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Regulated antisense transcription controls expression of cell-type specific genes in yeast

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ABSTRACT

Transcriptome profiling studies have recently uncovered a large number of non-coding RNA transcripts (ncRNAs) in eukaryotic organisms and there is a growing interest in their role in the cell. For example, in haploid *Saccharomyces cerevisiae* cells, expression of an overlapping antisense ncRNA, referred to here as *RME2* (Regulator of Meiosis 2) prevents *IME4* expression. In diploid cells, the **a**1-α2 complex represses transcription of *RME2*, allowing *IME4* to be induced during meiosis. In this study we show that antisense transcription across the *IME4* promoter region does not block transcription factors from binding, nor is it required for repression. Mutational analyses found that sequences within the *IME4* ORF are required for repression mediated by *RME2* transcription. These results support a model where transcription of *RME2* blocks elongation of the full-length *IME4* transcript, but not initiation. We have found that another antisense transcript, called *RME3*, represses *ZIP2* in a cell-type specific manner. These results suggest that regulated antisense transcription may be a widespread mechanism to control gene expression, and may account for the roles of some of the previously uncharacterized ncRNAs in yeast.

INTRODUCTION

One of the main paradigms for the control of gene expression is through regulatory proteins binding to the promoter regions of genes to activate or repress transcription. However, it is now clear that non-coding RNAs (ncRNAs) also have important roles in gene regulation. For example, RNAi-mediated regulation controls gene expression in *Caenorhabditis elegans*, *Arabidopsis thaliana*, humans and many other organisms (16,

24). However, there also appear to be a large number of ncRNAs that do not appear to be involved in RNAi-mediated regulation. For example, there are over 900 ncRNAs expressed in the yeast *Saccharomyces cerevisiae* (11, 15, 33, 40, 45). Several of these ncRNAs act to regulate gene expression in yeast (2, 5, 18, 26). However, *S. cerevisiae* lacks the enzymes Dicer and Argonaute, which are required for RNAi, and therefore it must utilize different mechanisms for ncRNA-mediated regulation (12). In this paper we investigate how two antisense ncRNAs regulate expression of genes required for meiosis in yeast.

Under starvation conditions, diploid yeast undergoes meiosis and sporulation to form four haploid spores. This process involves the expression of over 500 genes that are highly regulated in a coordinated manner (7, 28). Entry into the meiotic pathway is controlled by expression of *IME1*, which is the master initiator of meiosis (20, 29). There are two signals that regulate *IME1* expression (Fig. 1A). One signal is through the nutritional status of the cell, which activates *IME1* expression under starvation for both nitrogen and a fermentable carbon source (14, 37). The second signal is through cell-type specific regulation, which only allows expression of meiotic genes in **a**/α diploid cells. Cell-type specific regulation is controlled by the **a**1-α2 repressor complex, which regulates *IME1* expression through two different pathways. One pathway involves the haploid-specific repressor Rme1, which binds to the promoter of *IME1,* preventing its expression (10, 30). In diploid cells, the **a**1-α2 complex binds to the *RME1* promoter, repressing its transcription, thereby relieving repression of *IME1*. The second form of cell-type specific control is mediated through Ime4, which is required for full level expression of *IME1* (38). *IME4* is repressed in haploid cells and the **a**1-α2 repressor

complex is required for its expression in diploid cells. Rme1 does not regulate *IME4,* so it was hypothesized that a different haploid-specific repressor regulates *IME4* (38).

To identify **a**1-α2 target sites in the yeast genome and possibly the factor regulating *IME4* expression, we used an algorithm that combined **a**1-α2 binding site preference data with cell-type specific microarray data (34). One of the strong **a**1-α2 binding sites identified in the search is downstream of the *IME4* ORF**.** This site has an indirect role in controlling the expression of *IME4* through the regulation of an antisense ncRNA (17). This ncRNA, which we refer to here as *RME2* (Regulator of Meiosis 2), is expressed in haploid cells and blocks expression of *IME4* (Fig. 1B). In diploid cells the **a**1-α2 complex represses *RME2* expression, allowing *IME4* to be induced under starvation conditions. This system appears to function only in a *cis*-configuration because *RME2* is unable to repress an adjacent copy of *IME4* (17).

We show in this paper that expression of another antisense ncRNA regulates the meiosis-specific *ZIP2* gene in a manner similar to *IME4*. This result suggests that regulated expression of antisense ncRNAs may be a conserved mechanism of gene regulation in yeast. Interestingly, there are specific elements within the *IME4* ORF that are required for repression mediated by *RME2*. Antisense expression does not inhibit transcription factors from binding to the *IME4* and *ZIP2* promoters and therefore may block transcription elongation of the coding genes.

MATERIALS AND METHODS

Plasmid and strain construction. Plasmid pBG1 contains a 600 bp PCR-generated fragment of bp +1400 to +2000 bp from *IME4* translation initiation site cloned into the

TOPO TA vector (Invitrogen). Site-directed mutagenesis was used to change four base pairs in the **a**1-α2 binding site (WT: GTGTATTTTTTTACATCA; mu: GTcgATTTTTTTACggCA) to produce plasmid pBG7. Plasmid pBG113 contains a 2.9-kb PCR fragment of bp -450 to +400 bp flanking the *IME4* ORF cloned into the TOPO TA vector (Invitrogen). This plasmid was digested with XbaI and HindIII to clone into the same sites in pRS415 and pRS405 to generate pBG112 and pBG129, respectively (39). The *HOP1-urs1* mutant (pBG157) and 225-675 flip (pBG166) plasmids were generated by gap repair of pBG112 (32). All other *IME4* mutants were generated by site-directed mutagenesis of pBG129. The *rme2-s1* mutation changes bp +447 relative to the start of the *IME4* ORF from an A to a T and is silent in terms of coding for the *IME4* protein. The *rme2-s2* mutant changes bps -23 and -24 relative to the start of the *IME4* ORF from GA to TT.

Plasmid pJM532 contains a 3.1-kb PCR generated fragment of bp -450 to +400 bp flanking *ZIP2* genomic DNA cloned into the TOPO TA vector (Invitrogen). This plasmid was digested with SpeI and ApaI to clone *ZIP2* into the same sites in pRS405, to generate pJM533. Site directed mutagenesis was used to change four base pairs in the downstream **a**1-α2 binding site (as indicated above, for the *IME4* site) to produce pJM535.

A list of the strains used in this study is shown in Table 1. Strains YBG111 and YBG112 were constructed by transforming W303 derivative strains LNY315 and LNY316 with a PCR fragment amplified from pFA6a-KanMX6 with the *KanMX* cassette and 50 bp flanking homology, to delete the -450 to +400 bp flanking the *IME4* ORF (43). These strains were mated to produce a *ime4Δ/ime4Δ* diploid strain, YBG115. *IME4*

deletion and polyA terminator mutants were integrated at the *LEU2* locus by digesting the listed plasmids with XcmI and transforming the linearized DNA into strain YBG111. Transformants were selected on media lacking leucine and confirmed by PCR.

Strains JMY076 and JMY077 were constructed by using a *Candida albicans URA3* cassette from pGEM-*CaURA3* to delete the **a**1-α2 binding site. These strains were transformed with a PCR product from pBG7 in the presence of 5-FOA to recombine the mutant binding site at the wild-type locus, to generate strains JMY081 and JMY082. JMY081 and JMY082 were mated to generate JMY084, a homozygous diploid with the **a**1-α2 mutation downstream of *IME4*.

The native *ZIP2* gene was deleted by transformation with a *KanMX* PCR fragment amplified from the Yeast Deletion Strain collection (Research Genetics) in strains LNY392 and LNY433 to generate JMY104 and JMY105. JMY104 and JMY105 were transformed with XcmI-linearized pJM533 to generate JMY108 and JMY109, which were mated to produce JMY110. JMY104 and JMY105 were also transformed with XcmI-linearized pJM535 to generate JMY111 and JMY112, which were mated to produce JMY113. All genomic integrations were confirmed by PCR.

RT-PCR assays. Expression of the RNA transcripts was assayed by Reverse-Transcriptase PCR (RT-PCR) and performed on two to four replicate samples. Single colonies of yeast strains (from separate transformations for plasmid bearing strains) were grown under rich nutrient (YEPD or SD) or sporulation inducing (SPM) conditions (3 hours in SPM for *IME4* assays, and 5 hours for *ZIP2* assays) and total RNA was extracted by hot acid-phenol extraction, as previously described (1). Normalized RNA

samples were treated with Turbo DNA Free DNase (Ambion) and PCR amplification of the DNase treated RNA with the *IME4*/*RME2* or *ZIP2*/*RME3* primer set was performed to verify the absence of contaminating DNA. cDNA of the *IME4*, *RME2, URA3, ZIP2, RME3, HSP26, YFL012W* and *ACT1* genes was synthesized for each RNA sample with sense and antisense specific primers, using Omniscript RT (Qiagen). Different cDNA sample concentrations (1-4 µl) were assayed to verify that the reaction was in the linear range. PCR reactions using different concentrations of cDNA as a template were amplified in 50 µl reactions containing 10 pmols of each amplicon primer set, 1X Amplitaq Taq Buffer II, 2.5 U of Amplitaq Taq polymerase (Applied Biosystems). The amplifications were carried out for 30 cycles of 94°C for 30 secs, 52°C for 1 min, and 72°C for 30 secs. Samples were run on 1.4% agarose TAE gels, and images were photographed with a Fluorochem 8800 camera.

Chromatin Immunoprecipitation Assay (ChIP). ChIP assays were performed using a modified version of protocols described elsewhere (27, 34). Cultures of LNY392 and YBG144, or YBG111 and YBG115 strains carrying the indicated *HOP1pr::IME4* constructs were grown to midlog (O.D. $_{600}$ = .500) in the appropriate media (YEPD, SD-Leu, or SPM); 50 ml of the cultures was fixed with a final concentration of 1% formaldehyde for 15 min at 22°C, washed with 1 ml TBS, and frozen at -80°C for a minimum of 12 h. Cell pellets were suspended with 400 µl FA lysis buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% Na-Deoxycholate) plus 1mM PMSF, 1X Protease Inhibitor Cocktail from Roche (1873580), and 50 µl Sigma Protease Inhibitor Cocktail (P8215). To this, 200 µl of glass beads was added and cells were lysed by vortexing at full speed for 40 min at 4°C. The lysate was then

centrifuged for 5 min at 12,000 X *g* at 4°C. The supernatant was transferred to a new tube, and the beads were washed with 500 µl of FA lysis buffer; the supernatant from this wash was added to the original supernatant fraction. DNA was sonicated at 30% output for 6 x 5 sec cycles to give an average chromatin fragment size of 500 bp. Sonicated lysates were centrifuged at 12,000 X *g*, for 5 min and 50 µl of the sonicated DNA was reserved as a Total Chromatin (TC) sample. The remaining DNA was pre-cleared by the addition of 25 µl of protein G agarose beads, nutated for 1 hour at 4°C; and the lysates were cleared by centrifugation at 12,000 X *g* for 5 min at 4°C. To immunoprecipitate TBP- or Abf1-bound DNA, 10 µl or 1 µl of TBP yN-20 polyclonal antibody (Santa Cruz, sc-26141) or Abf-1 yC-20 polyclonal antibody (Santa Cruz, sc-6679) was added and nutated for 16 h at 4°C. The protein G beads centrifugation, washes, DNA elution, crosslinking reversal, and proteinase treatment, were all performed as previously described (34, 36). Frozen TC samples were brought up to 500 µl in volume with elution buffer. A Qiagen PCR Purification kit was used to purify the amplified DNA. Multiple quantities of DNA input were used for the PCR. Typically, 2 ul of total chromatin (TC) samples (500-fold dilution and 50-fold dilution) and immunoprecipitated (IP) samples (1 and 3 µl) was used in 50 µl reactions for 30 cycles. The PCR products were run on 1.5% agarose gels. Samples were quantified using ImageJ software and normalizing to the *ACT1* signal.

RESULTS

***IME4* is repressed by cell-type specific antisense transcription.**

Chromatin immunoprecipitation (ChIP) assays had previously shown that the **a**1-α2 repressor complex is bound to a site downstream of *IME4* (34). Similar to what has been previously reported, we found that a mutation in this site allows expression of the *IME4* antisense transcript, *RME2*, and prevents *IME4* expression in diploid cells (17) (data not shown).

To determine if *RME2* is required for repression of *IME4,* we constructed a mutation, *ime4Δ3',* which deletes the *RME2* promoter. Haploid cells with this mutation failed to express *RME2* and *IME4* was derepressed (Fig 2, lane 3). This suggests that expression of *RME2* is required for repression of *IME4*.

Expression of *RME2* from the native locus did not repress transcription of *IME4* from the *ime4Δ3'* mutant integrated at distant locus (Fig 2, lane 4). This indicates that *RME2* transcription is only able to repress *IME4* in a *cis*-acting manner. Our results using a different mutation in the **a**1-α2 site and a different assay for expression of the sense and antisense genes are consistent with previous work on the regulation of *IME4* by an antisense transcript (17).

Antisense transcription is a conserved model of gene regulation in yeast

Since *IME4* is regulated by transcription of a ncRNA in the antisense direction, we wanted to determine if other genes are regulated by a similar mechanism. We therefore re-evaluated our previous data identifying **a**1-α2 sites in the yeast genome by relaxing the sequence requirements for **a**1-α2 sites and specifically searching for sites that are downstream of cell-type specific genes (34). We then used the Yeast Transcriptome Database to search for the presence of antisense transcription of the gene

upstream of the **a**1-α2 site (11). In addition to *IME4,* this search identified the *HSP26, YFL012W*, and *ZIP2* genes as potential targets for antisense-mediated regulation. RT-PCR assays for the expression of sense and antisense transcripts from these genes showed that the *HSP26* and *YFL012W* are not regulated in a cell-type manner by antisense transcription (data not shown). However, analysis of *ZIP2*, which was previously identified as a meiosis-specific component of the synaptonemal complex, suggested the sense and antisense transcripts are regulated in a cell-type specific manner (Fig 3A) (8). In agreement with gene expression profiling experiments during meiosis, the *ZIP2* (sense) transcript was expressed only in diploid cells under conditions of sporulation (Fig. 3B, lane 4) (7). In contrast, haploid cells under the same conditions expressed a ncRNA antisense to *ZIP2*, which we will refer to as *RME3* (Regulator of Meiosis 3) (Fig. 3B, lane 3).

The expression pattern of *ZIP2* and *RME3* is similar to that observed for *IME4* and *RME2.* We were therefore interested if the *RME3* transcript was responsible for antisense-mediated regulated expression of *ZIP2*. Mutation of four bases in the **a**1-α2 site downstream of *ZIP2* caused derepression of *RME3* in diploid cells (Fig. 3C, lane 4). Expression of the *ZIP2* transcript was inhibited in the mutant diploid strain (Fig. 3C, lane 4). This shows that like *IME4*, *ZIP2* is repressed by antisense transcription.

To test if *RME3* represses *ZIP2* transcription in a *cis*- or *trans*-acting manner, we constructed a diploid strain heterozygous for the *ZIP2* locus. One of the *ZIP2* alleles in this strain is wild type, while the other contains the 4 bp mutation in the **a**1-α2 site that allows *RME3* expression in diploid cells. If *ZIP2* is repressed by *RME3* in *trans*, we would have expected to see a decrease in the level of *ZIP2* in the heterozygote compared

to a wild type homozygous strain, similar to the mechanism of RNAi (9, 16). However, the level of *ZIP2* expression was the same in the mutant heterozygote and wild-type homozygous diploid strains, indicating that expression of *RME3* in *trans* did not inhibit *ZIP2* transcription (Fig. 3D, lane 2 vs. lane 4). This indicates that *RME3* regulates *ZIP2* in a *cis*-dependant configuration, similar to the *IME4/RME2* regulatory system.

Changing the termination site of *RME2* affects ability to regulate *IME4*

The observation that both *IME4* and *ZIP2* are repressed in haploid cells by antisense transcription suggests that this may be a common mechanism of gene regulation. We wanted to determine how the expression of an antisense transcript in *cis* prevent expression of the sense transcript? One of the most economical models for this form of regulation is that antisense transcription through the sense promoter prevents transcription factor binding and activation. This model is similar to the mechanisms proposed for the *SER3* and *ADH1* genes, which are regulated by the upstream ncRNAs, *SRG1* and *ZRR1*, respectively (3, 25, 26). Transcription of the upstream ncRNAs through the promoter of the coding gene prevents binding of the transcriptional activators required for expression. If *IME4* is regulated by a similar mechanism as *SER3* and *ADH1*, then premature termination of the *RME2* transcript would allow expression of *IME4* in haploid cells. To test this model, two mutations, *rme2-s1* and *rme2-s2*, were constructed that truncate the *RME2* transcript by the introduction of a eukaryotic poly(A) signal sequence, AAUAAA. The *rme2-s1* mutation introduced a poly(A) site in the direction of *RME2* at 447 bp downstream from the *IME4* ATG and this change is silent with respect to the *IME4* coding sequence. The *rme2-s2* mutation introduced the same

polyA site 23 bp upstream from the *IME4* ATG and it does not overlap with the presumptive *IME4* TATA box or alter the spacing of the promoter. Because transcription extends past polyA sites by roughly 100 bp, these mutations shorten *RME2* from 700-800 bp in the case of *rme2-s1*, and 300-400 bp in the case of *rme2-s2* (Fig. 4A) (4). A similar approach was used to truncate the *Kcnq1ot1* transcript in mouse and investigate its role in antisense-mediated regulation (19). To assay for premature termination of *RME2,* primer sets within the *IME4* promoter (Pr), the 5' end of *IME4* (5'), and the middle of the ORF (Mid) were used to differentially detect the wild type, *rme2-s1* and *rme2-s2* transcripts (Fig. 4A). In wild-type cells, the *RME2* transcript was detected by all three primer sets (Fig. 4B, lane 1). In contrast, the *rme2-s1* transcript was only detected by the Mid primer set, indicating that the transcript was prematurely terminated before the 5' end of the *IME4* ORF. Similarly, *rme2-s2* was only detected by the Mid and 5' primer sets but not the Pr set, indicating that transcription did not extend through the entire *IME4* promoter and upstream region, as in the wild-type haploid (Fig. 4B, *RME2* lane 3). Despite this change, the *rme2-s2* mutation did not appear to affect the repression of *IME4* (Fig. 4B, *IME4* lane 3). In contrast, the truncated *rme2-s1* transcript, which does not extend into the promoter or the 5' end of *IME4,* was unable to repress expression of *IME4* (Fig. 4B, *IME4* lane 2). In **a**/α diploid cells, the *rme2-s1* and *rme2-s2* mutants expressed *IME4* at wild-type levels, indicating they did not have an effect on TBP or polymerase binding at the *IME4* promoter (data not shown). These results suggest that transcription of *RME2* through the 5' end of the *IME4* ORF is essential for repression, but extension of the antisense transcript through the *IME4* promoter region is not required.

***RME2* transcription can repress a heterologous promoter.**

Previous research showed that high-level expression of *IME4* from the *GAL1* promoter was able to override repression by *RME2* in haploid cells (17). It is possible that the *GAL1* promoter was not repressed by *RME2* transcription due to differences in promoter specificity. For example, antisense transcription may disrupt binding of specific transcription factors to the *IME4* promoter. In contrast, the transcriptional activator of *GAL1,* the Gal4 protein and its cofactors, may be insensitive to this form of regulation. To test this model we assayed for the ability of *RME2* to repress a heterologous promoter with similar expression activity to the *IME4* promoter. We constructed a strain in which the *IME4* promoter was substituted with a derivative of the *HOP1* promoter, *HOP1-urs1*, that is constitutively active in both haploid and diploid cells (42). Diploid cells grown under sporulation conditions expressed similar levels of *IME4* from either the *IME4* or *HOP1-urs1* promoters (Fig. 5, lanes 2 and 4). In haploid cells, both of the promoters were repressed by *RME2* transcription (Fig. 5, lanes 1 and 3). To further test this result, we mutated the **a**1-α2 site downstream of *IME4* in the context of the *HOP1-urs1* promoter to allow expression of *RME2* in haploid and diploid cell types (Fig. 5, lanes 5 and 6). In the mutant strain, the *HOP1-urs1* promoter was repressed by *RME2* in diploid cells (Fig. 5, lane 6). This is consistent with previous work, where a similar **a**1-α2 site mutant was shown to permit *RME2* expression and prevent meiosis in diploids (17). These results indicate that *RME2* transcription is able to repress a heterologous promoter with similar activity and that there are unlikely to be specific elements or factors bound at the *IME4* promoter that make it sensitive to antisense transcription.

TBP Binding at the sense and antisense promoters of *IME4* and *ZIP2*

The observation that the *rme2-s2* mutant is able to repress expression of *IME4* suggests that antisense transcription across the promoter is not required for repression. Although *RME3* is able to repress *ZIP2* expression, transcriptome profiling experiments suggest that the *RME3* transcript does not extend through the entire ORF and into the *ZIP2* promoter (11). Even though *rme2-s2* or *RME3* do not extend through the promoters, they could still work through a mechanism that blocks transcription factors from binding and activating transcription. To test this model, we used ChIP assays to monitor transcription factor binding at both the sense and antisense promoter regions. The recruitment of TATA-Binding Protein (TBP) can be used to determine if a ncRNA disrupts transcription factor binding at a promoter (25). TBP binding to the *IME4* promoter (5'), middle of the ORF (Mid) and to the *RME2* promoter (3') was assayed in haploid and diploid cells. The *RME2* promoter was bound by TBP in haploid cells with 16.9-fold higher affinity than in diploid cells (Fig. 6A, 3' *IME4,* lanes 3 vs. 4). As expected, TBP was bound to the *IME4* promoter in diploid cells (Fig. 6A, 5' *IME4,* lane 4). Surprisingly, despite repression of *IME4* by *RME2*, TBP was also bound to the *IME4* promoter with almost equal affinity (0.90-fold difference) in haploid and diploid cells (Fig. 6A, 5' *IME4,* lanes 3 and 4). In addition, Abf1, the *HOP1* activator protein, and TBP were bound to the *HOP1-urs1* promoter fusion even when repressed by *RME2* in haploid cells (data not shown) (13). These results indicate that *RME2* transcription does not disrupt occupancy of the *IME4* promoter by transcription factors.

We also examined the binding of TBP at the *ZIP2* locus to determine if the mechanism of *RME3*-mediated repression is similar to *RME2*. Like *RME2*, the *RME3* promoter was bound by TBP in haploid cells with 16.1-fold higher affinity than in diploid cells (Fig. 6B, *ZIP2* 3' lane 3 vs. 4). Similar to *IME4*, TBP bound to the *ZIP2* promoter with roughly the same affinity in both haploid and diploid cells (Fig. 6B, *ZIP2* 5' lane 3, 4). The wild type *HOP1* gene, which is activated at a similar time point in meiosis as *ZIP2*, was only bound by TBP in diploid cells (Fig. 6B, *HOP1* lane 3 vs. 4). This shows that unlike *IME4* and *ZIP2*, the meiosis-specific *HOP1* promoter is not bound by TBP when it is repressed according to cell-type. These results suggest that *RME2* and *RME3* do not repress *IME4* and *ZIP2* through mechanisms that interfere with factors binding to their promoters.

A specific region within the *IME4* ORF is required for antisense-mediated repression.

The observation that early termination of the *rme2-s1* transcript blocked repression suggests the *IME4* ORF sequence may have a role in regulation by *RME2*. To determine if regions within the *IME4* ORF are required for antisense-mediated repression, we performed an internal deletion analysis of the gene. Specific deletions of the *IME4* ORF were assayed for expression of the *IME4* and *RME2* transcripts in haploid cells. The *ime4Δ1-900* deletion caused derepression of the *IME4* transcript in haploid cells (Fig. 7, lane 3 vs. lane 1). In contrast, a deletion of bp 901-1800 had no effect on *IME4* repression (data not shown). It was possible that the loss of *IME4* transcriptional repression in the *ime4Δ1-900* mutant was due to premature termination of the *RME2*

transcript. However, RT-PCR assays confirmed that *RME2* was expressed and that the transcript extended across the *IME4* promoter region (Fig. 7, lane 3). Taken together, these results suggest that the first 900 bp of the *IME4* ORF is required for antisense-mediated repression.

To further define what region is required for repression of *IME4* by *RME2*, a series of smaller deletions were made within the first 900 bp of the *IME4* ORF. The *ime4Δ1-224* and *ime4Δ676-900* deletions had no effect on the repression of *IME4* in haploid cells (Fig. 7, lanes 6 and 7). In contrast, the *ime4Δ1-450* and *ime4Δ451-900* deletions caused derepression of *IME4* (Fig. 7, lanes 4 and 5). This indicates that a DNA element within bp 225-675 is required for proper antisense-mediated repression.

Orientation of a portion of *IME4* is involved in strand specific expression.

Deletion analysis of the *IME4* ORF showed that a region from bp 225-675 is required for *RME2*-mediated repression of *IME4* in haploid cells. It is possible that transcription of this region in the antisense direction creates a chromatin structure that prevents effective extension of the full-length sense transcript. If this occurs, then there may be an orientation-specific requirement of this DNA element. To test for this, the segment of DNA from bp 225-675 was flipped to the opposite orientation within the context of the *IME4* ORF (Fig. 8, Top). This mutation had no effect on the expression of *IME4* in diploid cells (Fig. 8, lane 3 vs. lane 4), showing that it is silent with respect to expression and stability of the *IME4* transcript. However, in haploid cells, this mutation causes derepression of the *IME4* transcript (Fig. 8, lane 1 vs. lane 2). This result suggests

that transcription of *RME2* across this element may set up orientation-specific termination of *IME4*.

Discussion

Research on eukaryotic transcriptomes has uncovered the presence of large numbers of ncRNAs that are expressed from either intergenic regions or regions that overlap with coding genes in either the sense and antisense directions (11, 21, 31, 35, 40). In higher eukaryotes, many of these ncRNAs regulate gene expression through *trans*-acting micro-RNA and si-RNA pathways that are dependent on Dicer and Argonaute (16, 24). Since the yeast *S. cerevisiae* lacks the genes coding for these proteins it is unable to conduct RNAi-mediated regulation through mechanisms similar to higher eukaryotes (12). However, work by several labs has shown that some of the over 900 ncRNAs in yeast do have regulatory functions. For example, *trans*-acting antisense regulatory ncRNAs function to silence transcription and transposition of the Ty1 retrotransposon, and expression of *PHO84* (2, 6). Expression of other ncRNAs, such as the *SRG1* and *ZRR1* ncRNAs, which are transcribed from upstream of the *SER3* and *ADH1* promoters, respectively, repress expression of the coding genes in a *cis*-dependent manner (3, 25). Expression of an antisense ncRNA transcript, *RME2*, prevents *IME4* expression in haploid cells (17) (Fig. 2B). We have now identified another *cis*-acting antisense ncRNA, *RME3*, which functions to repress transcription of the meiosis-specific *ZIP2* gene in haploid cells. This suggests that overlapping sense/antisense transcripts may be a common form of regulation in *S. cerevisiae.*

RME2 and *RME3* appear to function strictly in *cis-;* a single extra *trans-* copy of the non-coding gene, expressed from a distant or adjacent locus, failed to repress sense transcription (Fig 2B and 3D; (17)). The *SRG1* and *ZRR1* transcripts are similarly non-functional when supplied in *trans* (3, 25). This is unlike *PHO84*, where a second copy has a *trans*-acting effect on the native gene (6). When taken together, these cases suggest that while ncRNA-mediated repression may be prevalent in yeast, different sense-antisense gene pairs are regulated through at least two distinct mechanisms.

Previous work on the *IME4* gene showed that using a *GAL1* promoter fusion to express *IME4* at a high level could overcome repression by *RME2* in haploid cell-types (17). The induced *GAL1* promoter is therefore insensitive to antisense-mediated repression. One model for this finding is that there is a specific level of sense transcription that can be repressed by antisense transcription. In this model, the high level of *IME4* expressed from the *GAL1* promoter may not be repressed by antisense transcription from the weaker *RME2* promoter. Another possible explanation for this result is that only specific promoters, such as *IME4* and *ZIP2,* are sensitive to disruption by antisense transcription. However, a derivative of the *HOP1* promoter is repressed by *RME2* in a similar manner as the wild-type *IME4* promoter. Therefore, the specificity of the promoter does not appear to be an integral part of the mechanism for repression by *RME2,* but activity level of the repressed promoter may play a role.

One hypothesis for the mechanism of *cis*-acting antisense repression is that the transcription of ncRNA somehow acts to block the binding of the transcription factors required for expression of the coding gene. This would be similar to the mechanism of regulation proposed for the *SRG1* and *ZRR1* ncRNAs, which are transcribed from

upstream of the *SER3* and *ADH1* promoters, respectively, and repress expression of the coding genes by inhibiting the binding of transcription factors to the downstream promoters (3, 25). Repression of the upstream ncRNA genes allows the factors to bind the downstream promoter to activate transcription of the coding genes. It is possible that *RME2* regulates *IME4* through a similar mechanism. However, expression of the *rme2-s2* transcript, which terminates before reaching the *IME4* promoter, is able to repress *IME4.* This shows that transcription through the *IME4* promoter is not required for repression. Antisense transcription through the *ZIP2* promoter is also not likely required for repression because the *RME3* transcript appears to naturally terminate in the *ZIP2* ORF (11, 31). These results suggest that ncRNA-mediated repression of *IME4* and *ZIP2* is not through a mechanism of promoter interference, as has been observed for *SER3* and *ADH1*. Our results are similar to the analysis of the *Mus musculus* gene *Kcnq1* (*Lit1*), which is repressed by the expression of an internal antisense RNA, *Kcnq1ot1* (41). When the *Kcnq1ot1* transcript is shortened by the insertion of a premature polyadenylation site, repression of *Kcnq1* is reduced (19). One difference between the mouse and yeast genes that are regulated by antisense transcription is that large regions of the *Kcnq1* locus are transcriptionally inactivated through chromatin modification. In contrast, transcriptome profiling experiments shows that genes adjacent to *IME4* and *ZIP2 (HOS2/COX13* and *RMR1/PDE1*, respectively) are not co-repressed when *RME2* and *RME3* are expressed (11). Further evidence against the promoter interference model was obtained in the ChIP assays for the presence of TBP at *IME4* and *ZIP2* promoters (Fig 6). If antisense transcription regulates through a mechanism of promoter interference, we would have expected to find TBP bound to the *IME4* and *ZIP2* promoters only in diploid cells, when

RME2 and *RME3* are repressed. However, we found that TBP remained bound to these promoters in both haploid and diploid cells. This result suggests that repression by *RME2* and *RME3* does not work through interference with transcription factors binding at the sense promoters. Our results are similar to regulation by the *trans*-acting *PHO84* antisense transcript, which does not interfere with binding of TBP at the sense promoter (5, 25). This further highlights that the mechanisms of ncRNA-mediated regulation are different for different gene pairs.

A 450 bp region (bp 225-675) within *IME4* is essential for *RME2*-mediated repression. It is possible that this region contains target sites for protein complexes that have a role in blocking the extension of the full length *IME4* transcript. *RME2* transcription may expose these sites, allowing chromatin remodeling and/or modification that prevent full-length *IME4* expression. It has been shown that directional chromatin remodeling represses antisense transcription from cryptic sites (44). Therefore, it is possible that deleting this region of *IME4* removes a target site for the required chromatin remodeling/modifying enzymes, resulting in the expression of both the sense and antisense transcripts. It has been previously shown that expression of the internal *cis*-antisense ncRNA *Kcnq1ot1* alters the surrounding chromatin state to inhibit sense expression of the mouse gene *Kcnq1* (41). *RME2* and *RME3* may remodel chromatin across the *IME4* and *ZIP2* loci to prevent full-length transcription of the coding genes.

Another possible mechanism for antisense regulation by *RME2* is that the region (bp 225-675) within *IME4* is particularly sensitive to allowing transcription in one direction to disrupt transcription in the opposite direction. When this region of the *IME4* ORF was deleted, both sense and antisense were expressed simultaneously (Fig 7). This

result is corroborated by the effect of the *rme2-s1* mutation on regulation (Fig 4). The truncated *rme2-s1* transcript does not cross the entire bp 225-675 region of the ORF, and this results in full-length transcription of *IME4*. This DNA sequence may therefore only be transcribed in a single direction at a given time. In contrast, other sequences permit bidirectional transcription. This appears to be the case for the nested antisense gene pair of *YGR031W* and *NAG1*, two overlapping genes that appear to be transcribed simultaneously in vegetative media (23).

We also observed that there is an orientation-specific requirement of *IME4* bp 225-675 for antisense-mediated repression. Recent work has shown that abortive transcription upstream of *IMD2* blocks the start site of the coding gene (18, 22). When a region of *IMD2* required for the termination of these short transcripts is reversed in orientation, termination does not occur, and the *IMD2* gene is no longer repressed. These experiments provide evidence that specific DNA architecture can play a role in gene regulation. Although *RME2* represses expression of the full-length *IME4* transcript, short incomplete *IME4* transcripts have been detected (17) (*data not shown*). This suggests that the transcription of *RME2* across this region of DNA causes premature termination of *IME4*.

There is no readily apparent homology between the 225-675 bp region of *IME4* and the region of *ZIP2* overlapped by *RME3*. It is therefore possible that these two gene pairs use different mechanisms for antisense-mediated repression. Alternatively, it is possible that degenerate sequences within each gene pair are required for transcriptional repression. If there are similarities in the mechanism of regulation between the *IME4/RME2* and *ZIP2/RME3* gene pairs, then it is possible that other overlapping

antisense transcripts may also use a similar mechanism to regulate one another. Transcriptome profiling experiments have identified over 350 ncRNAs expressed in an antisense orientation to coding genes (31). It is possible that some of these antisense ncRNAs regulate coding genes in a manner similar to *RME2* and *RME3.* These gene pairs may explain the role for the expression of some of the over 900 ncRNAs in yeast.

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Tables:

TABLE 1. Yeast Strains

Strain	Genotype	Source
LNY315	*MAT**a** ade2-1 trp1-1 his3-11,15 can1-100 ura3-1 leu2-3,112*	L. Neigeborn
LNY316	*MATα ade2-1 trp1-1 his3-11,15 can1-100 ura3-1 leu2-3,112*	L. Neigeborn
LNY392	*MAT**a** ade2-1 TRP1 his3-11,15 can1-100 ura3-1 leu2-3,112*	L. Neigeborn
LNY433	*MATα ADE2 trp1-1 his3-11,15 can1-100 ura3-1 leu2-3,112*	L. Neigeborn
YBG111	*MAT**a** ade2-1 trp1-1 his3-11,15 can1-100 ura3-1 leu2-3,112 ime4Δ::kanMX4*	this study
YBG112	*MATα ade2-1 trp1-1 his3-11,15 can1-100 ura3-1 leu2-3,112 ime4Δ::kanMX4*	this study
YBG115	*MAT**a**/MATα ade2-1/ade2-1 trp1-1/trp1-1 his3-11,15/his3-11,15*	
	can1-100/can1-100 ura3-1/ura3-1 leu2-3,112/leu2-3,112	this study
	ime4Δ::kanMX4/ ime4Δ::kanMX4	this study
YBG144	*MAT**a**/MATα ade2-1ADE trp1-1/TRP his3-11,15/his3-11,15*	this study
	can1-100/can1-100 ura3-1/ura3-1 leu2-3,112/leu2-3,112	
JMY076	LNY392 with *IME4—**a**1-α2::CaURA3*	this study
JMY077	LNY433 with *IME4—**a**1-α2::CaURA3*	this study
JMY081	LNY392 with *IME4—**a**1-α2 mut*	this study
JMY082	LNY433 with *IME4—**a**1-α2 mut*	this study
JMY084	YBG144 with *IME4—**a**1-α2 mut/IME4—**a**1-α2 mut*	this study
JMY104	LNY392 with *zip2::KanMX*	this study
JMY105	LNY433 with *zip2::KanMX 2*	this study
JMY108	LNY392 with *zip2::KanMX leu2::ZIP2*	this study
JMY109	LNY433 with *zip2::KanMX leu2::ZIP2*	this study
JMY110	YBG144 with *zip2::KanMX/ zip2::KanMX leu2::ZIP2/leu2::ZIP2*	this study
JMY111	LNY392 with *zip2::KanMX leu2::ZIP2-**a**1-α2 mut*	this study
JMY112	LNY433 with *zip2::KanMX leu2::ZIP2-**a**1-α2 mut*	this study
JMY113	YBG144 with *zip2::KanMX leu2::ZIP2-**a**1-α2 mut*	this study
YBG145	YBG111 with *leu2::ime4Δ3'*	this study
YBG147	YBG111 with *leu2::IME4*	this study
YBG149	YBG115 with *leu2::IME4/leu2::IME4*	this study
YBG150	LNY315 with *leu2::ime4Δ3'*	this study
YBG158	YBG144 with *zip2::KanMX/zip2::KanMX*; *leu2::ZIP2-**a**1-α2 mut/leu2::ZIP2*	this study
YBG159	YBG111 with *leu2::rme2-s1*	this study
YBG160	YBG111 with *leu2::ime4Δ1-900*	this study
YBG161	YBG111 with *leu2::ime4Δ1-450*	this study
YBG162	YBG111 with *leu2::ime4Δ451-900*	this study
YBG183	YBG111 with *leu2::ime4Δ1-224*	this study
YBG184	YBG111 with *leu2::ime4Δ676-900*	this study
YBG202	YBG111 with *leu2::rme2-s2*	this study

Figure Legends

FIG. 1. Model for regulation of *IME1*. (A) Cell-type specific regulation of *IME1* is mediated in part by the **a**1-α2 complex, which prevents expression of *RME1*, a repressor of *IME1*. The *IME4* gene is also required to fully activate *IME1*. *IME4* expression requires the **a**1-α2 complex, which represses the haploid-specific ncRNA *RME2* in diploid cells. (B) Expression of the haploid-specific antisense *RME2* transcript represses *IME4*. In diploid cells, the **a**1-α2 complex binds downstream of *IME4* and prevents *RME2* transcription, allowing expression of *IME4* under sporulation inducing conditions. An "X" indicates that the transcript is not expressed.

FIG. 2. (A) Cartoon illustrating the wild type and *ime4Δ3'* constructs. The *ime4Δ3'* construct contains a deletion of the *RME2* promoter region (+1809 to +2209) as indicated by the dashed line. An "X" indicates that the transcript is not expressed from that copy of the *IME4* locus. (B) Deletion of the *RME2* promoter region allows expression of *IME4* in haploid cells, and *RME2* expression fails to repress *ime4Δ3' in trans*. RT-PCR assays of the *IME4* and *RME2* transcripts were performed on strains *ime4Δ* (YBG111, lane 1), WT (YBG147, lane 2), *ime4Δ3'* (YBG145, lane 3) and WT + *ime4Δ3'* (YBG150, lane 4) in haploid strains, under sporulation inducing conditions. The –RT row are PCR amplifications of DNase treated RNA with the *IME4* primer set to control for contamination of genomic DNA. RT-PCR of *ACT1* expression is used as a loading control. Genomic DNA (gDNA, lane 5) was included as a control for PCR amplification.

FIG. 3. Expression analyses of the *ZIP2* gene and antisense transcript *RME3* show cell-type specific regulation. (A) Schematic of the *ZIP2* and *RME3* transcripts, and relative position of the **a**1-α2 binding site downstream of the meiosis-specific gene *ZIP2*, and upstream of the non-cell-type specific gene *PDE1*. (B) RT-PCR assays of *ZIP2* and *RME3* from haploid (LNY392; lanes 1,3) and diploid cells (YBG144; lanes 2,4), grown in YEPD (Veg, lanes 1,2) or sporulation inducing (Spo, lanes 3,4) media. (C) RT-PCR assays of *ZIP2* and *RME3* from haploid and diploid cells grown under sporulation conditions for 5 hours, with either a wild-type (WT) haploid (JMY108, lane 1) or diploid (JMY110, lane 3) strains or **a**1-α2 binding site mutant (Mu) haploid (JMY108 lane 2) or diploid (JMY110 lane 4) strains. (D) RT-PCR assays of *ZIP2* and *RME3* from wild-type (JMY110 lanes 1,2) or heterozygous diploid cells (YBG158, lanes 3,4) grown in Veg. or Spo media. Assays and controls were performed as described in Fig. 2.

FIG. 4. Altering the polyadenylation and termination of the *RME2* transcript alters its ability to repress *IME4* in haploid cells. (A) Illustration of the three PCR amplicons (*IME4* Promoter, Pr; 5' end of the sense ORF, 5'; and middle of the ORF, Mid) used to detect both *IME4* and *RME2* transcripts, up- and downstream of the introduced termination sites in *rme2-s1* and *rme2-s2*. Primers down-stream (relative to *RME2*) of each amplicon were used to generate strand-specific cDNA. (B) RT-PCR assays of wild type (YBG147, lane 1), *rme2-s1* (YBG159, lane 2) and *rme2-s2* haploid strains (YBG202, lane 3). Assays and controls were performed as described in Fig. 2. The -RT control was performed for all assay primer pairs.

FIG. 5. The *HOP1-urs1* promoter is regulated by antisense transcription in a manner similar to the native *IME4* promoter. RT-PCR assays of *IME4* and *RME2* from haploid (1n) and diploid (2n) cells, after 3 hours in Sporulation-inducing media. *IME4* (sense) transcription is driven by either the native *IME4* promoter (LNY392 lane 1; YBG144 lane 2) or a *HOP1* promoter containing a mutation in the URS1 site (YBG111 + pBG157 lane 3; YBG115 + pBG157 lane 4). The expression of the constitutive *HOP1-urs1* mutant promoter, combined with a mutant **a**1-α2 binding site downstream of the ORF was assayed under the same conditions in haploid (YBG111 + pBG167 lane 5) and diploid (YBG115 + pBG167 lane 6) cells. Assays and controls were performed as described in Fig. 2.

FIG. 6. Antisense transcription does not disrupt TBP binding at the *IME4* or *ZIP2* promoters. (A) ChIP assays for TBP bound at both the sense (5') and antisense (3') promoters in haploid WT (LNY392 lanes 1,3); diploid WT (YBG144 lanes 2,4) cells. Assays were performed after 3 hours in SPM media. The middle of the *IME4* and *ACT1* ORF's, which are not precipitated with TBP-antibody were included as negative controls, and the constitutive *ACT1* promoter is included as a positive control for antibody binding. TC is the Total Chromatin sample (lanes 1-2), and IP is DNA Immunoprecipitated with TBP yN-20 antibody (lanes 3,4). Numbers to the right represent the fold-change in TBP binding in haploid over diploid cells (B) Amplification of the *ZIP2* and *RME3* promoters from haploid (LNY392; lanes 1, 3) and diploid cells (YBG144; lanes 2,4). Similar to the *IME4* ChIP in (A), TBP binding was assayed with the 5', Mid-, and 3' amplicons, using the listed concentrations of purified TC and IP DNA. The native *HOP1* promoter was

tested as a meiosis-specific, cell-type dependant promoter. ChIP assays were also performed on the *ZIP2* and *RME3* promoters in vegetative cultures of these haploid and diploid cells, to confirm that these regions are not constitutively bound by TBP (Fig. 6B, Veg. *ZIP2* 5' and *ZIP2* 3' lanes 3, 4)

FIG. 7. Deletion analysis of *IME4* reveals a region within the ORF is required for antisense-mediated regulation. The cartoon on top shows the location of the deletions and how deletion of the hatched region leads to loss of *IME4* repression. RT-PCR assays of *IME4* and *RME2* expression are shown from haploid (lane 1), diploid (lane 2), and haploid cells with the indicted bp of the *IME4* ORF deleted (lanes 3-7), grown in Spo media. *IME4* expression was monitored using an amplicon within the ORF and *RME2* expression was monitored using an amplicon in the *IME4* promoter region. Deletion strains are listed in Table 1.

FIG. 8. Reversing the orientation of the bp 225-675 region of *IME4* prevents proper regulation by *RME2*. RT-PCR assays of *IME4* and *RME2* from haploid (YBG111, lanes 1,2) or diploid (YBG115, lanes 3,4) cells, carrying *IME4* on a plasmid with bp 225-675 in either the wild-type (lanes 1,3) or "flipped" (antiparallel, relative to WT) orientation (lanes 2,4).