Molecular and Cellular Biology, Apr. 2011, p. 1701–1709 Vol. 31, No. 8
0270-7306/11/$12.00 doi:10.1128/MCB.01071-10
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Regulated Antisense Transcription Controls Expression of Cell-Type-Specific Genes in Yeast[▽]

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Received 13 September 2010/Returned for modification 14 October 2010/Accepted 31 January 2011

Transcriptome profiling studies have recently uncovered a large number of noncoding RNA transcripts (ncRNAs) in eukaryotic organisms, and there is growing interest in their role in the cell. For example, in haploid *Saccharomyces cerevisiae* cells, the expression of an overlapping antisense ncRNA, referred to here as *RME2* (Regulator of Meiosis 2), prevents *IME4* expression. In diploid cells, the a1-α2 complex represses the 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 and is not required for repression. Mutational analyses found that sequences within the *IME4* open reading frame (ORF) are required for the repression mediated by *RME2* transcription. These results support a model where transcription of *RME2* blocks the elongation of the full-length *IME4* transcript but not its 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 for the control of gene expression and may account for the roles of some of the previously uncharacterized ncRNAs in yeast.

One of the main paradigms for the control of gene expression is that regulatory proteins bind to the promoter regions of genes to activate or repress transcription. However, it is now clear that noncoding RNAs (ncRNAs) also play important roles in gene regulation. For example, RNA interference (RNAi)-mediated regulation controls gene expression in *Caenorhabditis elegans*, *Arabidopsis thaliana*, humans, and many other organisms (16, 24). However, a large number of ncRNAs do not appear to be involved in RNAi-mediated regulation. For example, more than 900 ncRNAs are 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 the 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 more than 500 genes that are highly regulated in a coordinated manner (7, 28). Entry into the meiotic pathway is controlled by the expression of *IME1*, the master initiator of meiosis (20, 29). There are two signals that regulate *IME1* expression (Fig. 1A). One signal relates to the nutritional status of the cell, activating *IME1* expression when the cell is starved of both nitrogen and a fermentable carbon source (14, 37). The second signal operates through cell-type-specific regulation, which allows the expression of meiotic genes only 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-cell-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 and thereby relieving the repression of *IME1*. The second form of cell-type-specific control is mediated through Ime4, which is required for full 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 has been hypothesized that a different haploid-cell-specific repressor regulates *IME4* (38).

To identify **a**1-α2 target sites, and possibly the factor regulating *IME4* expression, in the yeast genome, 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* open reading frame (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 the 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, since *RME2* is unable to repress an adjacent copy of *IME4* (17).

We show in this paper that the expression of another antisense ncRNA regulates the meiosis-specific *ZIP2* gene in a manner similar to the regulation of *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 expres-

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▽ Published ahead of print on 7 February 2011.

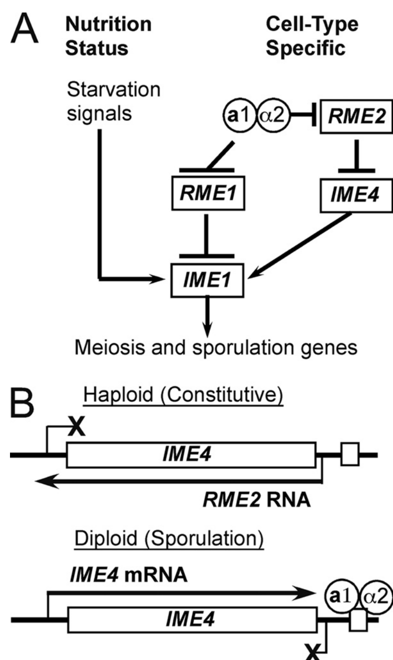

FIG. 1. Model for the regulation of *IME1*. (A) Cell-type-specific regulation of *IME1* is mediated in part by the **a**1-α2 complex, which prevents the expression of *RME1*, a repressor of *IME1*. The *IME4* gene is also required for the full activation of *IME1. IME4* expression requires the **a**1-α2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

sion 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, consisting of bp +1400 to +2000 from the *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 (wild-type [WT] sequence, GTGTATTTTTTTACATCA; mutant [Mu] sequence, GTcgA TTTTTTTACggCA) to produce plasmid pBG7. Plasmid pBG113 contains a 2.9-kb PCR fragment, consisting of bp −450 to +400 flanking the *IME4* ORF, cloned into the TOPO TA vector (Invitrogen). This plasmid was first digested with XbaI and HindIII, and *IME4* was cloned 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 site 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 bp −23 and −24 relative to the start site of the *IME4* ORF from GA to TT.

Plasmid pJM532 contains a 3.1-kb PCR-generated fragment, consisting of bp −450 to +400 flanking *ZIP2* genomic DNA, cloned into the TOPO TA vector (Invitrogen). This plasmid was digested with SpeI and ApaI, and *ZIP2* was cloned 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 given in Table 1. Strains YBG111 and YBG112 were constructed by transforming the W303 derivative strains LNY315 and LNY316 with a PCR fragment amplified from pFA6a-KanMX6 with the *KanMX* cassette and 50 bp of flanking homology so as to delete bp −450 to +400 flanking the *IME4* ORF (43). These strains were mated to produce an *ime4*Δ/*ime4*Δ diploid strain, YBG115. *IME4* deletion and poly(A) terminator mutants were integrated at the *LEU2* locus by digesting the plasmids listed above with XcmI and transforming the linearized DNA into strain YBG111. Transformants were selected on a medium lacking leucine and were 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-fluoroorotic acid (5-FOA) to recombine the mutant binding site at the wild-type locus, generating 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), which was performed on two to four replicate samples. Single colonies of yeast strains (from separate transformations for plasmid-bearing strains) were grown under rich nutrient (yeast extract-peptone-dextrose [YEPD] or synthetic dextrose [SD] medium) or sporulation-inducing (sporulation medium [SPM]) conditions (3 h in SPM for *IME4* assays; 5 h for *ZIP2* assays), and total RNA was extracted by hot acid-phenol extraction, as described previously (1). Normalized RNA samples were treated with Turbo DNA-Free DNase (Ambion), and the DNase-treated RNA was amplified by PCR with the *IME4*/*RME2* or *ZIP2*/*RME3* primer set to verify the absence of contaminating DNA. cDNAs of the *IME4*, *RME2*, *URA3*, *ZIP2*, *RME3*, *HSP26*, *YFL012W*, and *ACT1* genes were synthesized for each RNA sample with sense and antisense specific primers by using Omniscript RT (Qiagen). Different cDNA sample concentrations (1 to 4 μl) were assayed to verify that the reaction was in the linear range. PCRs using different concentrations of cDNA as a template were amplified in 50-μl reaction mixtures containing 10 pmol of each amplicon primer set, 1× AmpliTaq *Taq* Buffer II, and 2.5 U of AmpliTaq *Taq* polymerase (Applied Biosystems). The amplifications were carried out for 30 cycles of 94°C for 30 s, 52°C for 1 min, and 72°C for 30 s. Samples were run on 1.4% agarose–Tris-acetate-EDTA (TAE) gels, and images were photographed with a Fluorochem 8800 camera.

ChIP assays. Chromatin immunoprecipitation (ChIP) assays were performed using a modified version of protocols described elsewhere (27, 34). Cultures of strains LNY392 and YBG144 or strains YBG111 and YBG115 carrying the *HOP1pr*::*IME4* constructs indicated in Fig. 5 were grown to mid-log phase (optical density at 600 nm [OD_{600}], 0.500) in the appropriate medium (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 Tris-buffered saline (TBS), and frozen at −80°C for a minimum of 12 h. Cell pellets were suspended with 400 μl lysis buffer (50 mM HEPES [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate) plus 1 mM phenylmethylsulfonyl fluoride (PMSF), 1× protease inhibitor cocktail from Roche (catalog no. 1873580), and 50 μl Sigma protease inhibitor cocktail (catalog no. 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 × *g* and 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 5-s cycles to give an average chromatin fragment size of 500 bp. Sonicated lysates were centrifuged at 12,000 × *g* for 5 min, and 50 μl of the sonicated DNA was reserved as a total-chromatin (TC) sample. The remaining DNA was precleared by the addition of 25 μl of protein G agarose beads and was nutated for 1 h at 4°C, and the lysates were cleared by centrifugation at 12,000 × *g* for 5 min at 4°C. To immunoprecipitate TATA-binding protein (TBP)- or Abf1-bound DNA, 10 μl or 1 μl of the anti-TBP polyclonal antibody yN-20 (sc-26141; Santa Cruz) or the anti-Abf-1 polyclonal antibody yC-20 (sc-6679; Santa Cruz) was added, and the mixture was nutated for 16 h at 4°C. Centrifugation with protein G beads, washes, DNA elution, cross-linking reversal, and proteinase treatment were all performed as described previously (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 μl of TC samples (a 500-fold dilution and a 50-fold dilution) or immunoprecipitated (IP) samples (1 and 3 μl) was used in 50-μl reaction

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	*MAT*α *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	*MAT*α *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 ime4*Δ*::kanMX4*	This study
YBG112	*MAT*α *ade2-1 trp1-1 his3-11,15 can1-100 ura3-1 leu2-3,112 ime4*Δ*::kanMX4*	This study
YBG115	*MAT***a**/*MAT*α *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 ime4*Δ*::kanMX4/ime4*Δ*::kanMX4*	This study
YBG144	*MAT***a**/*MAT*α *ade2-1/ADE trp1-1/TRP his3-11,15/his3-11,15 can1-100/can1-100 ura3-1/ura3-1 leu2-3,112/leu2-3,112*	This study
JMY076	LNY392 with *IME4–***a***1-*α*2::CaURA3*	This study
JMY077	LNY433 with *IME4–***a***1-*α*2::CaURA3*	This study
JMY081	LNY392 with *IME4–***a***1-*α*2 mut*	This study
JMY082	LNY433 with *IME4–***a***1-*α*2 mut*	This study
JMY084	YBG144 with *IME4–***a***1-*α*2 mut/IME4–***a***1-*α*2 mut*	This study
JMY104	LNY392 with *zip2::kanMX4*	This study
JMY105	LNY433 with *zip2::kanMX4*	This study
JMY108	LNY392 with *zip2::kanMX4 leu2::ZIP2*	This study
JMY109	LNY433 with *zip2::kanMX4 leu2::ZIP2*	This study
JMY110	YBG144 with *zip2::kanMX4/zip2::kanMX4 leu2::ZIP2/leu2::ZIP2*	This study
JMY111	LNY392 with *zip2::kanMX4 leu2::ZIP2-***a***1-*α*2 mut*	This study
JMY112	LNY433 with *zip2::kanMX4 leu2::ZIP2-***a***1-*α*2 mut*	This study
JMY113	YBG144 with *zip2::kanMX4 leu2::ZIP2-***a***1-*α*2 mut*	This study
YBG145	YBG111 with *leu2::ime4*Δ*3′*	This study
YBG147	YBG111 with *leu2::IME4*	This study
YBG149	YBG115 with *leu2::IME4/leu2::IME4*	This study
YBG150	LNY315 with *leu2::ime4*Δ*3′*	This study
YBG158	YBG144 with *zip2::kanMX4/zip2::kanMX4 leu2::ZIP2-***a***1-*α*2 mut/leu2::ZIP2*	This study
YBG159	YBG111 with *leu2::rme2-s1*	This study
YBG160	YBG111 with *leu2::ime4*Δ*1-900*	This study
YBG161	YBG111 with *leu2::ime4*Δ*1-450*	This study
YBG162	YBG111 with *leu2::ime4*Δ*451-900*	This study
YBG183	YBG111 with *leu2::ime4*Δ*1-224*	This study
YBG184	YBG111 with *leu2::ime4*Δ*676-900*	This study
YBG202	YBG111 with *leu2::rme2-s2*	This study

mixtures for 30 cycles. The PCR products were run on 1.5% agarose gels. Samples were quantified using ImageJ software and normalization 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). In agreement with previous reports, we found that a mutation in this site allows expression of the *IME4* antisense transcript *RME2* and prevents *IME4* expression in diploid cells (17; also data not shown).

To determine whether *RME2* is required for the repression of *IME4*, we constructed a mutation, *ime4*Δ*3′*, that deletes the *RME2* promoter. Haploid cells with this mutation failed to express *RME2*, and *IME4* was derepressed (Fig. 2B, lane 3). This suggests that *RME2* expression is required for the repression of *IME4*.

Expression of *RME2* from the native locus did not repress the transcription of *IME4* from the *ime4*Δ*3′* mutant integrated at the distant locus (Fig. 2B, lane 4). This indicates that *RME2* transcription is able to repress *IME4* only in a *cis*-acting manner. Our results are consistent with previous work that used a different mutation in the **a**1-α2 site and a different assay for expression of the sense and antisense genes (17).

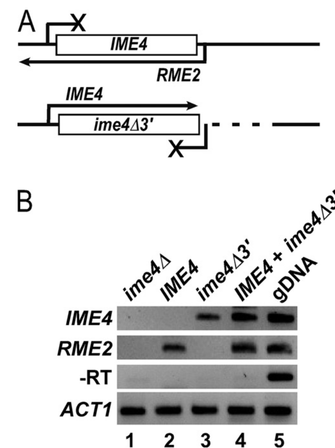

FIG. 2. (A) Cartoon illustrating the wild-type and *ime4*Δ*3′* constructs. The *ime4*Δ*3′* construct contains a deletion of the *RME2* promoter region (+1809 to +2209), as indicated by the dashed line. "X" indicates that the transcript is not expressed from that copy of the *IME4* locus. (B) Deletion of the *RME2* promoter region allows the expression of *IME4* in haploid cells, and *RME2* expression fails to repress the *ime4*Δ*3′* mutation in *trans*. RT-PCR assays of the *IME4* and *RME2* transcripts were performed on *ime4*Δ (YBG111) (lane 1), WT (YBG147) (lane 2), *ime4*Δ*3′* (YBG145) (lane 3), and WT + *ime4*Δ*3′* (YBG150) (lane 4) haploid strains under sporulation-inducing conditions. The −RT row shows PCR amplifications of DNase-treated RNA with the *IME4* primer set to control for the contamination of genomic DNA. RT-PCR of *ACT1* expression was used as a loading control. Genomic DNA (gDNA) (lane 5) was included as a control for PCR amplification.

Antisense transcription is a conserved model of gene regulation in yeast. Since *IME4* is regulated by transcription of an ncRNA in the antisense direction, we wanted to determine if other genes are regulated by a similar mechanism. We therefore reevaluated 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 *HSP26* and *YFL012W* are not regulated in a cell-type-specific 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 that 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 sporulation conditions (Fig. 3B, lane 4) (7). In contrast, haploid cells under the same conditions expressed an ncRNA antisense to *ZIP2*, which we 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 in determining whether 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 whether *RME3* represses *ZIP2* transcription in a *cis*- or a *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 mutation of four base pairs in the **a**1-α2 site that allows *RME3* expression in diploid cells. If *ZIP2* were repressed by *RME3* in *trans*, we would have expected to see a lower level of *ZIP2* in the heterozygote than in a wild-type homozygous strain, a mechanism similar to RNAi (9, 16). However, the level of *ZIP2* expression was the same in the mutant heterozygous and wild-type homozygous diploid strains, indicating that expression of *RME3* in *trans* did not inhibit *ZIP2* transcription (Fig. 3D, lane 2 versus lane 4). This indicates that *RME3* regulates *ZIP2* in a *cis*-dependent configuration, a pattern similar to that of the *IME4*/*RME2* regulatory system.

Changing the termination site of *RME2* affects its ability to regulate *IME4*. The observation that both *IME4* and *ZIP2* are repressed by antisense transcription in haploid cells suggests that this may be a common mechanism of gene regulation. We wanted to determine how the expression of an antisense transcript in *cis* prevents the 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 the binding of the transcriptional activators required for expression. If *IME4* is regulated by a mechanism similar to those for *SER3* and *ADH1*, then premature termination of the *RME2* transcript would allow the expression of *IME4* in haploid cells. To test this model, two mutations, *rme2-s1* and *rme2-s2*, that truncate the *RME2* transcript by the introduction of a eukaryotic poly(A) signal sequence, AAUAAA, were constructed. The *rme2-s1* mutation introduced a poly(A) site in the direction of *RME2* at 447 bp downstream of the *IME4* ATG; this change is silent with respect to the *IME4* coding sequence. The *rme2-s2* mutation introduced the same poly(A) site 23 bp upstream of the *IME4* ATG; it does not overlap with the presumptive *IME4* TATA box or alter the spacing of the promoter. Because transcription extends past poly(A) sites by roughly 100 bp, the *rme2-s1* mutation shortens *RME2* by 700 to 800 bp, and *rme2-s2* shortens *RME2* by 300 to 400 bp (Fig. 4A)

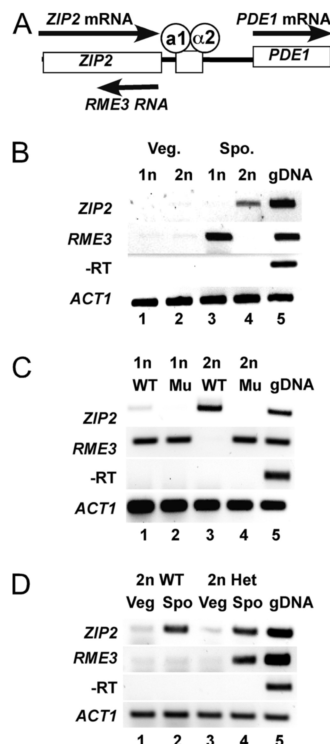

FIG. 3. Expression analyses of the *ZIP2* gene and the antisense transcript *RME3* show cell-type-specific regulation. (A) Schematic of the *ZIP2* and *RME3* transcripts and the relative position of the **a**1-α2 binding site downstream of the meiosis-specific gene *ZIP2* and upstream of the non-cell-type-specific gene *PDE1*. (B) RT-PCR assays of *ZIP2* and *RME3* from haploid (LNY392) (lanes 1 and 3) and diploid (YBG144) (lanes 2 and 4) cells grown in YEPD (Veg) (lanes 1 and 2) or sporulation-inducing (Spo) (lanes 3 and 4) medium. (C) RT-PCR assays of *ZIP2* and *RME3* from haploid and diploid cells grown under sporulation conditions for 5 h. Either a wild-type (WT) haploid (JMY108) (lane 1) or diploid (JMY110) (lane 3) strain or an **a**1-α2 binding site mutant (Mu) haploid (JMY108) (lane 2) or diploid (JMY110) (lane 4) strain was used. (D) RT-PCR assays of *ZIP2* and *RME3* from wild-type (JMY110) (lanes 1 and 2) or heterozygous (YBG158) (lanes 3 and 4) diploid cells grown in Veg or Spo medium. Assays and controls are described in the legend to Fig. 2.

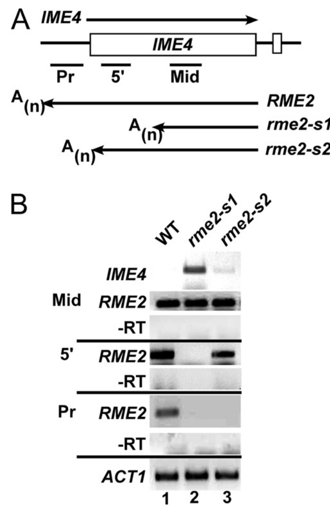

FIG. 4. Alteration of the polyadenylation and termination of the *RME2* transcript alters its ability to repress *IME4* in haploid cells. (A) Illustration of the three PCR amplicons (Pr, *IME4* promoter; 5′, 5′ end of the sense ORF; Mid, middle of the ORF) used to detect both *IME4* and *RME2* transcripts upstream and downstream of the termination sites introduced in *rme2-s1* and *rme2-s2.* Primers downstream (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* (YBG202) (lane 3) haploid strains. Assays and controls are described in the legend to Fig. 2. The −RT control was performed for all assay primer pairs.

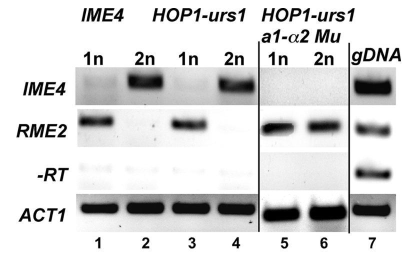

FIG. 5. The *HOP1-urs1* promoter is regulated by antisense transcription in a manner similar to the regulation of the native *IME4* promoter. Shown are results of RT-PCR assays of *IME4* and *RME2* from haploid (1n) and diploid (2n) cells after 3 h in sporulation-inducing medium. *IME4* (sense) transcription is driven either by the native *IME4* promoter (LNY392 [lane 1] and YBG144 [lane 2]) or by a *HOP1* promoter containing a mutation in the URS1 site (YBG111/pBG157 [lane 3] and 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 are described in the legend to Fig. 2.

(4). A similar approach was used to truncate the *Kcnq1ot1* transcript in mouse and to investigate its role in antisense-mediated regulation (19). To assay for premature termination of *RME2*, primer sets targeting regions 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 detected only by the Mid primer set, indicating that the transcript was prematurely terminated before the 5′ end of the *IME4* ORF. Similarly, *rme2-s2* was detected only by the Mid and 5′ primer sets, not by the Pr primer set, indicating that transcription did not extend through the entire *IME4* promoter and upstream region, as in the wild-type haploid strain (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 the 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 that they had no effect on TATA-binding protein (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 the 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 expression activity similar to that of the *IME4* promoter. We constructed a strain in which the *IME4* promoter was replaced 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 the *HOP1-urs1* promoter (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 the expression of *RME2* in haploid and diploid cell types (Fig. 5, lanes 5 and 6). For the mutant, 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 to prevent meiosis in diploid cells (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 the 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* and *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

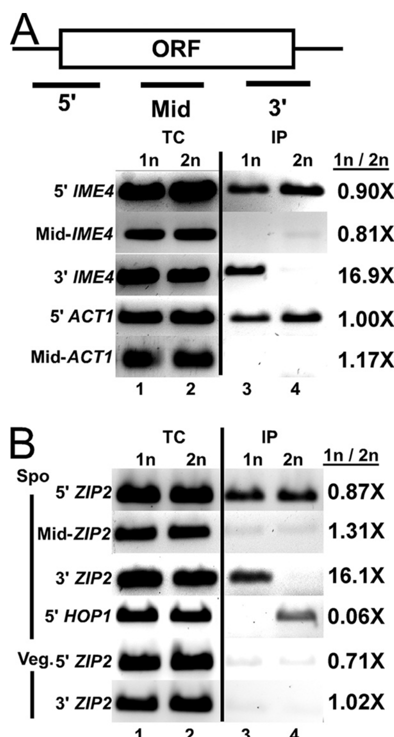

FIG. 6. Antisense transcription does not disrupt TBP binding at the *IME4* or *ZIP2* promoter. (A) ChIP assays for TBP bound at both the sense (5′) and antisense (3′) promoters in haploid WT (LNY392) (lanes 1 and 3) and diploid WT (YBG144) (lanes 2 and 4) cells. Assays were performed after 3 h in SPM. The middle regions (Mid-) of the *IME4* and *ACT1* ORFs, which are not precipitated with an anti-TBP antibody, were included as negative controls, and the constitutive *ACT1* promoter was included as a positive control for antibody binding. TC, total-chromatin sample (lanes 1 and 2); IP, DNA immunoprecipitated with the anti-TBP antibody yN-20 (lanes 3 and 4). The fold change in TBP binding in haploid versus diploid cells is given on the right. (B) Amplification of the *ZIP2* and *RME3* promoters from haploid (LNY392) (lanes 1 and 3) and diploid (YBG144) (lanes 2 and 4) cells. As in the *IME4* ChIP assay for which results are shown in panel A, TBP binding was assayed with the 5′, Mid, and 3′ amplicons by using the concentrations of purified TC and IP DNA given in Materials and Methods. The native *HOP1* promoter was tested as a meiosis-specific, cell-type-dependent promoter. ChIP assays were also performed on the *ZIP2* and *RME3* promoters in vegetative cultures of these haploid and diploid cells in order to confirm that these regions are not constitutively bound by TBP (Veg. 5′ *ZIP2* and Veg. 3′ *ZIP2*, lanes 3 and 4).

monitor transcription factor binding at both the sense and antisense promoter regions. The recruitment of TBP can be used to determine whether an ncRNA disrupts transcription factor binding at a promoter (25). TBP binding to the *IME4* promoter (5′), the middle of the ORF (Mid), and 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*, lane 3 versus lane 4). As expected, TBP was bound to the *IME4* promoter in diploid cells (Fig. 6A, 5′ *IME4*, lane 4). Surprisingly, despite the 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 IME4* promoter fusion even when *IME4* was repressed by *RME2* in haploid cells (data not shown) (13). These results indicate that *RME2* transcription does not disrupt the occupancy of the *IME4* promoter by transcription factors.

We also examined the binding of TBP at the *ZIP2* locus in order to determine if the mechanism of *RME3*-mediated repression is similar to that of *RME2*-mediated repression. Like the *RME2* promoter, the *RME3* promoter was bound by TBP in haploid cells with 16.1-fold higher affinity than in diploid cells (Fig. 6B, 3′ *ZIP2*, lane 3 versus lane 4). As with *IME4*, TBP bound to the *ZIP2* promoter with roughly the same affinity in haploid and diploid cells (Fig. 6B, 5′ *ZIP2*, lanes 3 and 4). The wild-type *HOP1* gene, which is activated at a time point in meiosis similar to that for *ZIP2*, was bound by TBP only in diploid cells (Fig. 6B, 5′ *HOP1*, lane 3 versus lane 4). This shows that, unlike the *IME4* and *ZIP2* promoters, 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 that the *IME4* ORF sequence may play a role in regulation by *RME2*. To determine whether regions within the *IME4* ORF are required for antisense-mediated repression, we performed an internal deletion analysis of the gene. Constructs with 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 versus lane 1). In contrast, deletion of bp 901 to 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 are required for antisense-mediated repression.

To further define the region required for the 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 to 675 is required for proper antisense-mediated repression.

The 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 to 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 for this DNA element. To test for this, the segment of DNA from bp 225 to 675 was flipped to the opposite orientation within the context of the *IME4* ORF (Fig. 8, top). This mutation had no effect on the expres-

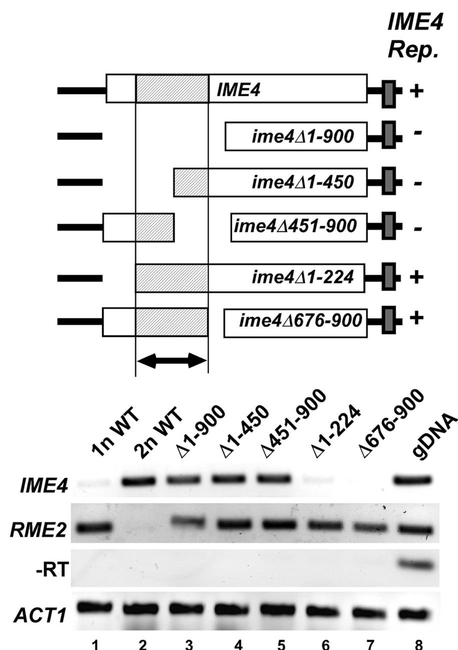

FIG. 7. Deletion analysis of *IME4* reveals the region within the ORF that is required for antisense-mediated regulation. (Top) Cartoon showing the locations of the deletions and whether deletion of the hatched region leads to the loss of *IME4* repression (Rep.). +, repression; −, loss of repression. (Bottom) RT-PCR assays of *IME4* and *RME2* expression from wild-type haploid (lane 1) and diploid (lane 2) cells and from haploid cells with the indicted base pairs of the *IME4* ORF deleted (lanes 3 to 7), grown in sporulation-inducing medium. *IME4* expression was monitored using an amplicon within the ORF, and *RME2* expression was monitored using an amplicon in the *IME4* promoter region. gDNA, genomic DNA. Deletion strains are listed in Table 1.

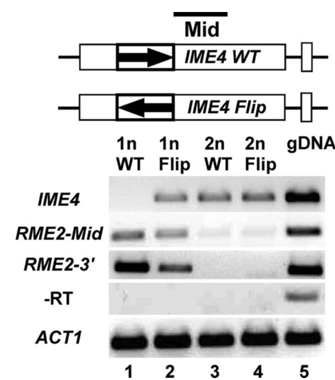

FIG. 8. Reversing the orientation of the region of *IME4* from bp 225 to bp 675 prevents proper regulation by *RME2*. Shown are results of RT-PCR assays of *IME4* and *RME2* from haploid (YBG111) (lanes 1 and 2) or diploid (YBG115) (lanes 3 and 4) cells carrying *IME4* on a plasmid with bp 225 to 675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

sion of *IME4* in diploid cells (Fig. 8, lane 3 versus lane 4), showing that it is silent with respect to the expression and stability of the *IME4* transcript. However, in haploid cells, this mutation caused derepression of the *IME4* transcript (Fig. 8, lane 1 versus 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 or the antisense direction (11, 21, 31, 35, 40). In higher eukaryotes, many of these ncRNAs regulate gene expression through *trans*-acting micro-RNA and small interfering RNA (siRNA) 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 those of higher eukaryotes (12). However, work by several labs has shown that some of the >900 ncRNAs in yeast do have regulatory functions. For example, *trans*-acting antisense regulatory ncRNAs function to silence the transcription and transposition of the Ty1 retrotransposon and the 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 the 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*, that functions to repress the 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 noncoding 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 nonfunctional when supplied in *trans* (3, 25). This contrasts with *PHO84*, where a second copy has a *trans*-acting effect on the native gene (6). 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 the use of 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 manner similar to that of 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 the 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 the 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 the 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 also is not likely required for repression, because the *RME3* transcript appears to terminate naturally in the *ZIP2* ORF (11, 31). These results suggest that ncRNA-mediated repression of *IME4* and *ZIP2* does not occur through a mechanism of promoter interference such as that observed for *SER3* and *ADH1.* Our results are similar to those of 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, the 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 show that genes adjacent to *IME4* and *ZIP2* (*HOS2*/*COX13* and *RMR1*/*PDE1*, respectively) are not corepressed 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 the *IME4* and *ZIP2* promoters (Fig. 6). If antisense transcription regulated 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 those for regulation by the *trans*-acting *PHO84* antisense transcript, which does not interfere with the binding of TBP at the sense promoter (5, 25). This further highlights the fact that the mechanisms of ncRNA-mediated regulation are different for different gene pairs.

A 450-bp region (bp 225 to 675) within *IME4* is essential for *RME2*-mediated repression. It is possible that this region contains target sites for protein complexes that play 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 prevents full-length *IME4* expression. It has been shown that directional chromatin remodeling represses antisense transcription from cryptic sites (44). Therefore, it is possible that deletion of 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 shown previously 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 possibility for the mechanism of antisense regulation by *RME2* is that the region (bp 225 to 675) within *IME4* that is essential for its repression 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 transcripts 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-to-675 region of the ORF, and this truncation results in full-length transcription of *IME4*. This DNA sequence may therefore be transcribed only 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 *YGR031W* and *NAG1*, two overlapping genes that appear to be transcribed simultaneously in vegetative medium (23).

We also observed that there is an orientation-specific requirement of *IME4* bp 225 to 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 the expression of the full-length *IME4* transcript, short, incomplete *IME4* transcripts have been detected (17; also 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 bp 225-to-675 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 more than 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 that of *RME2* and *RME3.* These gene pairs may explain the role of some of the >900 ncRNAs in yeast.

ACKNOWLEDGMENTS

We thank Chuck Martin for providing the *CaURA3* clone.

This work was supported by the Charles and Johanna Busch Fellowship (Rutgers University) to B.G. and by grants from the National Institutes of Health to A.M.S. (HG03470) and A.K.V. (GM49265).

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