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Analysis of a Meiosis-Specific URS1 Site: Sequence Requirements and Involvement of Replication Protein A

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URS1 is a transcriptional repressor site found in the promoters of a wide variety of yeast genes that are induced under stress conditions. In the context of meiotic promoters, URS1 sites act as repressor sequences during mitosis and function as activator sites during meiosis. We have investigated the sequence requirements of the URS1 site of the meiosis-specific *HOP1* gene ($URS1_H$) and have found differences compared with a URS1 site from a nonmeiotic gene. We have also observed that the sequence specificity for meiotic activation at this site differs from that for mitotic repression. Base pairs flanking the conserved core sequence enhance meiotic induction but are not required for mitotic repression of *HOP1*. Electrophoretic mobility shift assays of mitotic and meiotic cell extracts show a complex pattern of DNA-protein complexes, suggesting that several different protein factors bind specifically to the site. We have determined that one of the complexes of $URS1_H$ is formed by replication protein A (RPA). Although RPA binds to the double-stranded $URS1_H$ site in vitro, it has much higher affinity for single-stranded than for double-stranded $URS1_H$, and one-hybrid assays suggest that RPA does not bind to this site at detectable levels in vivo. In addition, conditional-lethal mutations in RPA were found to have no effect on $URS1_H$-mediated repression. These results suggest that although RPA binds to $URS1_H$ in vitro, it does not appear to have a functional role in transcriptional repression through this site in vivo.

In the yeast *Saccharomyces cerevisiae*, the process of meiosis and spore formation is carried out by a highly regulated and complex pathway. The initiation of meiosis requires a specific set of genetic and nutritional conditions (32). Once these conditions are met, a cascade of regulatory events leads to the specific expression of target genes that are required for various meiosis-specific processes such as pairing of the chromosomes, meiotic recombination, reductional segregation of the homologs, and spore formation (14). All of these events require proteins that are specific to the process of meiosis, and often the genes that encode these proteins are expressed at a precisely defined point in the meiotic pathway. Many of these genes appear to be regulated in a coordinated fashion to ensure that they are expressed at the proper level and time during meiosis. Improper expression of these proteins may block specific steps in meiosis and produce incomplete or inviable spores (4, 20, 24, 37). The appropriate regulation of these genes is therefore critical to the process of meiosis.

We have chosen to study the transcriptional regulation of the meiosis-specific *HOP1* gene to determine which proteins bind to meiosis-specific promoters and regulate their expression. Hop1 is expressed early during the meiotic pathway and is required for the formation of the synaptonemal complex, prior to the first meiotic division (20, 21). Two regulatory sites, $URS1_H$ and UAS_H, which are required for the proper regulation and expression of *HOP1* during mitosis and meiosis have been identified in the promoter region of *HOP1*. The UAS_H site functions as a constitutive activator site and is required for the full expression of *HOP1* during meiosis (36, 46). The general transcription factor Abf1 has been purified as the binding activity of the UAS_H site, and mutational analysis of UAS_H has shown that it is indeed an Abf1-binding site (15). During vegetative growth, transcriptional activation of the *HOP1* promoter by Abf1/UAS_H is repressed by the factors that interact at the $URS1_H$ site. However, after cells enter meiosis, $URS1_H$ switches its function and becomes an activator site, which together with Abf1/UAS_H, is required for high level expression of *HOP1*.

Sites with strong sequence similarity to the $URS1_H$ site have been identified in almost every early meiotic gene, as well as in a wide variety of nonmeiotic genes, in yeast (5, 10, 39, 43, 46). Analyses of the URS1 sites from the meiosis-specific genes *SPO13*, *HOP1*, and *IME2* have shown that they function as repressor sites during mitotic growth and either are derepressed or serve as activator sites during meiosis (6, 10, 46). The canonical and perhaps most extensively studied URS1 site is part of the promoter of the nonmeiotic gene *CAR1*, a gene which codes for arginase and is required for nitrogen metabolism (26, 43). This site, which we will refer to as $URS1_C$, functions to repress *CAR1* expression in the absence of arginine and also works as a strong repressor element in heterologous promoters. $URS1_C$ consists of a 9-bp palindromic sequence (5′-TCGGCGGCT-3′); there is an 8-of-9-bp identity between the *HOP1* and *CAR1* URS sites within this core region, suggesting that the same proteins may function at both of these sites (26, 43, 46). This prediction is supported by the observation that the *CAR1*, *SPO13*, and *IME2* URS1 sites require the presence of Ume6, also known as Car80, to repress transcription (6, 34, 42). Ume6 contains a Cys_6 cluster, as does the DNA-binding domain of the transcriptional activator Gal4, and purified Ume6 protein has been shown to directly bind to the *SPO13* URS1 ($URS1_{SPO13}$) site (3, 42). The Ume6 protein is also required for the URS1 site to function as a meiosis-specific activator site (6, 40, 42). Two-hybrid experiments have shown that the major inducer of meiosis, the Ime1 protein, and

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TABLE 1. Strains used

Strain	Genotype	Reference or source
YV16	**a**/α *TRP1/trp1 ura3-52/ura3-x CAN1/can1 LEU1/leu1 CYH2/cyh2 ade2-1/ade2-R8 HIS7/his7*	42
RSY270	**a** *can1 his4-519 leu2-3,112 lys2 trp1 ume6-5 ura3*	38
RSY271	**a** *can1 his4-519 leu2-3,112 lys2 trp1 ura3*	38
LNY273	**a**/α *leu2-hisG/leu2-hisG trp1-hisG/trp1-hisG lys2-SK1/lys2-SK1 gal80-LEU2/gal80-LEU2 IME1-14-TRP1/+ ura3-SK1/ura3-SK1 ho::LYS2/ho::Lys2*	L. Neigeborn
LNY2	α *ura3 leu2::hisG trp1::hisG lys2 ho::LYS2*	L. Neigeborn
LNY3	**a**/α *ura3/ura3 leu2::hisG/leu2::hisG trp1::hisG/trp1::hisG lys2/lys2 ho::LYS2/ho::LYS2*	L. Neigeborn
W303-1a	**a** *ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100*	41
HMY357	W303-1a *rfa2::TRP1 HIS3::RFA2*	28
HMY344	W303-1a *rfa2::TRP1 HIS3::rfa2-55*	28
HMY343	W303-1a *rfa2::TRP1 HIS3::rfa2-210*	28
HMY345	W303-1a *rfa2::TRP1 HIS3::rfa2-214*	28
HMY350	W303-1a *rfa2::TRP1 HIS3::rfa2-215*	28
HMY346	W303-1a *rfa2::TRP1 HIS3::rfa2-C100*	28
HMY353	W303-1a *rfa3::TRP1 HIS3::rfa3-313*	28
HMY347	W303-1a *rfa3::TRP1 HIS3::rfa3-N70*	28
PCY2	α *Δgal4 Δgal80 URA3::GAL1-lacZ lys2-801 his3-Δ200 trp1-Δ63 leu2 ade2-101*	10
PCY2U	PCY2 *ura3*	This study

the protein kinase Rim11 interact with Ume6 to form a transcriptional activator complex (38). Although Ume6 clearly plays a major role in mitotic repression and meiotic activation through the URS1 site, it is possible that other proteins function at URS1 sites as well. For example, electrophoretic mobility shift assays (EMSAs) of $URS1_{SPO13}$ show that there are at least four shifts that are not dependent on Ume6/Car80, suggesting that other proteins may recognize and function at this site (34, 42). A second binding activity, called Buf, that binds to a nonmeiotic URS1 site has been identified (27). This binding activity has been purified to homogeneity and was identified to be replication protein A (RPA) (26). RPA consists of three subunits encoded by the *RFA1*, *RFA2*, and *RFA3* genes (encoding 69-, 36-, and 13-kDa proteins, respectively) and was first shown to be an essential, single-stranded, nonspecific DNA-binding protein involved in DNA replication (8, 9, 19). However, recent work has shown that RPA also binds to double-stranded URS1 sites, and it has been implicated in transcriptional regulation (39, 44). It is also possible that different proteins function at the URS1 sites in meiotic and nonmeiotic promoters.

In this study, we investigated whether the sequence requirements of a meiotic URS1 site are the same as those of a URS1 site found in a nonmeiotic promoter. Although the *HOP1* site has sequence requirements similar to those of the nonmeiotic *CAR1* site, we find some differences between the sites. We also investigated whether the sequence specificity for mitotic repression by the $URS1_H$ site is the same as for meiotic activation. Our genetic and biochemical results show that there are changes in the sequence requirements at the $URS1_H$ site during mitosis and meiosis. Finally, we find that the RPA complex binds with relatively low affinity to the meiotic $URS1_H$ site in vitro, that RPA does not appear to bind to this site with high affinity in vivo, and that *rfa* mutants have no effect on $URS1_H$-mediated repression. These results suggest that RPA does not play an important role in *HOP1* regulation.

MATERIALS AND METHODS

Yeast strains, media, and culture conditions. Strains used in this study are described in Table 1. Strains containing temperature-sensitive *rfa2* and *rfa3* mutations are described by Maniar et al. (31). Synthetic growth medium, liquid sporulation medium, and YEPD have been described previously (46). YEPAc media contained 1% yeast extract (Difco), 2% Bacto Peptone (Difco), and 2% potassium acetate. Transformations were performed by the lithium acetate procedure (22). All yeast strains were grown at 30°C except for the *rfa* mutant strains, which were maintained at the permissive temperature of 25°C, unless otherwise noted.

Oligonucleotide synthesis. All oligonucleotides were synthesized on a model 392-5 Applied Biosystems DNA synthesizer. Oligonucleotides used for cloning were cleaved on the instrument, deprotected, dried, resuspended in Tris-EDTA, and used without further purification. Oligonucleotides used in DNA-binding and competition assays were purified by C_{18} reverse-phase high-pressure liquid chromatography. The oligonucleotide containing the top strand of the wild-type $URS1_H$ site used in these studies has the sequence 5′-tcgacTTAACCTGGGCGGCTAAATTc and corresponds to bp −179 to −160 upstream of the start site of translation in the *HOP1* promoter. Lowercase letters are bases added to the site for cloning purposes, and the underlined bases indicate the core element of the URS1 site. All $URS1_H$ mutants described in this study were constructed in this sequence background. The oligonucleotide containing the top strand of the $URS1_C$ site has the sequence 5′-tcgacGACCCCTCGGCGGCTACCGCc. This site is identical to the site used in the analysis of the $URS1_C$ site (26).

Construction of plasmids. To construct *HOP1-lacZ* reporter promoters with mutant $URS1_H$ sites, synthetic oligonucleotides containing the top and bottom strands with the desired substitutions were annealed and ligated to the *Xho*I site of plasmid pAV124. pAV124 contains a 207-bp region of the *HOP1* promoter and the region coding for the first 115 residues of the protein fused in frame to the *lacZ* gene. Five base pairs within the $URS1_H$ site in the promoter were mutated to create a *Xho*I site into which the $URS1_H$-containing oligonucleotides were cloned (46). Oligonucleotides containing mutant $URS1_H$ sites were also cloned into the *Sal*I site of pUC18, and the 58-bp *Hin*dIII/*Bam*HI fragments containing these sites were used in the EMSAs described below. Plasmids pAV73, pΔSS, and pTBA30 are derivatives of pLGΔ312S and contain a *CYC1-lacZ* fusion under the control of the *CYC1* promoter (1, 17, 46). These plasmids differ only by the presence (pAV73) or absence (pΔSS and pTBA30) of the endogenous *CYC1* UAS sites. Mitotic repression of the *CYC1-lacZ* reporter was measured with pAV138-1, which contains one $URS1_H$ site cloned between the UAS_{CYC} and TATA elements (46). Meiotic activation of the *CYC1-lacZ* reporter was measured in constructs which contain tandem URS1 sites cloned upstream of the TATA element in pΔSS. All plasmid constructions were transformed into *Escherichia coli* DH5α, screened by restriction digest, and verified by dideoxy sequencing.

Plasmids for the one- and two-hybrid experiments were based on the Gal4 DNA-binding domain (GBD) fusion vector, pPC97, and the Gal4 activation domain (GAD) fusion vector, pPC86 (11). Before insertion of the *RFA1* or *RFA2* sequences, the selectable markers of these two plasmids were switched by exchanging their *Apa*I/*Not*I fragments to create pGDB (*TRP1*) and pGAD (*LEU2*) fusion vectors. Full-length *RFA1* and *RFA2* inserts were then prepared by PCR amplification, using oligonucleotides with unique *Sal*I and *Bam*HI sites, and inserted into the *Sal*I and *Bgl*II polylinker sites of pGDB and pGAD to create pRFA1-GDB, pRFA2-GDB, pRFA1-GAD, and pRFA2-GAD. The resulting plasmids express the *GAL4* sequences fused to the fourth amino acid of RPA. Plasmids pRFA1-GAD and pRFA2-GAD were shown to complement the respective *rfa* null mutation as described by Philipova et al. (35).

β-Galactosidase assay. β-Galactosidase activities of the various *HOP1-lacZ* constructs were determined in strains YV16 and LNY273 as described previously (15, 46). $URS1_H$-mediated repression in *rfa* mutant strains was measured by assaying *lacZ* expression of transformants with a heterologous *CYC1-lacZ* re-

porter promoter that contains a $URS1_H$ site (pAV138-1) or a *CYC1-lacZ* construct (pAV73) that does not contain the site (46). Transformants were grown at 25°C on SD-Ura medium to saturation, diluted 1:10 in the same medium, grown overnight at 25°C, and then assayed for β-galactosidase activity. Cells were then shifted to the nonpermissive temperature (37°C) and assayed after 5 h for β-galactosidase activity. β-Galactosidase activities in the two-hybrid assays were determined in strain PCY2 (11). One-hybrid assays were performed in PCY2U, a Ura^- derivative of strain PCY2 that was selected for the loss of the *URA3* marker on medium containing 5-fluoro-orotic acid. All β-galactosidase assays were performed with at least three independent transformants of each construct, and each experiment was repeated twice.

Preparation of yeast cell extracts and RPA for DNA-binding studies. Saturated cultures of diploid (LNY3, LNY273, and YV16) and haploid (LNY2 and RSY271) strains were inoculated at a 1:50 dilution into YEPD medium, grown for 12 h at 30°C, then diluted 1:10 into YEPAc medium, and grown overnight at 30°C. Extracts were made as described previously (15) except that the lysis buffer was modified to include 20 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, 1 mM dithiothreitol, 1 mM $MgCl_2$, 0.1 mM phenylmethylsulfonyl fluoride, 30 μM tolylsulfonyl phenylalanyl chloromethyl ketone, 2 mM leupeptin, 1.5 mM aprotinin, 1 μM pepstatin A, 2 mM benzamidine, and 10% (wt/vol) glycerol (1 g of cells/ml of extraction buffer). Protein concentration was determined by the method of Bradford (7) (Bio-Rad protein assay), and all extracts were normalized to a protein concentration of 2 mg/ml. Protein extracts made from *rfa* mutant strains were prepared from cells grown to log phase in YEPD at 25°C and from cells which have been additionally incubated at 37°C for 5 h.

The trimeric RPA was purified from yeast as described previously (9). Recombinant RPA was purified from *E. coli* BL21(DE3) carrying plasmid pJM126, which expresses the three *RFA* genes from separate phage T7 promoters, as described in He et al. (18).

EMSAs. EMSAs of the $URS1_H$ sites were performed with radiolabeled *Bam*HI/*Hin*dIII fragments from pUC18 derivatives containing the URS1 sites. Plasmids were digested with *Hin*dIII and *Bam*HI, and the 5′ overhangs were filled in with [α-^{32}P]dATP by using Klenow polymerase. The 62-bp probe was purified on a 6% nondenaturing polyacrylamide gel. Oligonucleotide probes were end labeled with [γ-^{32}P]ATP by using T4 polynucleotide kinase and purified by using a Nensorb column (NEN) as instructed by the manufacturer. The oligonucleotides were made double stranded by the addition of a threefold excess of the unlabeled matching strand, incubated at 90°C for 20 min, and then slowly cooled to 25°C overnight in a water bath. Double-stranded oligonucleotides used in the competition experiments were made by mixing the strands at equal molar concentrations. The degree of annealing and the amount of double-stranded DNA were monitored by running the oligonucleotides on a 15% nondenaturing polyacrylamide gel and comparing the size and intensity with similar amounts of the single-stranded oligonucleotides.

Binding reactions for the $URS1_H$-binding factor(s) were carried out as described previously (15). In competition experiments, unlabeled competitor oligonucleotides were mixed with the probe before the addition of the cell extract. Antibody supershift experiments were performed by premixing the DNA probe with 5 μl of a 1:100 dilution of RPA antiserum against the 69-kDa subunit (9) before adding purified RPA or yeast extracts.

RESULTS

A URS1 site from a nonmeiotic gene can function as a meiosis-specific regulatory site. It was previously shown that a 5-bp mutation in the $URS1_H$ site of the *HOP1* promoter results in failure of this site to mediate repression during mitosis and activation of expression during meiosis (46). To investigate the effects of point mutations in the $URS1_H$ site and to assay other URS1 sites for meiosis-specific activity, we first demonstrated that we could reconstitute wild-type *HOP1* regulation by cloning a 26-bp oligonucleotide containing the wild-type $URS1_H$ site into the mutant promoter (see Materials and Methods). *HOP1-lacZ* expression regulated by the wild-type, mutant, and reconstituted promoters was measured by assaying β-galactosidase activity during mitotic growth and after meiotic induction. The reconstituted promoter (pCC51) functions like the wild-type promoter (pAV79), since it is fully repressed during mitosis and is activated to wild-type levels under meiotic conditions (Fig. 1). These results show that we can use this reconstitution system to assay the effect of various mutations within the $URS1_H$ site on repression and activation of *HOP1* transcription.

Sequences similar to the $URS1_H$ site have been identified in the promoter regions of other meiosis-specific genes, as well as nonmeiotic genes such as *CAR1* (10, 39, 43, 46). Although it has been shown that $URS1_C$ activates *lacZ* expression under meiotic conditions in the context of the heterologous *CYC1* promoter (6), we wanted to investigate whether the *CAR1* site can replace the function of the *HOP1* site in the context of a meiosis-specific promoter. We cloned the $URS1_C$ site into the mutant *HOP1* promoter described above and assayed the expression of *HOP1-lacZ* under both mitotic and meiotic conditions (Fig. 1, pCC71). The *CAR1* site fully represses transcription of the *HOP1* promoter during mitotic growth. During meiosis, this site also activates transcription, although at a slightly lower level than the $URS1_H$ site. The $URS1_C$ site therefore behaves like the $URS1_H$ site in regulation of *HOP1* transcription, which suggests that *CAR1* and *HOP1* utilize some common transcription regulatory factors at URS1 to control gene expression. In haploid cells, these promoters did not activate *lacZ* expression above the background level when the cells were switched to sporulation conditions or under conditions that would induce *CAR1* expression (data not shown). These results indicate that induction by both sites is meiosis specific in the context of the *HOP1* promoter.

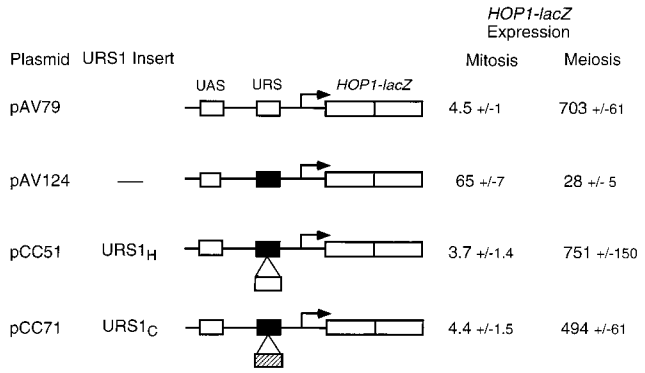


FIG. 1. Reconstitution of $URS1_H$ function. pAV79B contains the wild-type *HOP1* promoter regulating expression of a *HOP1-lacZ* fusion. Plasmid pAV124 contains a 5-bp mutation in the $URS1_H$ site (black box) which created an *Xho*I site. The reconstituted promoters were made by cloning a 26-bp oligonucleotide containing the wild-type $URS1_H$ (pCC51) or $URS1_C$ (pCC71) site into the *Xho*I site of pAV124. Constructs were transformed into strain LNY273 and assayed for β-galactosidase activity under mitotic and meiotic conditions as described in Materials and Methods. β-Galactosidase activity is expressed in Miller units, and values represent the averages from at least three independent transformants.

Sequence requirements for $URS1_H$ repression and activation. To determine if the sequence requirements for meiosis-specific activation are the same as those for repression during mitosis, we performed a detailed mutational analysis of the $URS1_H$ site. We synthesized a series of $URS1_H$ sites containing single or double base pair substitutions, inserted these sites into the reconstituted *HOP1* promoter described above, and assayed their effects on the regulation of *HOP1-lacZ* expression during mitosis and meiosis (Fig. 2).

A mutation in the $URS1_H$ site may effect either mitotic repression, meiotic activation, or both. To distinguish between these possible effects, the level of *lacZ* expression for each mutant was compared with that of a promoter lacking a functional $URS1_H$ site (pAV124) and a promoter containing a reconstituted wild-type site (pCC51) during vegetative growth and meiosis. To simplify the interpretation of our data, we defined a base pair that is required for mitotic repression as one whose substitution results in greater than twofold derepression of the promoter (i.e., more than 8 U of β-galactosidase for a mutated site, compared to 4.2 U for the wild-type site). A fully derepressed site yields approximately 65 U of β-galactosidase. A residue was designated as being critical for

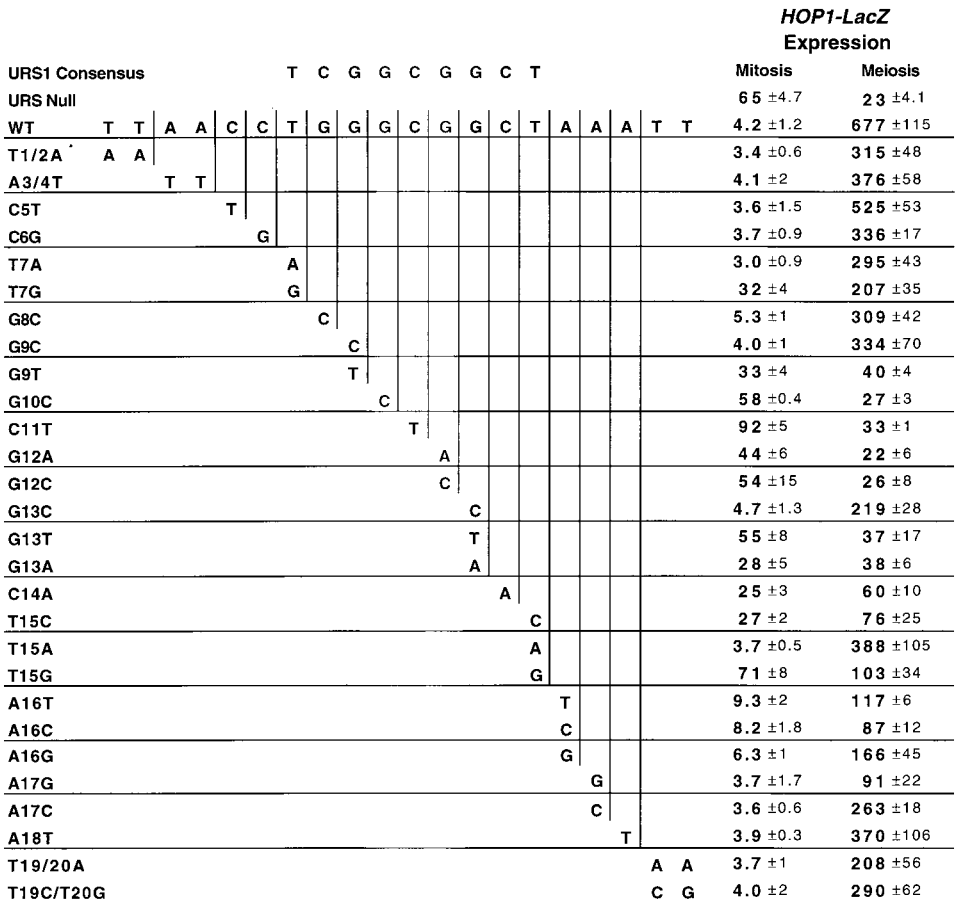


FIG. 2. Effects of base pair substitutions in the $URS1_H$ site on the mitotic repression and meiotic activation of a *HOP1-lacZ* fusion. $URS1_H$ sites with base pair substitutions were cloned into the *HOP1* promoter (pAV124) and were assayed for β-galactosidase activity under mitotic and meiotic conditions as described for Fig. 1. The sequence of wild-type (WT) $URS1_H$ is shown in the top line.

meiosis-specific activation when the activity decreased more than threefold compared to that of the wild-type site (i.e., 225 U or less, compared to 677 U for the wild-type reconstituted control).

Mutations in positions 7 to 15 within the core of the $URS1_H$ site (5′-TGGGCGGCT-3′) have a dramatic effect on repression during mitosis. Substitutions at position 16 only slightly but reproducibly derepress transcription of the promoter. In contrast, substitutions of the positions outside the core (positions 1 to 6 and 17 to 20) did not show any significant effect on mitotic repression. This result indicates that the core of the $URS1_H$ site is sufficient for the binding of the proteins required for repression during mitosis. Most of the substitutions in the *HOP1* site have the same effect on the level of repression as was observed for identical substitutions made in the analysis of the *CAR1* site in the context of the heterologous *CYC1* promoter (26). However, we observed several significant differences between the two sites. For example, it was reported that a G-to-C substitution at position 12 in $URS1_C$ caused a 10-fold increase in transcription over a nonrepressed promoter, indicating that this substitution created a strong activator site (*CAR-0*$^-$ mutation) (43). An identical substitution at the same position of $URS1_H$ (G12C) did not create a strong activator site and resulted only in failure of the site both to repress transcription during mitosis and to activate transcription during meiosis. Another difference between the two studies is that a substitution at position 7 (T7A) in $URS1_C$ in the context of the *CYC* promoter caused a fourfold defect in the level of mitotic repression, but the same substitution in $URS1_H$ in the context of the *HOP1* promoter had essentially no effect on repression or activation. Conversely, a substitution of G to A at position 13 in $URS1_C$ showed wild-type levels of repression, but the identical substitution in $URS1_H$ was completely defective in repression (G13A). A substitution of G to C (G13C) at the same position leads to a fivefold decrease in repression of $URS1_C$ but shows no effect on mitotic repression in $URS1_H$.

The sequence requirements for meiosis-specific activation appear to be more complex than for repression. Most mutations in the $URS1_H$ core positions (9 to 15) that decrease mitotic repression also significantly decrease meiosis-specific activation. However, the substitution at position 7 (T7G), which has a large effect on mitotic repression, shows only a relatively weak effect on meiotic activation. In contrast, substitutions at other positions, such as A16C, A16G, A16T, A17G, and to some extent TT19/20AA and G13C, have an effect on meiotic activation with little or no effect on mitotic repression. Although there are strong similarities within the core sequence in base pair specificities for mitotic repression and meiotic activation at the URS1 site, these results suggest that flanking sequences 3′ to the core enhance meiosis-specific activation without affecting mitotic repression. In summary, these results show that there are not only differences between sequence requirements for mitotic repression and meiotic activation but

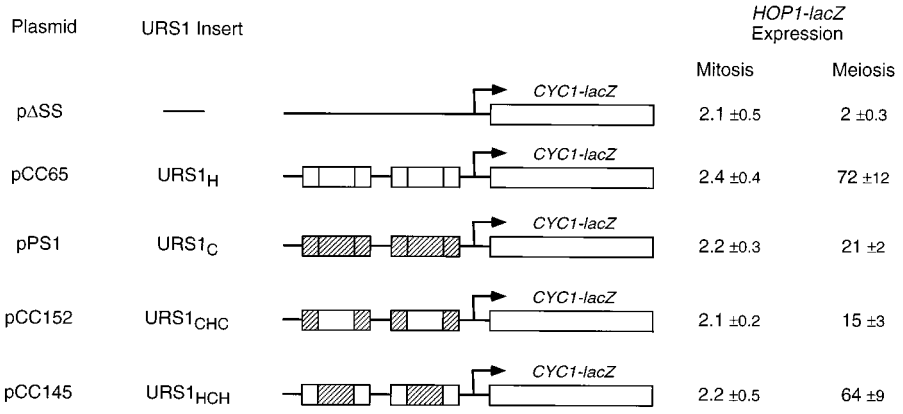


FIG. 3. Importance of flanking regions for URS1-mediated meiosis-specific activation. pΔSS contains the *CYC1-lacZ* gene under the control of the *CYC1* promoter without endogenous *CYC1* UAS sites. Tandem $URS1_H$ sites (core and flanking regions [open boxes]; plasmid pCC65) and two $URS1_C$-sites (core and flanking regions [shaded boxes]; plasmid pPS1) were cloned upstream of the TATA box in pΔSS. The origins of core and flanking regions in the hybrid URS1 sites ($URS1_{CHC}$ and $URS1_{HCH}$) are marked by open (*HOP1*) or shaded (*CAR1*) boxes (pCC152 and pCC145). Constructs were transformed into strain LNY273 and assayed as described in Materials and Methods and the legend to Fig. 1.

also differences between the *CAR1* and *HOP1* sites during mitotic repression.

$URS1_H$ flanking regions contribute to meiosis-specific activation. To verify that sequences outside the core region of $URS1_H$ are required for meiosis-specific activation, we cloned the *HOP1* and *CAR1* URS1 sites in the context of the heterologous *CYC1-lacZ* promoter (Fig. 3). The two sites have a common core sequence (except for one naturally occurring single base pair substitution) but vary in their flanking sequences. These sites are duplicated because, as was previously shown in the analysis of the *IME2* URS1 site, we have observed that meiosis-specific activation of the heterologous *CYC1* promoter in the absence of other activator sites requires multiple URS1 sites (6) (data not shown). In this context, we found that the $URS1_H$ site is threefold more efficient than the $URS1_C$ site for meiosis-specific transcriptional activation. A hybrid URS1 site containing the *HOP1* core and the *CAR1* flanking sequences also shows the relatively weak activation compared to $URS1_H$ ($URS1_{CHC}$, in pCC152). However, a hybrid containing the *CAR1* URS1 core and the *HOP1* flanking regions generated an activator site that is comparable to $URS1_H$ ($URS1_{HCH}$, in pCC145). These results strongly support the conclusions of our mutational analysis, suggesting a specific role for the flanking regions of $URS1_H$ in meiosis-specific activation.

EMSAs of the $URS1_H$ site during mitosis, meiosis, and starvation. Our mutational analysis of $URS1_H$ indicates that there are different sequence requirements within this site for mitotic repression and meiotic activation. This observation suggests that either different proteins bind to this site under mitotic and meiotic conditions or $URS1_H$-binding proteins are modified during meiosis to bind DNA differently or to interact with other factors. To characterize the DNA-binding activities of the $URS1_H$ site, we performed EMSAs using as a probe labeled $URS1_H$ incubated with extracts from diploid and haploid yeast cells from different strain backgrounds under vegetative and starvation (sporulation) conditions (Fig. 4). Extracts from mitotic and meiotic diploid cells (Fig. 4A, lanes 2 to 6) produced at least 12 different DNA-protein complexes. Strich et al. (42) reported that six different DNA-protein complexes (C1 to C6) can be observed by incubating yeast extracts with $URS1_{SPO13}$ and have shown that Ume6 is required for the formation of C1 and C2. It was also shown that there are at least five complexes formed on $URS1_C$ (34). The ladder of complexes 1 to 12 shown in the present report probably overlap with those defined for the $URS1_C$ and $URS1_{SPO13}$ sites. All of the complexes are $URS1_H$ specific, except for complexes 8 and 11, which cannot be competed by addition of unlabeled $URS1_H$ DNA (Fig. 4A, lanes 7 to 14). Therefore, complexes 8 and 11 might not represent biologically relevant $URS1_H$-binding proteins. We observe an increase in intensity of complexes 3, 4, 5, 6, 7, 9, and 10 during meiosis (lanes 2 to 6). The increase in intensity of these complexes is not meiosis specific, because it can be observed during starvation in haploid cells (Fig. 4B, lanes 2 to 6). Although we do not observe a complex 12 shift in haploid cells, the presence of this complex appears to be preparation dependent. Taken together, these results suggest that $URS1_H$-specific DNA-binding activities are present in both diploid and haploid cells and that their appearance depends on the nutritional status of the cell.

DNA binding to mutant $URS1_H$ sites. The effects of mutations in $URS1_H$ on mitotic repression or meiotic activation of *HOP1-lacZ* might also be reflected by a change in the DNA-protein complex pattern in an EMSA using mutant $URS1_H$ probes. We tested different $URS1_H$ sites in EMSAs and compared the shift pattern with that of the wild-type *HOP1* site (Fig. 5, lanes 6 to 10). Except for a reduction in the intensity of complex 8, we do not find a significant difference between the $URS1_H$-specific complexes binding to $URS1_H$ and $URS1_C$ (lanes 1 to 5). A $URS1_H$ site with the mutation G12C, which shows a strong effect on both mitotic repression and meiotic activation, fails to form complexes 7, 9, and 10 (lanes 11 to 15). A site containing the G13C mutation, which shows no effect on repression but a slight effect on activation, is missing complexes 7, 8, 9, and 10 (lanes 16 to 20). This same pattern was also observed for the A16T mutation (lanes 21 to 25). This mutation is located outside the core sequence and has only a slight effect on mitotic repression but a stronger effect on meiotic activation. The mutation A18T, which affects neither mitotic repression nor meiotic activation, displays a shift pattern identical to that of the wild-type *HOP1* site (lanes 26 to 30). The fact that mutant sites G12C, G13C, and A16T, which fail to activate transcription, are all missing complexes 7, 9, and 10 suggests that the proteins forming these complexes may play a role in meiotic activation.

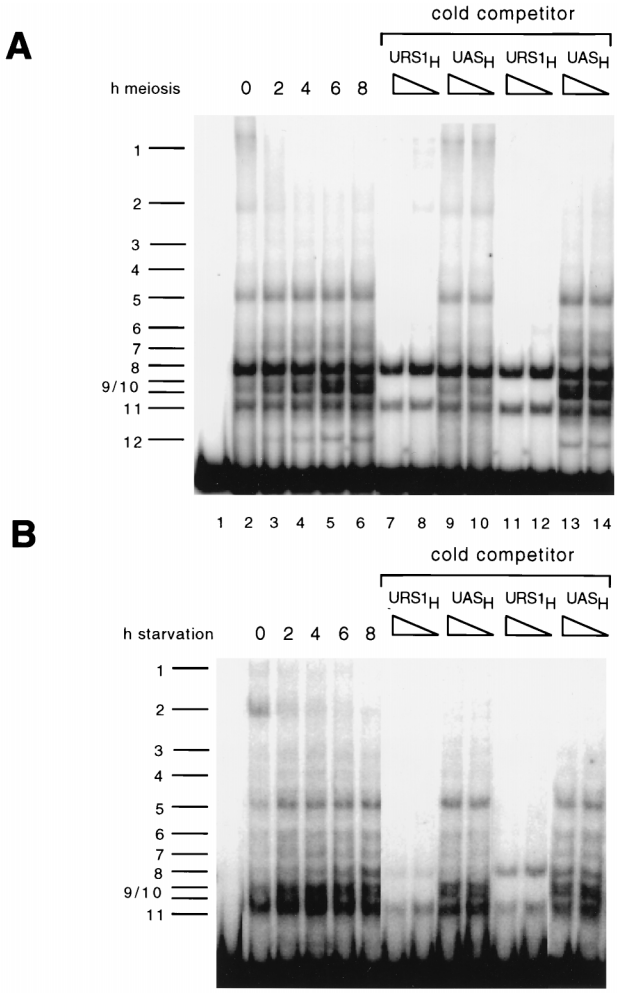


FIG. 4. DNA-binding activities of the $URS1_H$ site during meiosis and starvation. EMSAs of yeast extracts from diploid cells (A; LNY3) and haploid cells (B; RSY271) with the $URS1_H$ site during vegetative growth (lanes 2, 0 h of meiosis [A] or starvation [B]) and under starvation conditions (lanes 3 to 6, 2 to 8 h of meiosis [A] or starvation [B]) are shown. Unlabeled competitor was added in lanes 7 to 14 in 500-fold excess (lanes 7, 9, 11, and 13) and 50-fold excess (lanes 8, 10, 12, and 14). Unlabeled $URS1_H$ was added in lanes 7, 8, 11, and 12, and unlabeled UAS_H (Abf1) was added in lanes 9, 10, 11, and 12. In lanes 7 to 10, cell extracts at 0 h of meiosis (A) or starvation (B), and in lanes 11 to 14, cell extract at 8 h of meiosis (A) or starvation (B), were used; 30 μg (A) or 2 μg (B) of the cell extracts was used per lane. Lanes 1 show free probe alone. Protein-DNA complexes are marked by numbers 1 to 12 on the left side.

RPA binding to single-stranded and double-stranded $URS1_H$. The complicated shift pattern on $URS1_H$ suggests that multiple factors bind to this site. The involvement of Ume6 in mediating at least two of these shifts and its role in mitotic repression and meiotic activation in vivo is already well established (6, 34, 42). In vitro experiments have also suggested that Buf1, which has been shown to be identical to the heterotrimer RPA, has a role in URS1 function at nonmeiotic promoters (28, 39). We therefore tested whether RPA binds to a meiotic URS1 site and whether it plays a role in mitotic repression through $URS1_H$ in vivo.

To determine if one of the $URS1_H$-protein complexes contains RPA, we tested if antiserum against the yeast RPA 69-kDa subunit can supershift any of the observed protein-DNA complexes in an EMSA. The addition of RPA antibodies leads to a supershift of complex 2 (Fig. 6, lanes 2 and 3). This complex exhibits the same electrophoretic mobility as yeast RPA bound to double-stranded $URS1_H$ that was purified from *E. coli* (lanes 4 and 5). This result shows that RPA binds to the $URS1_H$ site and is a component of complex 2 from yeast extracts. Since RPA was originally isolated as a single-stranded DNA-binding protein involved in DNA replication (9), we examined the ability of RPA to bind to a single-stranded $URS1_H$ site. As shown in Fig. 6, RPA from yeast lysates, or purified recombinant RPA, binds to a single-stranded site at least 10-fold better than to a double-stranded $URS1_H$ site (compare lanes 7 and 12 with lanes 9 and 14). To test this result in greater detail, we compared the abilities of double-stranded and single-stranded $URS1_H$ to compete for binding of RPA. RPA binding to single-stranded $URS1_H$ is strongly competed by the single-stranded site. Competition by double-stranded $URS1_H$ is approximately 10-fold weaker, and there is little or no competition by nonspecific double-stranded DNA at concentrations used in this experiment (Fig. 7, lanes 3 to 8). RPA binding to double-stranded DNA is competed in the same manner; single-stranded $URS1_H$ competes best, followed by double-stranded $URS1_H$ and by double-stranded nonspecific DNA (lanes 11–16). Thus, RPA binds to single-stranded DNA better than to a double-stranded $URS1_H$ site (Fig. 6). RPA does show sequence specificity in binding to the $URS1_H$ site, since the URS double-stranded oligonucleotide competes better than the nonspecific oligonucleotide. However, mutations in the $URS1_H$ site, which completely destroy mitotic repression mediated by the site, have little or no effect on RPA binding (complex 2) (Fig. 5). Therefore, the sequence requirements for RPA binding and $URS1_H$-mediated repression do not strictly correlate.

RPA does not bind strongly to the $URS1_H$ site in vivo. Our data show that RPA does not bind strongly to the $URS1_H$ site in vitro. To determine if RPA binds to the $URS1_H$ site in vivo, we performed a one-hybrid assay and examined if Rfa1-GAD and Rfa2-GAD fusions activate transcription of a reporter promoter containing multiple $URS1_H$ sites (29). The *RFA1-GAD* and *RFA2-GAD* fusions complement *rfa1* or *rfa2* null mutants, respectively, indicating that these fusion constructs express functional Rfa1 and Rfa2 proteins (35). We also confirmed that the Rfa1-GAD and Rfa2-GAD fusions are capable of activating transcription in a two-hybrid system. Rfa1-GAD and Rfa2-GAD fusions activate expression of the *GAL1-lacZ* reporter if they are cotransformed with constructs expressing the Gal4 GDB fused to *RFA2* or *RFA1*, respectively (Fig. 8B). This control indicates that if the Rfa1-GAD or Rfa2-GAD fusions are brought to a promoter, they will activate transcription. The Rfa1-GAD and Rfa2-GAD fusions, however, are not capable of activating transcription of a promoter containing a single (pAV178) or tandem (pCC65) $URS1_H$ sites at higher levels than a reporter that did not contain the $URS1_H$ site (pTBA30) (Fig. 8A). These results show that the RPA-GAD fusions have undetectable binding to the $URS1_H$ site and suggests that RPA does not bind to the $URS1_H$ site in vivo.

RPA mutants do not affect $URS1_H$ repression. Although RPA binds poorly to a double-stranded $URS1_H$ site, it is possible that this weak binding is of biological significance. Therefore, we tested temperature-sensitive *rfa* mutants for the ability to influence $URS1_H$-mediated repression during mitotic growth (Fig. 9). The β-galactosidase activity of a *CYC1-lacZ* fusion containing a $URS1_H$ site in the promoter (pAV138-1) was compared with that of a promoter lacking the site (pAV73) at permissive (25°C) and nonpermissive (37°C) temperatures. All of the *rfa-2* and *rfa-3* mutants assayed showed essentially wild-type levels of repression at both the permissive and nonpermissive temperatures. Furthermore, we have determined

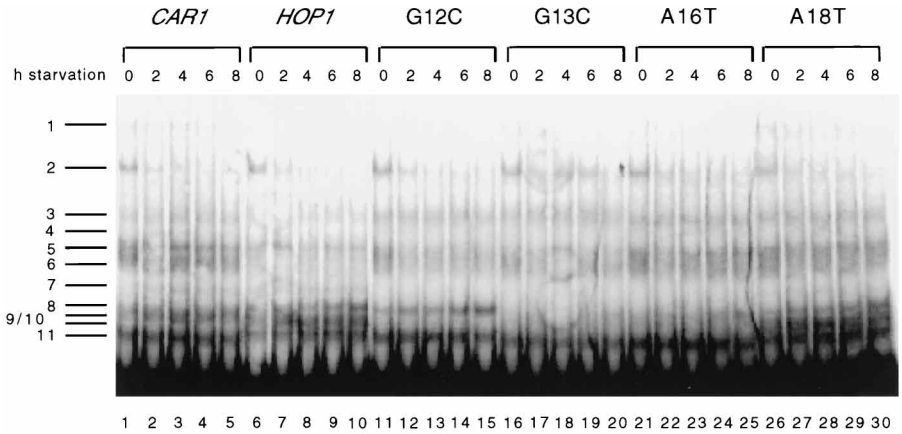


FIG. 5. Effects of substitutions in the $URS1_H$ site on the formation of DNA-protein complexes in extracts from haploid cells (RSY271). The $URS1_C$ was used in lanes 1 to 5, $URS1_H$ was used in lanes 6 to 10, $URS1_H$ with a G12C mutation was used in lanes 11 to 15, $URS1_H$ with a G13C mutation was used in lanes 16 to 20, $URS1_H$ with a A16T mutation was used in lanes 21 to 25, and $URS1_H$ with an A18T mutation was used in lanes 26 to 30. Extracts (2 μg per lane) were made at 0 to 8 h of starvation. Protein-DNA complexes are marked by numbers 1 to 11 on the left.

that several recently isolated temperature-sensitive *rfa1* mutants also have no effect on $URS1_H$-mediated repression (data not shown). The absolute level of repression appears to be lower at the nonpermissive temperature than at the permissive temperature, but this is most likely due to the overall drop in the level of expression of the derepressed promoter (pAV73) at the nonpermissive temperature. Comparison of the degrees of repression at nonpermissive temperature and permissive temperature reveals that there is no significant difference in repression between wild-type and mutant *rfa* strains.

We assayed the single-stranded and double-stranded $URS1_H$-binding activity of RPA derived from mutant strains (Fig. 10). Extracts from the *rfa2-55* and *rfa2-C100* mutant strains show impaired binding of RPA to both double-stranded and single-stranded $URS1_H$ (lanes 4, 5, 8, and 9), while binding activity in the *rfa3*-N70 mutant strain is comparable to that in extracts from wild-type cells (lanes 3, 6, 7, and 10). These results show that while at least two mutant alleles confer a $URS1_H$-binding defect, these mutants have no effect on transcriptional repression mediated by $URS1_H$.

DISCUSSION

We have investigated the sequence requirements of a meiosis-specific URS1 site for mitotic repression and meiotic activation in the context of its native promoter. We find by mutational analysis that only positions within the core sequence of $URS1_H$ (5′-T[G/C]GGCGGCT-3′), which are shared with the nonmeiotic *CAR1* and other URS1 sites, are critical to mediate mitotic repression. We have also shown that the URS1 core element is sufficient for partial meiotic induction, since $URS1_C$ can substitute for the $URS1_H$ site in the context of the *HOP1* promoter and allows for meiotic activation of the *CYC1* promoter. Meiosis-specific activation by $URS1_C$, however, is weaker than activation by $URS1_H$. The difference in the levels of activation by the two sites appears to be due to sequences outside the core element. The mutational analysis of the $URS1_H$ site shows that sequences adjacent to the 3′ side of the core element are required for full meiotic induction of the *HOP1* promoter. The importance of this region is further supported by the observation that the modest meiotic activation of *CYC1-lacZ* by $URS1_C$ can be increased by substitution of the *CAR1* flanking regions with the *HOP1* sequences. Taken together, these results show that the flanking regions of the site play a role in meiotic activation. Although the level of activation mediated by this flanking region is small in comparison with the level of activation mediated by the URS1 core element, it still has an important biological role. We have previously shown that as little as a fourfold reduction in the level of *HOP1* expression is sufficient to cause a *hop1* mutant phenotype (46). Small differences in the level of expression may therefore be critical for the function of other meiosis-specific genes as well.

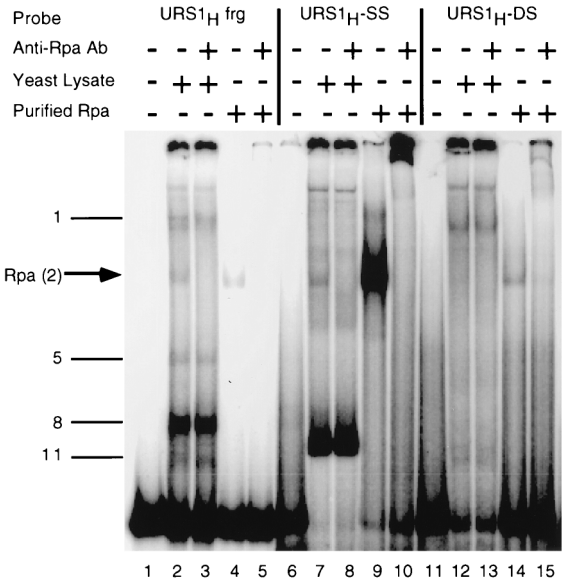


FIG. 6. Binding of RPA to the single-stranded and double-stranded *HOP1* $URS1_H$ sites. In lanes 1 to 5, a plasmid fragment containing the double-stranded $URS1_H$ site was used. In lanes 6 to 10, single-stranded oligonucleotide containing $URS1_H$ was used; and in lanes 11 to 15, the corresponding double-stranded oligonucleotide was used. Free probes are shown in lanes 1, 6, and 11. Seventy nanograms of purified RPA was used in lanes 9 and 10; 200 ng of purified RPA was used in lanes 4, 5, 14, and 15; 30 μg of mitotic cells extracts (LNY3) was used in lanes 2, 3, 7, 8, 12, and 13; 5 μl of 1:100-fold-diluted RPA antibody (Ab) (against the 69-kDa subunit) was added to the reaction mixture before the addition of DNA in lanes 3, 5, 8, 10, 13, and 15. For reference, protein-DNA complexes are marked by numbers on the left. Complex 2 is formed by the RPA protein. At high concentrations of extract a faint RPA-dependent shift of the double-stranded $URS1_H$ site is visible.

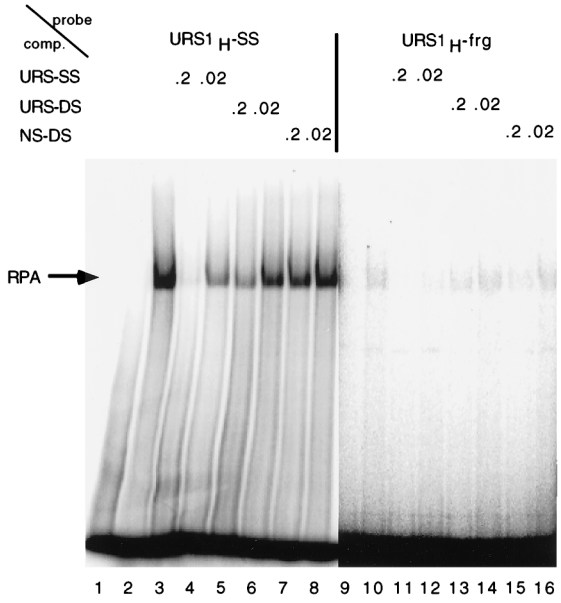


FIG. 7. Competition of RPA binding to the $URS1_H$ site by single- and double-stranded sites. Seventy nanograms of purified RPA was used per lane. $URS1_H$-SS represents single-stranded probe (lanes 2 to 8) or competitor (comp.) DNA, $URS1_H$-DS represents double-stranded probe (lanes 9 to 16) or competitor DNA. The competitor DNA used was in about 10- and 100-fold excess of the probe and is indicated in micrograms.

These small differences may be important distinguishing a meiotic URS1 site from a nonmeiotic site.

A sequence comparison of the URS1 sites from meiosis-specific promoters indicates that the core 3′ flanking regions are highly conserved (Fig. 11). For example, all meiosis-specific genes have an A at position 16 in their URS1 sites. We have shown that an A at this position is required for full meiotic activation of *HOP1*, and Bowdish and Mitchell (5) have shown that a mutation at this position in the *IME2* URS1 site affects meiotic activation of a *CYC1-lacZ* reporter. At position 17 an A is conserved in 11 of the 15 meiotic URS1 sites listed. Mutation at this position of $URS1_H$ to G or C reduces meiotic activation of *HOP1*. At position 18, in the meiotic URS1 sites, 14 of 15 of the bases are either an A or a T, and our mutational data show that either base can function at this position. Finally, at position 19, most of the meiotic URS1 sites contain a T, and our data show that a substitution at this position in the $URS1_H$ site causes a reduction in meiotic expression of *HOP1*. Taken together, the mutational analysis and the observation that these sequences are conserved among meiotic promoters suggest that this region enhances meiosis-specific activation. In comparison, the 3′ flanking regions of URS1 sites of nonmeiotic genes are considerably less conserved (26).

Our results show that the sequence requirements of the $URS1_H$ site for meiotic activation are different from those for mitotic repression. There are several possible explanations for these differences. It has been shown that Ume6 binds to the URS1 core sequence and is required for both mitotic repression and meiotic activation (6, 34, 40, 42). The extended sequence requirements of the URS1 site might be due to other cofactors that interact with Ume6 and bind DNA. It has been shown that the major meiotic inducer, Ime1, and the protein kinase Rim11 associate with Ume6 to form a transcriptional activator complex (38). Although neither Rim11 nor Ime1 contains any known DNA-binding motifs and both are probably unable to bind DNA on their own, it is possible that in complex with Ume6, one of these proteins makes contacts with the DNA (30). This mechanism of cooperative interactions is used by a variety of transcriptional activators. For example, the yeast Ste12 protein does not bind well on its own to isolated pheromone response element sequences but binds cooperatively with Mcm1 to activate **a**-specific genes at composite control elements containing an Mcm1 binding site and a nearby pheromone response element (12, 13). Likewise, the viral protein VP16, which does not bind well on its own, makes contacts to the DNA in complex with the Oct1 protein (16, 33, 41). Alternatively, it is imaginable that, in complex with its cofactors, Ume6 alters its conformation in such a way that it makes additional base-specific contacts. Although it is apparent that the 3′ flanking region of the URS1 site has a role in meiotic activation, it is clear that most of the base-specific requirements for binding affinity reside within the URS1 core element, since the nonmeiotic *CAR1* site can partially substitute for $URS1_H$, and mutations within the core have the largest effect on activation of transcription.

The examination of the $URS1_H$ site shows not only that there are differences in the sequence requirements for mitotic repression and meiotic activation but also that there are differences between a meiotic (*HOP1*) and a nonmeiotic (*CAR1*) URS1 site. One major difference is the failure of a G12C substitution in the $URS1_H$ site to create a strong mitotic activation site as was found for the same substitution in the $URS1_C$ site (26, 43). The differential effects of substitutions at position 12 have also been noted in other URS1 sites (47). We have also observed differences in the effects on repression between the *CAR1* and *HOP1* sites at positions 7 and 13. One explanation for these differences may be that some studies were performed

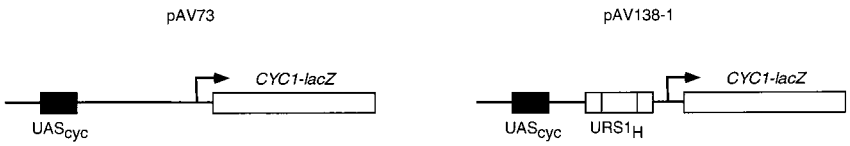
A One-Hybrid Assay

Activation Domain	Reporter	Units
pRFA1-GAD	pTBA30 — CYC1-lacZ	0.32
pRFA1-GAD	pAV178 — URS1H — CYC1-lacZ	0.20
pRFA1-GAD	pCC65 — URS1H URS1H — CYC1-lacZ	0.20
pRFA2-GAD	pTBA30 — CYC1-lacZ	0.40
pRFA2-GAD	pAV178 — URS1H — CYC1-lacZ	0.17
pRFA2-GAD	pCC65 — URS1H URS1H — CYC1-lacZ	0.16

B Two-Hybrid Control

DNA-Binding Domain	Activation Domain	Reporter	Units
pGDB	pRFA1-GAD	GAL1-lacZ	0.06
pRFA2-GDB	pRFA1-GAD	GAL1-lacZ	0.25
pGDB	pRFA2-GAD	GAL1-lacZ	0.06
pRFA1-GDB	pRFA2-GAD	GAL1-lacZ	2.84

FIG. 8. One-hybrid assay of RPA binding to the $URS1_H$ site in vivo. (A) Rfa1-GAD and Rfa2-GAD fusions were assayed for the ability to activate transcription from either a UAS-less *CYC1-lacZ* reporter promoter (pTBA30) or a *CYC1-lacZ* promoter containing one (pAV178) or two (pCC65) $URS1_H$ sites. (B) A two-hybrid control of the Rfa1-GAD and Rfa2-GAD fusions. Rfa1-GAD and Rfa2-GAD fusions were cotransformed with either an *RFA1* or *RFA2* fusion to the GDB or the GDB alone and assayed for the ability to activate transcription of a *GAL1-lacZ* reporter promoter. Units shown are the averages of β-galactosidase expression from three independent transformants.



	RT			37°C			
	ß-galactosidase units			ß-galactosidase units			
strain/allele	pAV73	pAV138-1	fold repression	pAV73	pAV138-1	fold repression	% repression compared to RT
HMY357/*wt RPA2*	306±36	3.0±0.2	102	191±14	2.8±0.2	68	67
HMY344/*rfa2-55*	323±59	4.2±0.6	77	208±35	3.9±0.3	53	69
HMY343/*rfa2-210*	217±16	3.8±0.2	57	131±7	3.0±0.1	44	77
HMY345/*rfa2-214*	319±8	3.3±0.1	97	196±5	3.6±0.4	54	56
HMY350/*rfa2-215*	180±6	3.2±0.3	56	110±9	4±0.3	28	50
HMY346/*rfa2-C100*	383±20	3.6±0.1	106	229±24	3.0±0	76	72
HMY353/*rfa3-313*	151±6	2±0.1	76	91±2	2.3±0.1	40	53
HMY347/*rfa3-N70*	289±43	3.7±0.7	78	229±24	4±0.7	57	73

FIG. 9. Effects of mutations in RPA on the repressor function of $URS1_H$. In strains with different temperature-sensitive alleles of RPA, β-galactosidase activities of the *CYC1-lacZ* fusion under the control of the endogenous UAS_{CYC} sites (pAV73) were measured during mitosis and compared with activities of the same construct with one $URS1_H$ site (pAV138-1) at permissive (room temperature [RT]) and nonpermissive (37°C) temperatures. *wt*, wild type.

in heterologous promoters, while others were kept within the context of their native promoter.

Multiple DNA-binding activities have been reported for binding to various URS1 sites (3, 25, 27, 34, 42, 47). The trimeric RPA has been purified as the $URS1_C$-binding factor (27, 28). Moreover, it was shown that the Ume6 (Car80) protein mediates transcriptional repression through $URS1_C$ (34) and that it binds to the URS1 site in *SPO13* (3, 42). DNA-binding activities in EMSAs have been reported for the *INO* URS1 and *TRK2* URS1 sites, called URSBf and URSF, respectively (25, 47). Binding of URSBf to the *INO* URS1 can not be competed by $URS1_C$ and might therefore not be identical to RPA (23). In addition, multiple uncharacterized DNA-protein complexes binding to $URS1_C$ and $URS1_{SPO13}$ have

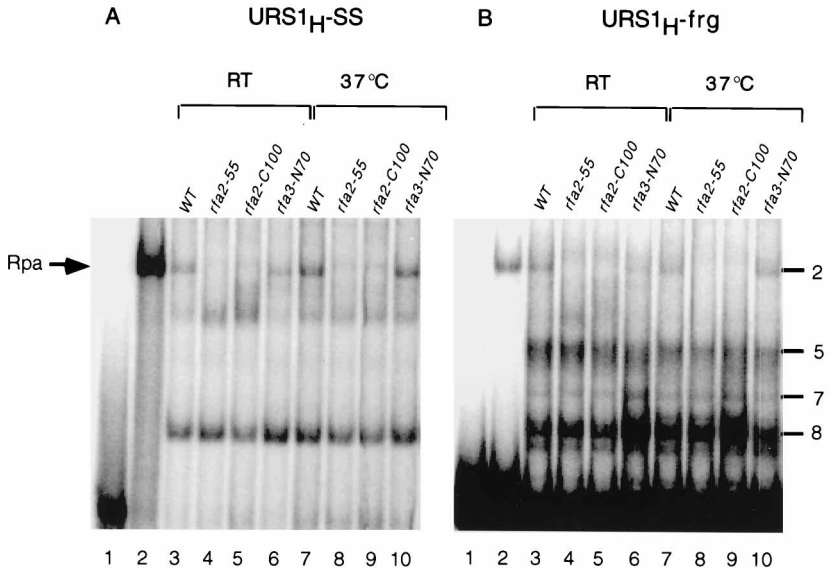


FIG. 10. Effects of mutations in RPA on binding to single-stranded and double-stranded $URS1_H$ sites. Protein was extracted from cells grown to log phase at the permissive temperature (lanes 3 to 6) and after being switched to 37°C for 5 h (lanes 7 to 10) and was incubated with single-stranded (A) and double-stranded (B) $URS1_H$ sites for EMSAs (see Materials and Methods). Lanes: 1, free probe; 2, purified recombinant yeast RPA (50 ng); 3 and 7, HMY357 (wild type [*WT*]); 4 and 8, HMY344 (*rfa2-55*); 5 and 9, HMY346 (*rfa2-C100*); 6 and 10, HMY347 (*rfa3-N70*). For reference, protein-DNA complexes are marked by numbers on the right. Complex 2 is formed by the RPA protein. RT, room temperature.

Position	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
$URS1_H$	T	T	A	A	C	C	T	G	G	G	C	G	G	C	T	A	A	A	T
Repression							+	?	+	+	+	+	+	+	+	+/-			
Activation							+/-	?	+	+	+	+	+	+	+	+	+	?	+/-
HOP1							T	G	G	G	C	G	G	C	**T**	**A**	**A**	**A**	**T**
REC114							T	G	G	G	C	G	G	C	**T**	**A**	**A**	**C**	**T**
SPO13							T	C	G	G	C	G	G	C	**T**	**A**	**T**	**T**	**T**
IME2							A	C	G	G	C	G	G	C	**T**	**A**	**T**	**T**	**T**
MER1							T	C	G	G	C	G	G	C	**T**	**A**	**A**	**A**	**A**
SPO16							T	G	G	G	C	G	G	C	**T**	**A**	**A**	**A**	**A**
SPO11							T	T	G	G	C	G	G	C	**T**	**A**	**A**	**T**	**T**
MEI4							T	G	G	G	C	G	G	C	**T**	**A**	**A**	**A**	**T**
MEK1							T	G	G	G	C	G	G	C	**T**	**A**	**A**	**A**	**T**
							A	T	G	G	C	G	G	C	**T**	**A**	**A**	**A**	**T**
REC104							T	T	G	G	C	G	G	C	**T**	**A**	**T**	**T**	**T**
DMC1							T	G	G	G	C	G	G	C	**T**	**A**	**T**	**T**	**T**
ZIP1							T	C	G	G	C	G	G	C	**T**	**A**	**A**	**A**	**T**
RED1							T	C	A	G	C	G	G	C	**T**	**A**	**A**	**A**	**T**
HSP82							T	G	A	G	C	G	G	T	**T**	**A**	**A**	**T**	**T**
CAR1							T	C	G	G	C	G	G	C	**T**	**A**	**C**	**C**	**G**

FIG. 11. Summary of the effects of mutations on repression and activation function of $URS1_H$ and an alignment of URS1 sites of meiosis-specific genes. +, position in which one or more substitutions strongly affect repression or activation; +/−, weak effect on repression or activation; ? position in which no effect was observed with the substitution tested. Below is shown an alignment of known or suggested URS1 regulatory sites from meiosis-specific genes (29). The nonmeiotic *CAR1* site is shown below for comparison. Positions in boldface denote the conserved 3′ flanking region that enhances meiotic activation.

also been observed in extracts of vegetatively grown haploid cells (34, 42). In the present study, we performed EMSAs of the $URS1_H$ site with protein extracts from haploid and diploid cells from different strain backgrounds grown under different conditions. We observed at least nine different specific DNA-binding activities, which were neither cell type nor meiosis specific. The relative intensities of some of these complexes changed at different time points after haploid or diploid cells were switched to starvation medium, indicating that the activity or level of these proteins may be nutrition dependent. We have used a large variety of protease inhibitors to minimize the possibility of proteolytic degradation of the URS_H-binding proteins. It is therefore unlikely that the nutrient-dependent complexes represent proteolytic degradation products. It is possible that these new shifts are caused by proteins that are required to inactivate the repressor activity of Ume6.

We determined by antibody supershift experiments that complex 2 of the $URS1_H$ site is the previously identified $URS1_C$-binding factor RPA. We investigated the binding of RPA to double-stranded and single-stranded $URS1_H$ and its role in $URS1_H$-mediated repression in vivo. It has been previously reported that single-stranded $URS1_C$ cannot compete in a binding assay with double-stranded $URS1_C$ for RPA binding (28). Moreover, it has been shown for the *MAG* URS2 (5′-TCGGTGGCGA-3′) that RPA binds to this double-stranded site with higher affinity than to a single-stranded oligonucleotide (39). In contrast to what has been found for the $URS1_C$ and *MAG* URS2 sites, we have observed that purified recombinant yeast RPA, as well as RPA in yeast extracts, binds more strongly to single-stranded than to double-stranded $URS1_H$. We have also shown that DNA binding of RPA is not influenced by point mutations in the core or flanking sequences of $URS1_H$. Taken together, these results indicate that RPA shows little sequence specificity at $URS1_H$ and prefers to bind to single-stranded DNA, as expected from previous studies (2, 8, 23). While these studies were in progress, it was reported that human RPA binds to a double-stranded DNA sequence in the HMTIIA promoter and functions to moderately repress transcription of the gene (44). Interestingly, purified recombinant human RPA did not have the same double-stranded DNA-binding activity as observed in nuclear extracts. It was suggested that this difference may be due either to modification of the RPA subunits or the fact that other proteins work in complex with RPA to bind double-stranded DNA. We observed a low level of RPA binding to double-stranded $URS1_H$ in crude extracts, and therefore a similar modification of RPA or association with other factors may occur in yeast.

Although RPA does bind to mitotic and meiotic URS1 sites in vitro, there has been no evidence as to whether the protein has a role in URS1-mediated repression (27, 28, 39). In the present study, we investigated for the first time the influence of mutations in RPA on URS1-mediated repression in vivo. We have demonstrated that mutations in any of the three RPA subunits have no effect on transcriptional repression mediated through the $URS1_H$ site, even though two mutant alleles (*rfa2-C100* and *rfa2-55*) show impaired DNA-binding activity and nucleotide excision repair in vitro, as well as DNA synthesis in vivo (Fig. 10) (18, 31). Consequently, although RPA binds to double-stranded $URS1_H$ in vitro, it does not appear to have a biological role in $URS1_H$-mediated repression. While it is possible that RPA binding to URS1 sites serves another biological purpose (e.g., DNA replication, repair, or recombination), we have shown that RPA does not bind strongly enough to the $URS1_H$ site in vivo to activate transcription in a one-hybrid assay. This result brings into question the biological significance of RPA binding to the $URS1_H$ site in vitro. Although our results suggest that RPA does not have a biological role at the $URS1_H$ site, it is still possible that RPA functions at $URS1_C$ or similar sites. We have shown that even though there is strong sequence and functional similarity between the *HOP1* and *CAR1* URS1 sites, there are also some significant differences. Moreover, the other sites that have been shown to be bound by RPA in yeast are highly diverged from $URS1_H$ (39). It is therefore possible that these sequence differences explain the differences in the binding of RPA to the various sites. Further experiments will be needed to show whether RPA has an in vivo role in binding to these sites in yeast.

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REFERENCES

1. **Acton, T. A., H. Zhong, and A. K. Vershon.** 1997. The DNA-binding specificity of Mcm1: operator mutations that alter DNA-bending by a MADS box protein. Mol. Cell. Biol. **17:**1881–1889.
2. **Alani, E., R. Thresher, J. D. Griffith, and R. D. Kolodner.** 1992. Characterization of DNA binding and strand-exchange stimulation properties of y-RPA, a yeast single-strand-DNA-binding protein. J. Mol. Biol. **227:**54–71.
3. **Anderson, S. F., C. M. Steber, R. E. Esposito, and J. E. Coleman.** 1995. *UME6*, a negative regulator of meiosis in *Saccharomyces cerevisiae*, contains a C-terminal Zn_2Cys_6 binuclear cluster that binds the URS1 DNA sequence in a zinc-dependent manner. Protein Sci. **4:**1832–1843.
4. **Bhargava, J., J. Engebrecht, and G. S. Roeder.** 1992. The *rec102* mutant of yeast is defective in meiotic recombination and chromosome synapsis. Genetics **130:**59–69.
5. **Bowdish, K., and A. Mitchell.** 1993. Bipartitite structure of an early meiotic upstream activation sequence from *Saccharomyces cerevisiae*. Mol. Cell. Biol. **13:**2172–2181.
6. **Bowdish, K., H. Yuan, and A. Mitchell.** 1995. Positive control of yeast meiotic genes by the negative regulator *UME6*. Mol. Cell. Biol. **15:**2955–2961.

7. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
8. **Brill, S. J., and B. Stillman.** 1989. Yeast replication factor-A functions in the unwinding of the SV40 origin of DNA replication. Nature (London) **342:** 92–95.
9. **Brill, S. J., and B. Stillman.** 1991. Replication factor-A from *Saccharomyces cerevisiae* is encoded by three essential genes coordinately expressed at S phase. Genes Dev. **5:**1589–1600.
10. **Buckingham, L. E., H.-T. Wang, R. T. Elder, R. M. McCarroll, M. R. Slater, and R. E. Esposito.** 1990. Nucleotide sequence and promoter analysis of *SPO13*, a meiosis-specific gene of *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **87:**9406–9410.
11. **Chevray, P. M., and D. Nathans.** 1992. Protein interactions cloning in yeast: identification mammalian proteins that react with the leucine zipper of Jun. Proc. Natl. Acad. Sci. USA **89:**5789–5793.
12. **Dolan, J. W., C. Kirkman, and S. Fields.** 1989. The yeast STE12 protein binds to the DNA sequence mediating pheromone induction. Proc. Natl. Acad. Sci. USA **86:**5703–5707.
13. **Errede, B., and G. Ammerer.** 1989. STE12, a protein involved in cell-type-specific transcription and signal transduction in yeast, is part of protein-DNA complexes. Genes Dev. **3:**1349–1361.
14. **Esposito, R. E., and S. Klapholz.** 1981. Meiosis and ascospore development, p. 211–287. *In* J. N. Strathern, E. W. Jones, and J. R. Broach (ed.), The molecular biology of the yeast *Saccharomyces cerevisiae*, life cycle and inheritance, vol. 1. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
15. **Gailus-Durner, V., J. Xie, C. Chintamaneni, and A. K. Vershon.** 1996. Participation of the yeast activator Abf1 in meiosis-specific expression of the *HOP1* gene. Mol. Cell. Biol. **16:**2777–2786.
16. **Gerster, T., and R. G. Roeder.** 1988. A herpesvirus trans-activating protein interacts with transcription factor OTF-1 and other cellular proteins. Proc. Natl. Acad. Sci. USA **85:**6347–6351.
17. **Guarente, L., and T. Mason.** 1983. Heme regulates transcription of the *CYC1* gene of *Saccharomyces cerevisiae* via an upstream activation site. Cell **32:**1279–1286.
18. **He, Z., J. M. S. Wong, H. S. Maniar, S. J. Brill, and C. J. Ingles.** 1997. Assessing the requirements for nucleotide excision repair proteins of *Saccharomyces cerevisiae* in an *in vitro* system. J. Biol. Chem. **271:**28243–28249.
19. **Heyer, W. D., M. R. Rao, L. F. Erdile, T. J. Kelly, and R. D. Kolodner.** 1990. An essential *Saccharomyces cerevisiae* single-stranded DNA binding protein is homologous to the large subunit of human RP-A. EMBO J. **9:**2321–2329.
20. **Hollingsworth, N. M., and B. Byers.** 1989. *HOP1*: a yeast meiotic pairing gene. Genetics **121:**445–462.
21. **Hollingsworth, N. M., L. Goetsch, and B. Byers.** 1990. The *HOP1* gene encodes a meiosis-specific component of yeast chromosomes. Cell **61:**73–84.
22. **Ito, H., Y. Fukuda, K. Murata, and A. Kimura.** 1983. Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. **153:**163–168.
23. **Kim, C., R. O. Snyder, and M. S. Wold.** 1992. Binding properties of replication protein A from human and yeast cells. Mol. Cell. Biol. **12:**3050–3059.
24. **Leem, S. H., and H. Ogawa.** 1992. The *MRE4* gene encodes a novel protein kinase homologue required for meiotic recombination in *Saccharomyces cerevisiae*. Nucleic Acids Res. **20:**449–457.
25. **Lopes, J. M., K. L. Schulz, J. W. Yates, J. P. Hirsch, and S. A. Henry.** 1993. The *INO1* promoter of *Saccharomyces cerevisiae* includes an upstream repressor sequence (URS1) common to a diverse set of yeast genes. J. Bacteriol. **175:**4235–4238.
26. **Luche, R. M., R. Sumrada, and T. G. Cooper.** 1990. A *cis*-acting element present in multiple genes serves as a repressor protein binding site for the yeast *CAR1* gene. Mol. Cell. Biol. **10:**3884–3895.
27. **Luche, R. M., W. Smart, and T. G. Cooper.** 1992. Purification of a heteromeric protein binding to the URS1 transcriptional repression site in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **89:**7412–7416.
28. **Luche, R. M., W. Smart, T. Marion, M. Tillman, R. Sumrada, and T. G. Cooper.** 1993. *Saccharomyces cerevisiae* BUF protein binds to sequences participating in DNA replication in addition to those mediating transcriptional repression (URS1) and activation. Mol. Cell. Biol. **13:**5749–5761.
29. **Luo, Y., S. Vijaychander, J. Stile, and L. Zhu.** 1996. Cloning and analysis of DNA-binding proteins by yeast one-hybrid and one-two-hybrid systems. Bio/Technology **20:**564–568.
30. **Mandel, S., K. Robzyk, and Y. Kassir.** 1994. *IME1* gene encodes a transcription factor which is required to induce meiosis in *Saccharomyces cerevisiae*. Dev. Genet. **15:**139–147.
31. **Maniar, H. S., R. Wilson, and S. J. Brill.** 1997. Roles of Replication Protein-A subunits 2 and 3 in DNA replication fork movement in *Saccharomyces cerevisiae*. Genetics **145:**891–902.
32. **Mitchell, A.** 1994. Control of meiotic gene expression in *Saccharomyces cerevisiae*. Microbiol. Rev. **58:**56–70.
33. **O'Hare, P., and C. R. Goding.** 1988. Herpes simplex virus regulatory elements and the immunoglobulin octamer domain bind a common factor and are both targets for virion transactivation. Cell **52:**435–445.
34. **Park, H.-D., R. M. Luche, and T. G. Cooper.** 1992. The yeast *UME6* gene product is required for transcriptional repression mediated by the *CAR1* URS1 repressor binding site. Nucleic Acids Res. **20:**1909–1915.
35. **Philipova, D., J. R. Mullen, H. S. Maniar, J. Lu, C. Gu, and S. J. Brill.** 1996. A hierarchy of SSB protomers in replication protein-A. Genes Dev. **10:**2222–2233.
36. **Prinz, S., F. Klein, H. Auer, D. Schweizer, and M. Primig.** 1995. A DNA binding factor (UBF) interacts with a positive regulatory element in the promoters of genes expressed during meiosis and vegetative growth in yeast. Nucleic Acids Res. **23:**3449–3456.
37. **Rockmill, B., and G. S. Roeder.** 1988. *RED1*: a yeast gene required for segregation of chromosomes during the reductional division of meiosis. Proc. Natl. Acad. Sci. USA **85:**6057–6061.
38. **Rubin-Bejerano, I., S. Mandel, K. Robzyk, and Y. Kassir.** 1996. Induction of meiosis in *Saccharomyces cerevisiae* depends on conversion of the transcriptional repressor Ume6 to a positive regulator by its regulated association with the transcriptional activator Ime1. Mol. Cell. Biol. **16:**2518–2526.
39. **Singh, K., and L. Samson.** 1995. Replication protein A binds to regulatory elements in yeast DNA repair and DNA metabolism genes. Proc. Natl. Acad. Sci. USA **92:**4907–4911.
40. **Steber, C. M., and R. E. Esposito.** 1995. *UME6* is a central component of a developmental regulatory switch controlling meiosis-specific gene expression. Proc. Natl. Acad. Sci. USA **92:**12490–12494.
41. **Stern, S., M. Tanaka, and W. Herr.** 1989. The Oct-1 homoeodomain directs formation of a multiprotein-DNA complex with HSV transactivator VP16. Nature (London) **341:**624–630.
42. **Strich, R., R. Surosky, C. Steber, E. B. Dubois, F. Messenguy, and R. Esposito.** 1994. *UME6* is a key regulator of nitrogen repression and meiotic development. Genes Dev. **8:**796–810.
43. **Sumrada, R. A., and T. G. Cooper.** 1987. Ubiquitous upstream repression sequences control activation of the inducible arginase gene in yeast. Proc. Natl. Acad. Sci. USA **84:**3997–4001.
44. **Tang, C.-M., A. E. Tomkinson, W. S. Lane, M. S. Wold, and E. Seto.** 1996. Replication Protein A is a component of a complex that binds the human metallothionein IIA gene transcription start site. J. Biol. Chem. **271:**21637–21644.
45. **Thomas, B. J., and R. Rothstein.** 1989. Elevated recombination rates in transcriptionally active DNA. Cell **56:**619–630.
46. **Vershon, A. K., N. M. Hollingsworth, and A. D. Johnson.** 1992. Meiotic induction of the yeast *HOP1* gene is controlled by positive and negative regulatory sites. Mol. Cell. Biol. **12:**3706–3714.
47. **Vidal, M., A. M. Buckley, C. Yohn, D. J. Hoeppner, and R. F. Gaber.** 1995. Identification of essential nucleotides in an upstream repressing sequence of *Saccharomyces cerevisiae* by selection for increased expression of *TRK2*. Proc. Natl. Acad. Sci. USA **92:**2370–2374.