencing.

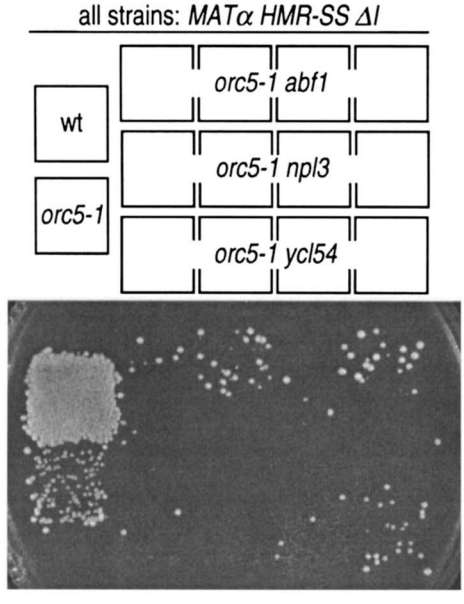

Figure 6.—Silencing defects caused by mutations in *ABF1*, *NPL3*, and *YCL54* when combined with *orc5-1*. In addition to the mutations indicated, all of the strains are *MATα* and have a synthetic silencer allele at *HMR* (*HMR-SS ΔI*). Because the mutant strains were not isogenic, four isolates of each double-mutant strain are shown.

In another view, Abf1p bound at the *HMR-E* silencer may help activate the *HMR-E* origin, either by binding ORC or by stimulating replication initiation. Interestingly, the *abf1-102* allele described in this work and the deletion of the Abf1p binding site of the synthetic silencer caused only a modest reduction in the synthetic silencer's ability to act as an ARS (Table 3; McNally and Rine 1991), yet both of these mutations cause as large a silencing defect as the *orc2-1* or *orc5-1* mutations, both of which dramatically reduce replication initiation at the synthetic silencer (Fox *al.* 1995). These observations suggest that Abf1p may play a role at the silencers that is not limited to replication initiation.

***NPL3*:** *NPL3* encodes a protein with strong homology to a class of RNA binding and processing proteins. Consistent with this predicted function, depletion of Npl3p causes rRNA processing defects (Russell and Tollervey 1992). It is not yet clear whether or how this role of *NPL3* is related to its silencing function. However, because the *npl3-95* mutation (and the other temperature-sensitive mutations described here) affected silencing of both **a** and *a* genes, it was unlikely that the mating phenotypes were due to effects on the splicing of the **a**1 mRNA. The lack of essentiality of *NPL3* in our studies may suggest that an extragenic modifier may be present in one strain but not in others.

Other temperature-sensitive mutations in *NPL3* are defective in nuclear protein localization (Bossie *et al.* 1992). These alleles are unable to localize efficiently histone H2B and other proteins to the nucleus. Because histone dosage is important for proper regulation of gene expression (Clark *et al.* 1988) and because histones H3 and H4 are important for silencing (Kayne *et al.* 1988; Johnson *et al.* 1990; Megee *et al.* 1990; Park

and SZOSTAK 1990; THOMPSON *et al.* 1994), a reasonable hypothesis would be that *NPL3*'s silencing defect was due to the mutant's inability to localize proteins such as the histones to the nucleus. This correlation is strengthened by the discovery that a *NPL3* null allele has neither silencing (Table 2) nor nuclear protein localization defects (BOSSIE *et al.* 1992).

***YCL54*:** Of the genes described in this report, the least is known about *YCL54*. Although *YCL54* and *E. coli ftsJ* share significant sequence homology, the only other information on either gene's function is that *ftsJ* mutants cause filamentous growth of *E. coli* and is part of an operon induced in cell division (OGURA *et al.* 1991). Therefore, little mechanistic information can be deduced from *YCL54*'s primary sequence. Interestingly, ribosomal RNA was underrepresented in total RNA isolated from *ycl54-1* cells (Figure 2). In what may be a related phenomenon, strains depleted of *NPL3* are defective in ribosomal RNA processing as well (RUSSELL and TOLLERVEY 1992). *SIR2* mutations enhance recombination within the DNA repeats encoding ribosomal RNAs (GOTTLIEB and ESPOSITO 1989). Thus one possibility is that *SIR2, NPL3,* and *YCL54* all have a role in nucleolar function, and that *npl3-95* and *ycl54-1* disrupt silencing by interfering with *SIR2* function. The null phenotype of *YCL54* is unknown.

The design of the genetic screen used in this work was based on weakening the redundancy among the binding sites at the *HMR-E* silencer. As predicted from the phenotypes of binding site mutations alone, mutations in genes encoding ORC subunits or Abf1p completed the derepression and hence were recovered in the screen. Thus in the case of the temperature-sensitive mutations in *ORC* genes or in *ABF1,* the combination of biochemical data and double-mutant analysis could narrow their sites of action to the ARS consensus sequence and the Abf1p binding site respectively.

In contrast, the enhanced silencing defects caused by *npl3-95* and *ycl54-1* with either the Rap1p or the Abf1p binding site mutations of the *HMR-E* silencer argued that neither of these genes function exclusively through the Rap1p and Abf1p binding sites. Although it is still possible that *NPL3* and *YCL54* are required for proper utilization of the ARS consensus sequence, this scenario seems unlikely because mutations in these genes caused neither defective cell cycle progression nor synthetic lethality or inviability when combined with the temperature-sensitive *orc5-1* mutation. Similarly, neither *npl3-95* nor *ycl54-1* mutants exhibited an elevated plasmid loss rate. In contrast, *orc5-1* double mutants with *orc2-1* or a variety of other *cdc* mutants thought to be involved in replication initiation are either inviable or exhibit greatly reduced growth rates (LOO *et al.* 1995). Thus the available data are most consistent with *NPL3* and *YCL54* affecting some step in silencing after silencer function.

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