An Antisense RNA-mediated Mechanism Eliminates a Meiosis-specific Copper-regulated Transcript in Mitotic Cells*

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Background: Convergent gene pairs produce sense and antisense transcripts that could interfere with the expression of either partner gene. In Schizosaccharomyces pombe, we found that the iss1+ gene produces two transcript isoforms, including a long antisense mRNA that is complementary to the meiotic cum1+ sense transcript, inhibiting cum1+ expression in vegetative cells. Inhibition of cum1+ transcription was not at the level of its initiation because fusion of the cum1+ promoter to the lacZ gene showed that activation of the reporter gene occurs in response to low copper conditions. Further analysis showed that the transcription factor Cufl and conserved copper-signaling elements (CuSEs) are required for induction of cum1+ lacZ transcription under copper deficiency. Insertion of a multiparticle polyadenylation signal immediately downstream of iss1+ led to the exclusive production of a shorter iss1+ mRNA isoform, thereby allowing accumulation of cum1+ sense mRNA in copper-limited vegetative cells. This finding suggested that the long iss1+ antisense mRNA could pair with cum1+ sense mRNA, thereby producing double-stranded RNA molecules that could induce RNAi.

Results: RNAi, sense, and antisense transcripts play a causal role in cum1+ gene silencing in vegetative cells.

Conclusion: This is the first report to show an antisense transcription control of a copper-regulated transcript.

Significance: Selective elimination of meiosis-specific transcripts prevents their aberrant expression during vegetative growth.

Sense and antisense transcripts produced from convergent gene pairs could interfere with the expression of either partner gene. In Schizosaccharomyces pombe, we found that the iss1+ gene produces two transcript isoforms, including a long antisense mRNA that is complementary to the meiotic cum1+ sense transcript, inhibiting cum1+ expression in vegetative cells. Inhibition of cum1+ transcription was not at the level of its initiation because fusion of the cum1+ promoter to the lacZ gene showed that activation of the reporter gene occurs in response to low copper conditions. Further analysis showed that the transcription factor Cufl and conserved copper-signaling elements (CuSEs) are required for induction of cum1+ lacZ transcription under copper deficiency. Insertion of a multiparticle polyadenylation signal immediately downstream of iss1+ led to the exclusive production of a shorter iss1+ mRNA isoform, thereby allowing accumulation of cum1+ sense mRNA in copper-limited vegetative cells. This finding suggested that the long iss1+ antisense mRNA could pair with cum1+ sense mRNA, thereby producing double-stranded RNA molecules that could induce RNAi.

We consistently found that mutant strains for RNAi (dcr1Δ, ago1Δ, rdplΔ, and clr4Δ) are defective in selectively eliminating cum1+ sense transcript in the G1 phase of the cell cycle. Taken together, these results describe the first example of a copper-regulated meiotic gene repressed by an antisense transcription mechanism in vegetative cells.

During most of its life cycle, the fission yeast Schizosaccharomyces pombe is haploid and undergoes mitotic proliferation (1). Under poor nitrogen supply conditions, S. pombe cells arrest in the G1 phase of the cell cycle and conjugate with cells of the opposite mating type. Conjugation leads to the formation of precursor diploid cells that can undergo the meiotic program. Once in meiosis, diploid cells replicate their genetic material, producing pairs of homologous chromosomes. After genetic recombination between homologous chromosomes, two successive nuclear divisions occur without an intervening S phase, generating four haploid sets of chromosomes, termed chromatids. Each set of chromatids is then enclosed in a forespore, resulting in four spores that are protected within an ascus. Although the meiotic program requires general components (e.g. spindles, centrosomes, and kinetochores) as the mitotic program, several genes encode proteins that are unique to meiosis (2, 3). Importantly, it has been shown that untimely expression of meiotic genes in cells undergoing mitotic proliferation could be detrimental (4, 5). Therefore, cells have developed different mechanisms to ensure elimination of meiosis-specific transcripts in cells undergoing mitotic growth.

One mechanism of mRNA elimination involves a region denoted DSR (determinant for selective removal) that is found in several meiosis-specific transcripts in fission yeast (6, 7). In cells undergoing mitotic growth, RNA-binding protein Mmi1 binds to a DSR region and then triggers transcript elimination with the aid of an RNA degradation system (5, 8, 9). In the case of cells entering into meiosis, meiotic protein Mei2 sequesters Mmi1 in a nuclear dot structure, preventing its action, thus allowing meiosis-specific transcripts to become stable and competent to be expressed in meiotic and sporulating cells.

An additional mechanism involves production of RNA molecules that are antisense to protein coding transcripts (mRNAs) (10, 11). The open reading frame or DNA region that produces an antisense transcript can be located in the neighborhood (e.g. on the opposite DNA strand) of the gene from which the sense mRNA strand is produced (12–14). An antisense transcript can also be produced at a distinct genomic locus from its sense partner RNA, thereby acting in trans to regulate sense transcription (13, 15). Control of gene expression by antisense transcripts could involve different regulators and modes of action. Studies have shown that transcription of an antisense strand inhibits transcription on a sense strand by blocking progression of a sense strand RNA polymerase (collision model) (16). Transcriptional interference could also function through the action of histone/chromatin-modifying enzymes (17, 18). Antisense/sense double-stranded RNAs could negatively affect splicing, stability, and translation of sense mRNAs (14, 18). Repression of sense transcription of meiotic genes in mitotic cells is also partially under the control of the forkhead transcription factor...
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Fkh2 (19). A genome-wide analysis has shown that 229 genes exhibit increased sense RNA levels in mitotic cells deficient in Fkh2 gene expression. More than 75% of these genes are normally expressed exclusively during middle-phase meiosis and not expressed during mitosis (19).

As observed in the case of several genomes of prokaryotes and eukaryotes, S. pombe genes are frequently organized into convergent pairs (20, 21). This arrangement is recognized when two genes are in proximity of one another with their transcription orientated one toward the other. When these convergent genes are transcribed in cis from opposing DNA strands, they produce sense and antisense transcripts that are often partially complementary to each other. In many cases, perturbation of expression of sense mRNA (from gene 1) occurs due to the presence of the corresponding antisense RNA (from gene 2). In fission yeast, sense/antisense RNA duplexes accumulate in G1 phase of the cell cycle, especially in regions where convergent genes are present (22). In G1, transcription of several convergent genes fails to terminate after their proximal cleavage and polyadenylation sites, thereby resulting in a transcriptional read-through that produces long sense/antisense transcripts. Accumulation of long sense/antisense RNA duplexes activates the RNA interference (RNAi) pathway, which leads to gene silencing and heterochromatin formation over convergent gene regions. Transient heterochromatin is then recognized by Swi6 (23). In subsequent S and G2 phases, Swi6 recruits cohesin loading complexes in regions between convergent genes. This mechanism has been found to promote proximal transcription termination of convergent gene pairs and significantly decrease transcriptional interference between convergent genes (22). In the case of some convergent genes, it has been shown that the mechanism of transcriptional interference is independent of the RNAi machinery. In those cases, however, the molecular basis of antisense-mediated repression of gene expression is unclear (19).

Cuf1 is a major regulator of copper homeostasis in S. pombe (24). When mitotic cells are grown under conditions of copper starvation, the transcription factor Cuf1 is located in the nucleus where it activates genes encoding components of the copper transport pathway, including Ctr4, Ctr5, and Ctr6 (25, 26). This transcriptional activation involves cis-acting promoter regulatory elements with the consensus sequence 5’-T(H/A)DDHGCTG-3’ (D = A, G, or T; H = A, C, or T), which are denoted CuSEs (copper signaling elements) (25). In contrast, in cells undergoing a transition from low to sufficient copper concentration, Cuf1 is inactivated, and that results in repression of copper transport genes. After its inactivation, Crm1 exportin interacts with Cuf1 via its nuclear export sequence, leading to export of the transcription factor to the cytoplasm (27).

A previous genome-wide study has identified a gene (SPAC22G7.11c), denoted cum1+, whose transcript is induced in copper-starved meiotic cells (28). cum1+ encodes a small polypeptide of 140 amino acid residues with a predicted molecular mass of 15.4 kDa. Cum1 is highly hydrophilic and contains two CON-6-type domains. CON-6-like proteins are mostly found in sporulating cells and conidia in fungi (29). Although their functions are still unclear, they may be activated under low-water conditions, which would favor adoption of their active conformation. Once activated, CON-6 proteins may play a role in resistance to desiccation stress.

Although a potential role of Cum1 in copper homeostasis has not yet been ascertained, in the present work we found that its copper-dependent regulation requires the presence of CuSEs and Cuf1. cum1+ is expressed exclusively during meiosis. However, results showed that when a reporter gene is expressed under the control of the wild-type cum1+ promoter, its copper-dependent expression is detected in mitotically growing cells. These findings supported the notion that an elimination of cum1+ mRNA occurs after its transcriptional initiation in mitotic cells. Northern blot analyses revealed that the iss1+ gene in proximity to cum1+ produces two antisense transcripts, including a longer one that is complementary to the sense cum1+ mRNA. We showed that the insertion of a multipartite polyadenylation signal (PAS) immediately downstream of iss1+ allows cum1+ sense mRNA expression during vegetative growth, markedly under low copper conditions. Using G1-synchronized wild-type and dcr1Δ, ago1Δ, rdplΔ, and cl4Δ mutant cells, we determined that components of the RNAi pathway are involved in antisense-mediated repression of cum1+ transcription, especially during the G1 phase of the cell cycle.

Experimental Procedures

Strains and Growth Conditions—Strains undergoing vegetative growth were isogenic derivatives of FY435 (Table 1). In the case of strains that entered into meiosis, we used two isogenic strains (JSY3 and JSY84) in which the pat1–114 temperature-sensitive mutation allowed synchronization of cells in terms of their entry into the meiotic program (Table 1). Under non-selective conditions, all strains were grown on YES medium containing 0.5% yeast extract, 3% glucose, and 225 mg/liter adenine, histidine, leucine, uracil, and lysine. Strains for which DNA fragment or plasmid integration was required were grown on synthetic Edinburgh minimal medium missing specific nutrients that allowed selection and maintenance of the integrated DNA fragment or plasmid (1). In the case of liquid cultures of cells proliferating in mitosis, unless otherwise stated cells were seeded to an A600 of 0.5, grown to exponential phase (A600 of ~1.0), and left untreated or were treated with ammonium tetrathiomolybdate (TTM) (250 μM) or CuSO4 (100 μM) for 90 min. Synchronous meiosis of pat1–114/pat1–114 diploid cells was carried out as described previously (28). Treatments of meiotic cells were performed using low concentrations of TTM (150 μM) and CuSO4 (50 μM) as described previously (30). Monitoring progression of meiotic cells throughout the differentiation program was performed as described previously (31).

Plasmids—DNA fragments containing 1608, 376, or 174 bp of the 5′-noncoding region and the first 10 codons of the cum1+ gene were isolated by PCR. The first set of primers was designed to generate BamHI and ApaI restriction sites at the 5′ termini of the PCR products, whereas the second set of primers was engi-
needed to generate an EcoRI restriction site at the 3′ end of the PCR-amplified DNA fragments. Each PCR product was purified, digested with BamHI and EcoRI, and then introduced into Yep357R vector (32). The indicated cum1+ promoter region was isolated from Yep357Rcum1+ -1608lacZ, Yep357Rcum1+ -376lacZ, and Yep357Rcum1+ -174lacZ after digestion with Apal and Bsu36I (a unique restriction site into lacZ). Each of these promoter regions was then swapped for the equivalent DNA restriction fragment in pBPade6cum1+ -737 from the initiator codon) and the first 10 codons of the lacZ gene into the same restriction sites in pBluescript SK (Stratagene). The resulting antisense RNA hybridizes to the region between positions +115 and +324 downstream of the A of the initiator codon of cum1+. 32P-Labeled antisense RNA probes were generated using either HindIII (pSKlacZ)- or BamHI-linearized plasmids (pSKctr4+ , and pSKact1+ ) were used to produce anti-sense RNA probes that served to determine lacZ, ctr4+, and act1+ mRNA levels, respectively (25, 37). pSKcum1+ was constructed by inserting a 199-bp BamHI-EcoRI fragment of the cum1+ gene into the same restriction sites in pBluescript SK (Stratagene). This study demonstrates that cum1+ is not detectable in pSKlacZ, pSKctr4+ , and pSKact1+ use to produce anti-sense RNA probes were generated using either HindIII (pSKlacZ)- or BamHI-linearized plasmids (pSKctr4+ , and pSKact1+ ).

**RNA Analysis—** Total RNA was extracted using a hot phenol method as described previously (35). RNase protection assays were performed as described previously (36). Plasmids pSKlacZ, pSKctr4+ , and pSKact1+ were used to produce antisense RNA probes that served to determine lacZ, ctr4+, and act1+ mRNA levels, respectively (25, 37). pSKcum1+ was constructed by inserting a 199-bp BamHI-EcoRI fragment of the cum1+ gene into the same restriction sites in pBluescript SK (Stratagene). The resulting antisense RNA hybridizes to the region between positions +115 and +324 downstream of the A of the initiator codon of cum1+. 32P-Labeled antisense RNA probes were generated using either HindIII (pSKlacZ) or BamHI-linearized plasmids (pSKctr4+ , and pSKact1+ ), [α-32P]UTP, and T7 RNA polymerase as described previously (36). In the case of Northern blot analysis, total RNA (25 μg per sample) was resolved on 1.25% agarose-formaldehyde gels and transferred to, and immobilized on nylon membranes. Prehybridization and hybridization were carried out in Church's buffer (1% bovine serum albumin, 1 mM EDTA, 0.5 M NaHPO4, pH 7.2, 7% SDS). In the case of prehybridization experiments, freshly denatured herring sperm DNA (50 μg/ml) was added to Church's buffer in which membranes were soaked. After the hybridization step with 32P-labeled DNA-specific probes, washes were performed in 2× SSC (0.15 M NaCl, 15 mM trisodium citrate, pH 7.0) containing 0.1% SDS and then in 0.1× SSC at the same SDS concentration (0.1%). AS1 and AS3 probes hybridized to regions between positions +292 and +525 and positions +1529 and +1790, respectively, downstream from A of the start codon of iss1+. In the case of S1 probe, it paired to the region between positions +142 and +428 downstream from A of the initiator codon of cum1+. The act1+ probe (internal control) hybridized to the coding region

**TABLE 1** S. pombe strains genotypes

| Strain   | Genotype                        | Source |
|----------|---------------------------------|--------|
| FY435    | h+ his-7-366 leu1-32 ura4-Δ18 ade6-M210 | (49)   |
| JSY484   | h+ pat1-114 ade6-M210            | (50)   |
| JSX3     | h+ pat1-114 ade6-M210 cuf1Δ::KAN  | (28)   |
| cuf1Δcuf1+ | h+ his-7-366 leu1-32 ura4-Δ18 ade6-M210 cuf1Δ::loxP cuf1+::ara4+ | This study |
| cuf1Δcuf1+ | h+ his-7-366 leu1-32 ura4-Δ18 ade6-M210 cuf1Δ::loxP cuf1+::ara4+ | This study |
| JSX17    | h+ his-7-366 leu1-32 ura4-Δ18 ade6-M210 dcr1Δ::KAN  | (27)   |
| AMY52    | h+ his-7-366 leu1-32 ura4-Δ18 ade6-M210 dcr1Δ::KAN  | (27)   |
| VNY3     | h+ his-7-366 leu1-32 ura4-Δ18 ade6-M210 dcr1Δ::KAN  | (27)   |
| VNY4     | h+ his-7-366 leu1-32 ura4-Δ18 ade6-M210 ago1Δ::KAN  | (27)   |
| cuf1Δcuf1+ | h+ his-7-366 leu1-32 ura4-Δ18 ade6-M210 dcr1Δ::KAN  | (27)   |
| VNY6     | h+ his-7-366 leu1-32 ura4-Δ18 ade6-M210 iss1+::ura4PAS::KAN | This study |
| VNY7     | h+ his-7-366 leu1-32 ura4-Δ18 ade6-M210 cuf1Δ::loxP iss1+::ura4PAS::KAN | This study |
| VNY8     | h+ his-7-366 leu1-32 ura4-Δ18 ade6-M210 clr4Δ::KAN | This study |
| VNY9     | h+ his-7-366 leu1-32 ura4-Δ18 ade6-M210 pat1-114 ade6-M210 clr4Δ::KAN | This study |

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between positions +472 and +702 downstream from A of the ATG codon of act1+.

ChIP Experiments—cuf1Δ cells expressing untagged or Myc12-tagged cuf1+ alleles were grown to logarithmic phase. Cells were harvested, washed, and aliquoted in 50-ml cultures that were grown in the presence of TTM (250 μM) or CuSO4 (100 μM) for 90 min. Formaldehyde treatment of cultures and their subsequent neutralization with glycine were performed as described previously (38, 39). Cell lysates were prepared and subsequently sonicated 10 times (10 s cycles at 20 amplitude microns (20%)) using a Branson 450 sonicator to shear chromatin DNA into fragments of ~400–800 bp. Immunoprecipitation of Myc12-tagged Cuf1 bound to chromatin, protein G-Sepharose beads washings and elution, reversed cross-linking, and DNA precipitation were performed as described previously (38, 40). Quantification of immunoprecipitated DNA was performed by real-time PCR (qPCR) using primers that spanned a proximal cuf1+ promoter region, which includes three elements that match the CuSE consensus motif (25). As positive controls, primers that encompassed a cuf4+ promoter region that contains functional CuSEs (25) were also used for qPCR analysis. Myc12-tagged Cuf1 density at the cuf1+ or cuf4+ promoter was calculated as the enrichment of the specific genomic cuf1+ or cuf4+ promoter region relative to a 185 ribosomal DNA coding region in which no CuSe was present. Primers were designated by the name of the gene promoter followed by the position of their 5’ end relative to that of the translational initiation codon: cuf1−344 (5’-GATCGATAGAGCTGCTAGTTTC-3’), cuf1−165 (5’-TTTCCATTCCCATCAGATTCC-3’), cuf1−737 (5’-GAATGCTACTGGGTAGTCTG-3’), and cuf1−584 (5’-CAGCTTCTTTTACACGGCAAT-3’). Two primers derived from a 185 ribosomal DNA coding region were used as internal background controls: 18S-1 (5’-GCCCTATCAACTTTCGTAGTGATT-3’) and 18S-2 (5’-GATGGTGGATCGCCCGTTCTC-3’). Each qPCR experiment was performed in triplicate, and all ChIP experiments were repeated at least three times using independent chromatin preparations.

Flow Cytometry Analysis—Cells were precultured in YES media. At log phase (A600 of ~1.0), cells were harvested, washed twice, and transferred to Edinburgh nitrogen-deficient medium to arrest cells at the G1 phase. After incubation for 24 h at 30 °C, cells were transferred to a nitrogen-replenished medium (YES) and divided into subcultures that were treated with TTM (250 μM), CuSO4 (100 μM), or left untreated. At this point, cells were handled and fixed for flow cytometry assays as described previously (41). Stained cells with propidium iodide were analyzed using a FACScan flow cytometer (BD Biosciences) with an argon laser tuned to 488 nm. The FL3 detector with a 670-nm long pass filter was used to collect propidium iodide fluorescence. Results were analyzed using the FlowJo v10 software (FlowJo LLC, Ashland, OR).

Results

cum1+ Is Regulated in a Copper- and Cuf1-dependent Manner in Meiotic Cells—Genome profiling studies have revealed that cum1+ exhibited higher levels of expression in copper-starved meiotic cells (28). To further characterize the expression profile of cum1+ during meiosis in relationship with copper and Cuf1 availability, pat1−/pat1− (cuf1+/cuf1+) and pat1−/pat1− (cuf1Δ/cuf1Δ) diploid strains were pre-synchronized in G1 meiotic phase by nitrogen starvation at 25 °C. The temperature was then shifted to 34 °C, triggering inactivation of Pat1 and allowing cells to undergo synchronous meiosis. Immediately before temperature shift, cells were treated with the copper chelator TTM (150 μM) or with CuSO4 (50 μM). Aliquots of cell cultures were taken 1, 3, 5, 7, and 9 h after meiotic induction, and steady-state levels of cum1+ mRNA were analyzed by RNase protection assays. Under low-copper conditions, transcript levels of cum1+ were induced in cuf1+/cuf1+ cells (Fig. 1). The magnitude of cum1+ gene expression increased throughout the meiotic program and was more robust under TTM conditions (Fig. 1). In contrast, mRNA levels of cum1+ remained very low under copper-replete conditions. RNA samples prepared from a pat1−/pat1− (cuf1Δ/cuf1Δ) deletion strain showed very weak cum1− mRNA levels in TTM-treated cells. Although in cuf1Δ/cuf1Δ cells cum1− mRNA levels were slightly increased after 9 h of meiotic induction (compared with levels observed after 1 h of meiotic induction), these levels corresponded to poor induction of cum1+ mRNA compared with levels observed with cuf1+/cuf1+ cells that had been exposed to TTM. Furthermore, cuf1−/cuf1+ cells showed loss of cum1+ expression under copper-replete conditions (Fig. 1). As a control and known copper- and Cuf1-regulated gene, cuf4+ expression was measured in the same strains under the conditions described above. Results showed that under low copper conditions, cuf4− mRNA levels were induced shortly after induction of meiosis and then were strongly reduced within ~5 h (Fig. 1) (28, 42). There was a lack of induction of cuf4+ mRNA under copper-replete conditions. Furthermore, cuf4+ transcripts were absent in mutant cells lacking Cuf1 (cuf1Δ/cuf1Δ) (Fig. 1) (28, 42). Collectively, results showed that under low copper conditions Cuf1 is required to robustly activate cum1+ gene expression during meiosis, especially in mid and late phases of the program.

cum1+ Transcripts Are Detected Exclusively during Meiosis—We next analyzed mRNA levels of cum1+ in mitotically growing cells as a function of copper and Cuf1 availability. Logarithmic phase cultures of wild type (cuf1+) and cuf1Δ strains were grown in the absence or presence of TTM (250 μM) or CuSO4 (100 μM) for 90 min. RNA analysis experiments failed to detect cum1+ transcripts regardless of cellular copper or Cuf1 status (Fig. 2). In the case of the expression of cuf4+ (assayed as a control), its steady-state mRNA levels were up- and down-regulated after treatment with TTM and copper, respectively, as compared with basal conditions (Fig. 2). In the absence of Cuf1 (cuf1Δ), cuf4+ mRNA was not detected because its expression is dependent of Cuf1 (28, 42). Together, the results revealed that cum1+ transcripts are found exclusively during meiosis.

Copper-responsiveness of cum1+ Promoter—Although cum1+ was not expressed in wild-type mitotically growing cells, four CuSEs containing the sequence 5’-(T/A)DDHGCTG-3’ (D = A, G, or T; H = A, C, or T) were identified within a promoter region of 1608 bp. Among these CuSEs, three were arranged as a direct repeat of 5’-(T/A)DDHGCTG-3’ between positions −337 to −330, −235 to −228, and −185 to −178 relative to the
translational initiator codon of \( \text{cum1}^+ \) (Fig. 3A). A fourth element was also oriented in the same direction relative to the transcription of \( \text{cum1}^+ \), but it was located farther upstream between positions \(-1584 \) to \(-1577 \) from the initiator codon. To test whether these CuSeS could confer copper-dependent regulation of gene expression in a different genomic context, three \( \text{cum1}^+/H11001 \) promoter segments were fused upstream of and in-frame with the \( \text{lacZ} \) gene in a reporter plasmid. Surprisingly, results showed that wild-type cells harboring \( \text{cum1}^+/-1608 \) and \( \text{cum1}^+/-376 \) reporter plasmids were able to induce \( \text{lacZ} \) mRNA expression under conditions of copper starvation (Fig. 3B and C). In contrast, \( \text{lacZ} \) mRNA was poorly expressed in wild-type cells treated with copper. When \( \text{cum1}^+/-174 \text{lacZ} \) fusion plasmid was transformed in wild-type cells, there was a lack of significant up- or down-regulation of \( \text{lacZ} \) mRNA levels as a function of copper availability (Fig. 3B and C). Intriguingly, we observed that the overall magnitude of constitutive \( \text{lacZ} \) expression was higher for this transformant as compared with other transformants (containing other \( \text{lacZ} \) fusion derivatives). When \( \text{cum1}^+/-1609 \text{lacZ} \) plasmid was expressed in \( \text{cuf1}/H9004 \) cells, \( \text{lacZ} \) mRNA expression was absent, revealing that Cuf1 was required for induction of \( \text{cum1}^+/-1608 \) \( \text{lacZ} \) transcription in response to copper starvation. In the case of \( \text{cum1}^+/-376 \text{lacZ} \) plasmid, its transformation in \( \text{cuf1} \Delta \) cells resulted in constitutive \( \text{lacZ} \) gene expression without any significant changes in response to the presence of TTM or copper (Fig. 3B and C). The fact that \( \text{lacZ} \) mRNA was constitutively expressed when it

**FIGURE 1.** \( \text{cuf1}^+ \) is required for maximal expression of \( \text{cum1}^+ \) in meiotic cells under copper-deficient conditions. A, representative expression profiles of \( \text{cum1}^+ \) and \( \text{ctr4}^- \) transcripts in \( \text{pat1}–114/\text{pat1}–114 \text{cum1}^+/ \) and \( \text{pat1}–114/\text{pat1}–114 \text{cum1}^+\Delta \) cells that were induced to undergo synchronous meiosis. Cells were incubated in the presence of TTM (150 \( \mu \text{M} \)) or \( \text{CuSO}_4 \) (50 \( \mu \text{M} \)). Total RNA was isolated from culture aliquots taken at the indicated time points. \( \text{cum1}^+ \), \( \text{ctr4}^- \), and \( \text{act1}^- \) steady-state mRNA levels were analyzed by RNase protection assays. \( \text{ctr4}^- \) mRNA steady-state levels were probed as a control transcript known to be induced in early meiosis under low copper conditions. B, graphic representations of quantification of the results of three independent RNase protection assays, including the experiment shown in panel A. Values indicate the normalized \( \text{cum1}^+ \) and \( \text{ctr4}^- \) transcript levels relative to \( \text{act1}^- \) mRNA levels. The histogram values represent the averages \( \pm \text{S.D.} \).
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**FIGURE 2. cum1* is expressed exclusively during meiosis.** Vegetatively logarthmic growing cells expressing or lacking cuf1* were incubated in the absence (−) or presence of TTM (250 μM) or CuSO₄ (Cu, 100 μM) for 90 min. Shown are representative RNase protection assays of cuf1*, cum1*, and act1* mRNA steady-state levels during mitosis. As a control for cum1* mRNA signal, azygotic meiotic cuf1*/+ cells were induced to undergo meiosis under low copper conditions. Five hours after meiotic induction, cum1* steady-state mRNA levels were analyzed by RNase protection assays (far right, meiosis).

was under control of the −376 cum* promoter may be due to the loss of negative cis-acting regulatory elements located between positions −1608 and −377 within the promoter. Similarly to −376 cum* lacZ fusion reporter, lacZ transcript levels from cum1Δ cells harboring −174 cum1* lacZ plasmid did not exhibit changes in response to variations in copper levels, except that the overall magnitude of lacZ expression was high. In fact, it was higher than the same fusion plasmid expressed in wild-type cells (Fig. 3, B and C). Results observed for −376 cum* lacZ and −174 cum1* lacZ fusion plasmids suggested that regulation of cum1* transcription is complicated and may involve additional trans-acting regulators.

To further characterize the ability of the proximal −376 cum* promoter to mediate copper starvation-dependent induction when it was dissociated from its own locus and fused to lacZ in the presence of Cuf1, a short DNA segment derived from the cum1* promoter (positions −100 to −351) was inserted upstream of the minimal promoter of the CYC1 gene fused to lacZ (Fig. 4A). This −351 cum1* CYC1-lacZ fusion containing three CuSEs (positions −337 to −330, −235 to −228, and −185 to −178) induced lacZ mRNA expression (−8.3-fold) under copper-limiting conditions compared with the transcript levels detected in the case of control (untreated) or copper-treated cells (Fig. 4, B and C). When these CuSEs were mutated in the fusion reporter, very weak expression of lacZ mRNA was observed in cuf1* cells irrespective of cellular copper status (Fig. 4, B and C). In the case of cuf1Δ mutant cells harboring the −351 cum1* CYC1-lacZ fusion plasmid containing wild-type CuSEs, results showed that transcript levels of lacZ mRNA were not observed under basal (untreated), copper-starved, or copper-replete conditions (Fig. 4, B and C). Taken together, these results strongly suggested that under low copper conditions CuSE promoter sequences found in the 5′ proximal untranslated region of cum1* are functional and could be recognized and used by the transcription factor Cuf1.

Cuf1 Interacts with the cum1* Promoter in Vivo under Low Copper Conditions—Despite the fact that there was no detection of cum1* transcripts in cells proliferating in mitosis under low copper conditions, we tested whether Cuf1 could possibly bind to cum1* promoter in vivo. cuf1Δ cells expressing an integrated untagged or Myc12-tagged cuf1* allele were precultured until they reached the logarithmic phase. Cells were harvested, washed, and resuspended in media containing TTM (250 μM) or CuSO₄ (100 μM) for 90 min. Results from ChIP analysis showed that in the case of cells treated with TTM, Cuf1-Myc12 occupied cum1* and cuf4* promoters at high levels with ~5.4- and ~5.8-fold enrichments, respectively, relative to a control 18S ribosomal DNA coding region that did not harbor CuSE (Fig. 4, A and B). Conversely, when cells were incubated in the presence of copper, very low levels of cum1* and cuf4* promoter fragments were immunoprecipitated. These low levels of immunoprecipitated chromatin were similar to the background signals observed when ChIP assays were performed in a cuf1Δ strain expressing an untagged cuf1* allele, which had been re-integrated (Fig. 4B).

To test whether insertion of the epitope Myc12 tag interfered with Cuf1 function, the untagged (cuf1*) and tagged (cuf1*+ Myc12) alleles were separately expressed in cuf1Δ cells. S. pombe cells harboring disrupted cuf1* gene (cuf1Δ) exhibited phenotypes linked with copper insufficiency, including the inability to grow on respiratory carbon sources due to a failure to provide copper to cytochrome c oxidase, resulting from a defect in copper acquisition. A cuf1Δ mutant strain in which an empty vector was transformed failed to grow in the presence of glycerol and ethanol as the sole sources of carbon (Fig. 5C). However, the growth defect was reversed when cuf1Δ mutant cells were transformed with wild-type or with Myc12 epitope-tagged cuf1* allele (Fig. 5C). These results indicated that expression of Cuf1 or Cuf1-Myc12 complemented the cuf1Δ growth defect in glycerol and ethanol as sources of respiratory carbon.

To further ensure that fusion of Myc12 to the C terminus of Cuf1 did not interfere with its function, we analyzed if copper starvation-dependent induction of cuf4* gene expression was restored in cuf1Δ cells carrying a wild-type or Myc12 epitope-tagged cuf1* allele. Results showed that cells expressing Cuf1-Myc12 fostered induction of cuf4* mRNA levels under low copper conditions in a manner similar to that of cells expressing an untagged Cuf1 (Fig. 5D). As expected, cuf4* expression was down-regulated in cells harboring the same alleles under untreated (basal) and copper-replete conditions. Analysis of Cuf1-Myc12 expressed in cells grown under low and high concentrations of copper for 90 min revealed that the fusion protein was stable and present in similar relative amounts irrespective of cellular copper status (Fig. 5E). Taken together, these results indicated that although there was no detection of cum1* transcripts in copper-starved mitotically growing cells, the cum1* promoter was occupied by Cuf1 under conditions of low copper levels.

**iss1** Produces Two Transcripts Including One That Is Partially Complementary with the cum1* Transcript—Based on genome-wide transcriptome and genome sequence data, iss1* and cum1* genes are arranged with their transcription orientated convergently (43, 44). Furthermore, a long 3′ untranslated region (UTR) of iss1* was predicted to overlap the cum1* open reading frame found in the opposite DNA strand (Fig. 6A). To validate the existence of the long iss1* transcript isoform, we used two distinct probes. The first probe (denoted AS1) hybridized in the coding sequence of iss1* (positions +292 to +525...
from the first base of the initiator codon), whereas the second probe (termed AS3) paired in the 3' UTR of iss1+ (positions +1529 to +1790) (Fig. 6A). Cells proliferating in mitosis were exposed to TTM (250 μM) or CuSO4 (100 μM) for 90 min. Total RNA was then isolated and analyzed for quantitation of steady-state mRNA levels of lacZ and act1+. RNA preparations probed with AS1 revealed two forms of iss1+ transcripts. RNA preparations probed with AS3 revealed a 2.0-kb transcript matched the predicted size of iss1+ transcript from genome-wide mapping studies (44). The 3' UTR of this antisense transcript overlapped with cum1+ sense mRNA. In addition, a shorter ~1.4-kb transcript was detected, revealing the presence of an alternative polyadenylation site that led to the production of a second transcript (Fig. 6A). Based on apparent absence of the cum1+ sense transcript, we predicted that long ~1.4-kb could form antisense transcripts with cum1+ sense mRNAs, thereby preventing their detection by an uncharacterized inhibitory mechanism. Potential mechanisms may include (i) that the antisense transcript could result in vivo degradation of the cum1+ transcript, (ii) that the antisense transcript could result in early transcriptional termination of the cum1+ transcript, or (iii) RNAi-mediated heterochromatin formation could decreased cum1+ transcription.

**cum1+ Transcripts Are Detected in Copper-starved Mitotic Cells When the Long iss1+ Antisense Transcript Is Absent**—To obtain further evidence that the long transcript isoform produced by iss1+ overlapped with cum1+ sense transcript, we generated mutant cells containing a PAS integrated immediately at the 3' end of the chromosomal locus of iss1+ (Fig. 7A). Results showed that this approach led to exclusive production of a short ~1.4-kb (Fig. 7B). When these
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A Role for the RNAi Machinery in Silencing cum1+ Transcription in the G1 Phase of the Cell Cycle—Transcription of several convergent genes in S. pombe produces overlapping transcripts, resulting in long double-stranded RNA molecules. Long double-stranded RNA molecules could be recognized and cut by Dicer (Dcr1), producing small interfering RNAs (siRNA) that activate the RNAi pathway, which could lead to gene silencing (22). Studies have shown that transcriptional interference between sense and antisense transcripts of convergent genes occurs mainly in the G1 phase of the S. pombe cell cycle (20, 22). To assess involvement of the RNAi machinery in silencing cum1+ transcription, we probed cum1+ mRNA levels in four mutants that affect the RNAi pathway. Cells were blocked in G1 using nitrogen starvation, and then they underwent synchronized mitosis in the presence of TTM or copper or were left untreated. These mutants were dcr1Δ (dicer), ago1Δ (argonaute), rdp1Δ (RNA-dependent RNA polymerase), and clr4Δ (histone H3 lysine methyltransferase). DNA content was determined by flow cytometry (FACS) analysis to assess that cell populations were largely restricted to G1 (1N) at the zero-point time and after 30 min of mitotic induction, whereas cells were primarily in G2 phase at 6 h (Fig. 8). Experiments using wild-type cells, either in the G1 or G2 phase, failed to detect cum1+ mRNA as a function of copper availability (Fig. 9). When dcr1Δ, ago1Δ, rdp1Δ, and clr4Δ mutant strains were blocked in G1 and then synchronously cultured in nitrogen-replenished medium under low copper conditions, sense cum1+ mRNA levels were detected after 30 min of mitotic entry, with an induction of ~2.9 (dcr1Δ), ~6.3 (ago1Δ), and ~5.1-fold (rdp1Δ) compared with that observed in the case of the wild-type strain (background threshold) (Fig. 9). In the case of clr4Δ mutant, cum1+ expression was observed ~2.4-fold after 15 min of mitotic induction. Sense cum1+ mRNA was also observed in the case of the above-mentioned mutants under basal and copper-replete conditions. However, its derepression was less in comparison with transcript levels observed in copper-starved mutant cells (Fig. 9). In the case of G2 cells at the 6-h time point, there was no significant increase of sense cum1+

cells (+PAS) in which Cuf1 (cuf1+) was expressed were assayed, results showed that cum1+ mRNAs were highly induced under conditions of copper starvation (~8-fold) (Fig. 7B). In contrast, cum1+ transcripts were repressed in cuf1+ cells grown under copper-replete conditions. To test whether Cuf1 was required for induction of cum1+ expression in copper-starved cells harboring PAS, we deleted the cuf1+ gene (cuf1Δ). Inactivation of cuf1+ resulted in strongly reduced (~7.5-fold) cum1+ mRNA levels in comparison with those seen with cuf1+ (iss1+PAS) cells grown under the same copper-limited conditions (Fig. 7B). When cuf1Δ cells containing PAS were incubated in the presence of copper, they exhibited very weak levels of cum1+ transcript that were similar to those observed with the same cells (cuf1Δ iss1+PAS) grown under copper starvation. Wild-type (cuf1+) and cuf1Δ mutant strains in which the long iss1+ transcript isoform was produced (no PAS) failed to generate significant amounts of cum1+ mRNAs when incubated in the presence of TTM and CuSO4 (Fig. 7B).

To validate the effect of the presence of PAS integrated at the 3’ end region of iss1+, we probed total RNA isolated from copper-deficient and copper-replete cells with AS1 probe. Cells bearing the iss1+-PAS allele produced a unique mRNA species that corresponded to a short transcript isoform (~1.4 kb) irrespective of cellular copper status (Fig. 7B). In the case of cells in which PAS was not integrated at the 3’ end region of iss1+, two forms of iss1+ transcripts were detected (~2.0 and ~1.4 kb) (Fig. 7B). When the same RNA preparations were hybridized with AS3 probe, we only detected the long transcript isoform (~2.0 kb) in cells lacking the PAS signal. As expected, cells expressing iss1+-PAS allele failed to produce the long transcript isoform (Fig. 7B). Taken together, these results revealed that production of the long iss1+ antisense transcript precludes detection of cum1+ sense mRNA in vegetative cells.

FIGURE 4. CuSE elements of cum1+ promoter are required for induction of gene expression in a Cuf1-dependent manner under copper-limiting conditions. A, scheme of a 251-bp cum1+ promoter DNA fragment and its mutant derivative that were inserted into the minimal promoter of the CYC1 gene fused to lacZ. Gray boxes indicate wild-type CuSE elements (5’-T/AIDDHGCTG-3’, D = A, G, or T; H = A, C, or T), whereas filled boxes depict mutated versions (5’-C/GDDHCTAT-3’). Nucleotide numbers refer to the positions relative to the translational initiator codon of cum1+. B, wild-type (cuf1+) and cuf1Δ cells were transformed with the indicated cum1+-CYC1-lacZ heterologous reporter plasmid. Total RNA was isolated from control (−), TTM (250 μM), or CuSO4 (Cu, 100 μM)-treated cultures. Shown is a representative RNase protection assay of lacZ and act1+ mRNA steady-state levels. C, the histogram shows quantification of lacZ mRNA levels relative to act1+ transcript levels after treatments shown in panel B. Values are the averages of triplicate determinations ± S.D.
transcript in dcr1Δ, rdp1Δ, and clr4Δ mutants under all three experimental conditions (TTM, CuSO4, and untreated) (Fig. 9). With respect to ago1Δ mutant, only a very weak level of sense cum1+ expression was observed after 6 h of mitotic induction. Using unsynchronized cells, cum1+ mRNA was also detected in ago1Δ and dcr1Δ mutant strains (Fig. 10). However, derepression of cum1+ observed in these cells could not be associated with a specific phase of the cell cycle. Taken together, these results revealed that RNAi components such as Dcr1, Ago1, Rdpl, and Clr4 are involved in the antisense repression of cum1+ transcription in mitotic cells, especially cells undergoing G1 cell-cycle phase. Due to the fact that cum1+ mRNA levels were induced in copper-starved RNAi mutant strains that were synchronously cultured in G1 phase, we sought to ascertain if this induction was Cuf1-dependent. To obtain evidence of the requirement of

FIGURE 5. Cuf1 binds to the cum1+ promoter in vegetative cells under copper-limiting conditions. A, schematic representation of ctr4+ and cum1+ promoter regions. Gray boxes represent CuSEs that are numbered relative to the initiator codon of each gene (ctr4+ or cum1+). Arrows indicate locations of the primers for qPCR analysis. B, ChIP analysis of ctr4+ and cum1+ promoters in a cuf1Δ mutant strain expressing an integrated untagged or Myc12-tagged cuf1+ allele. Cells were treated with TTM (250 μM) or CuSO4 (100 μM) for 90 min. Chromatin was immunoprecipitated using anti-c-myc antibodies. Specific regions of ctr4+ and cum1+ promoters were analyzed by qPCR to determine Cuf1 occupancy. Binding of Cuf1-Myc12 to ctr4+ and cum1+ promoters was calculated as the enrichment of specific ctr4+ and cum1+ promoter regions relative to a 18S ribosomal DNA coding region that lacked CuSE. ChIP data were calculated as values of the largest amount of chromatin measured (-fold enrichment). Results are shown as the averages ± S.D. of a minimum of three independent experiments. C, cells carrying a disrupted cuf1Δ allele were transformed with an empty vector (vector alone), cuf1+, and cuf1+Myc12. Cultures were spotted onto YES media containing glucose or glycerol/ethanol (Gly/EtOH). WT, isogenic parental strain FY435 (cuf1+). D, aliquots of the cultures described for panel C were grown to logarithmic phase in media containing glucose and then were left untreated (−) or incubated with TTM (250 μM) or CuSO4 (Cu, 100 μM) for 90 min. Total RNA was isolated, and steady-state ctr4+ and act1+ mRNA levels were analyzed by RNease protection assays. E, whole cell extracts were prepared from aliquots of cultures used in panel D and analyzed by immunoblotting using an anti-Myc antibody (α-Myc) and anti-tubulin antibody (α-tubulin). M, reference marker.
Cuf1, the cuf1+ gene was deleted in a dcr1Δ mutant strain, creating a dcr1 Δ cuf1Δ double mutant strain. In another series of experiments, a functional cuf1+ allele was reintegrated into dcr1Δ cuf1Δ cells. Wild-type (cuf1+ dcr1+), cuf1+ dcr1Δ, cuf1Δ dcr1Δ, and cuf1Δ dcr1Δ + cuf1+ strains were cultured in a way that they underwent synchronous mitosis. As expected, cum1+ gene expression in response to copper starvation (Fig. 12). When RNA samples were prepared from cultures treated with TTM (250 μM) or CuSO4 (Cu, 100 μM) and then analyzed by Northern blot assays. AS1, AS3, S1, and act1+ probes are indicated on the left, whereas approximate transcript sizes (in kilobases) are shown on the right.

**FIGURE 6. Expression of iss1+ produces two different sizes of transcripts.** A, diagram of the arrangement of iss1+ and cum1+ genes. Putative polyadenylation sites are indicated with arrowheads. White arrows indicate the direction of transcription. Filled rectangles show the positions of radiolabeled strand-specific probes (AS1, AS3, and S1) that were used to detect iss1+ and cum1+ mRNA steady-state levels. Numbers refer to the predicted sizes of transcripts. B, total RNA was isolated from cultures treated with TTM (250 μM) or CuSO4 (Cu, 100 μM) and then analyzed by Northern blot assays. AS1, AS3, S1, and act1+ probes are indicated on the left, whereas approximate transcript sizes (in kilobases) are shown on the right.

Cum1+ and the Long iss1+ RNA Isoform Are Expressed in the Opposite Direction Relative to Each Other during Meiosis—Because cum1+ is induced in copper-starved meiotic cells, we investigated its profile of expression compared with profiles of the two iss1+ RNA isoforms during meiosis. pat1–114/pat1–114 diploid cells were synchronized through meiosis in the absence or the presence of either TTM or CuSO4. Aliquots of cultures were picked up at representative time points after meiotic induction, and steady-state levels of cum1+ and iss1+ mRNAs were analyzed by Northern blot assays. Cum1+ expression was primarily detected in cells treated with TTM, peaking 9 h after meiotic induction (S1 probe, Fig. 12). There was a lack of induction of cum1+ mRNA under copper-replete conditions. When copper levels were low, the long iss1+ RNA isoform (~2.0-kb) was detected after 5 and 7 h of meiotic induction, and then its expression was strongly reduced within 9 h (AS1 and AS3 probes; Fig. 12). These results led us to observe that the abundance of the long iss1+ antisense transcript was anti-correlated with the abundance of cum1+ sense mRNA. Consistent with this observation, under copper-replete conditions, results showed that steady-state levels of the long iss1+ isoform remained high through the meiotic program (even after 9 h) (Fig. 12). In the case of the short iss1+ isoform (~1.4 kb), results showed that its expression changed in the opposite direction relative to the long iss1+ isoform under copper-starved and copper-replete conditions (Fig. 12).
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FIGURE 7. Elimination of the long iss1+ transcript leads to derepression of cum1+ mRNA in a TTM- and Cuf1-dependent manner. A, schematic arrangement of iss1+, iss1+-PAS, and cum1+ alleles. Putative polyadenylation or transcription termination sites are indicated with arrowheads. White arrows indicate the direction of transcription. Filled rectangles depict the positions of radiolabeled strand-specific probes (AS1, AS3, and S1) that were used to detect iss1+ and cum1+ transcripts. Numbers refer to the predicted sizes of transcripts. The PAS sequence derived from the ura4+ 3′ UTR was integrated at the chromosomal locus of iss1+ to trigger its immediate transcription termination at a proximal site downstream of the gene. B, logarithmic-phase cultures of wild-type (cuf1Δ) and cuf1Δ strains expressing the indicated iss1+ or iss1+-PAS allele were exposed to TTM (250 μM) or CuSO4 (Cu, 100 μM) for 90 min. After RNA isolation, iss1+ and cum1+ steady-state mRNA levels were analyzed by Northern blot assays. AS1, AS3, S1, and act1+ probes as well as approximate transcript sizes (in kilobases) are indicated on the left.

together, these results strengthened that production of the long iss1+ isoform negatively regulates cum1+ transcript abundance in vivo.

Discussion

cum1+ is a copper-regulated meiotic gene that is under the control of copper-dependent transcription factor Cuf1. Although cum1+ expression was detected exclusively in meiotic and sporulating cells, its promoter region was occupied by Cuf1 in mitotically growing cells under low copper conditions. The puzzling absence of cum1+ transcript in copper-starved growing vegetative cells prompted us to look for a mechanism responsible for its selective elimination. Based on genomic sequence analysis, it has been established that cum1+ and iss1+ genes are arranged in convergent orientation and are transcribed from opposing DNA strands. Furthermore, genome-wide transcriptome data suggested that iss1+ produces a long antisense transcript (~2.0 kb), which can hybridize with cum1+ sense mRNA. In S. pombe, iss1+ is an essential gene that is predicted to encode a critical subunit of the pre-mRNA polyadenylation factor complex (45). Our findings have uncovered not only the presence of a long transcript (~2.0 kb) but also the existence of a short transcript (~1.4 kb) that comes from the iss1+ gene. Results showed that the relative abundance of iss1+ mRNA isoforms is not significantly affected in response to changes in copper levels. This observation was different than that of the adh1+ antisense transcript (adh1AS) in S. pombe (11, 46). The adh1AS is generated at the adh1+ locus in zinc-limited cells. It serves to suppress sense RNA transcription of adh1+, which encodes an abundant zinc-binding protein that is required for the conversion of acetaldehyde to ethanol. As a result, adh1AS down-regulates adh1+ expression presumably to avoid a futile expenditure of energy in producing Adh1 that lacks its necessary cofactor (zinc) to function. Thus, the adh1AS transcript (induced under low zinc) shows a reciprocal expression pattern to that of the adh1+ sense mRNA (which is abundant under high zinc conditions). Although increased expression of the adh1AS transcript leads to reduced levels of the adh1+ sense transcript, the mechanism to eliminate this paired antisense/sense transcripts remains unclear. Another distinction between the two gene pairs, cum1+/iss1+ and adh1+/adhAS, is the nature of the biological process that is involved. In the case of adh1+/adhAS, the transcriptional interference mechanism is important for maintaining zinc homeostasis during vegetative growth (mitosis). In the case of cum1+/iss1+, action of the long iss1+ antisense transcript is important for maintaining tight vegetative (mitotic) repression of the meiosis-specific cum1+ gene.

In S. pombe, other examples of sense/antisense genes have been reported in which a continuous extension of a 3′ UTR of an annotated gene on the opposite strand covered the entire coding region of a meiosis-specific gene, thereby preventing sense transcription of the meiotic gene during vegetative growth (19). Examples include spo6+/SPBC1778.05c and mug28+/mrp17+. In a manner similar to cum1+/iss1+, insertion of a transcription terminator into SPBC1778.05c (the source of antisense RNA for spo6+) led to a corresponding increase in spo6+ sense mRNA levels in vegetative cells (19). However, the levels of spo6+ expression (in mitosis) remained low compared with levels of the spo6+ sense transcripts in meiotic cells. In the case of spo6+, this finding suggests that its maximum level of expression in meiosis requires additional an meiosis-specific transcription factor(s), and that the SPBC1778.05c antisense transcript in vegetative cells may only serve to prevent low level spo6+ expression.

To eliminate cum1+ sense transcripts in vegetative cells, the mechanism of antisense-mediated transcriptional repression should be efficient, especially under low copper conditions. In this situation, cum1+ mRNA levels are induced by Cuf1, which is active during both mitotic and meiotic cell division programs (28, 42). One possibility is that the long iss1+ antisense tran-
script is synthesized in sufficiently high concentrations to hybridize with all *cum1* sense mRNAs under all conditions, especially when copper is scarce. Other possibilities include the participation of additional mechanisms. One of them could involve RNA interference because pairing between *cum1* sense and *iss1* antisense transcripts could be processed into double-stranded small RNAs, which would induce the RNAi pathway. *S. pombe* possesses a canonical RNAi machinery that has been reported to be involved in processing sense/antisense RNA duplexes produced from convergent gene pairs. Therefore, we examined the effect of inactivating components of the RNAi pathway on the ability of *iss1* antisense RNA to inhibit *cum1* sense mRNA in the G1 phase of the cell cycle. Synchronization in the G1 phase revealed an increase of *cum1* sense transcripts in four mutants (dcr1Δ, ago1Δ, rpd1Δ, and clr4Δ) that affect the RNAi pathway as compared with a wild-type strain. These results were reminiscent of those observed with *mei4/act1* and *nmt2/avn2* convergent gene pairs for which dcr1 and ago1 mutations promote read-through transcription into the intergenic regions of convergent genes (22). In contrast, in the case of some convergent gene pairs (*spo6*/SPBC1778.05c and *mug28*/mrp17†), components of the RNAi machinery do not seem to be required for antisense transcript regulation (19). One caveat, however, is the fact that cells were not synchronized in the cases of *spo6*/SPBC1778.05c and *mug28*/mrp17† and that may have prevented detection of a functional contribution from RNAi in their regulation. Genomic RNA sequencing of small interfering RNAs has failed to detect significant small RNAs derived from meiotic genes that have antisense transcripts (47). It is possible that the short and transient G1 phase of the *S. pombe* cell cycle hinders detection of small RNAs derived from convergent genes. Furthermore, these
Experiments were not carried out under low copper conditions, which represent an optimal context to produce *cum1* sense mRNA that would hybridize with long *iss1* antisense transcript. In *Drosophila* it has been found that a number of small interfering RNAs were derived from some convergent genes (48). One condition to observe RNA interference is that sense mRNA levels relative to *act1* steady-state mRNA levels were analyzed by RNase protections assays. RNase protection analyses were quantified based on three (*n* = 3) independent experiments ± S.D. (including experiments shown in panels A–E). Values indicate the normalized *cum1* levels relative to *act1*. The highest value was assigned to 100%.
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RNA has to be transcribed to a detectable level so that the antisense RNA partner can pair with it (sense transcript), thereby generating enough double-stranded RNA substrates for their recognition by the RNAi machinery. In the cases of spo6\(^+\) and mug28\(^-\), their levels of sense transcription in vegetative cells were very low, suggesting that the levels were not high enough to allow robust sense/antisense duplex formation. In contrast, cum1\(^+\) sense transcription was highly induced in vegetative copper-starved cells. Under this latter condition, accumulation of cum1\(^+\) mRNA generated sufficient amounts of sense transcripts for their hybridization with iss1\(^+\) antisense transcripts, thereby allowing formation of RNA duplexes and recognition of these duplexes by the RNAi machinery. These possibilities lead to the proposal of a novel function for Cuf1, which is to ensure high levels of cum1\(^+\) transcription in vegetative cells under low copper conditions, therefore, fostering cum1\(^+\) mRNA elimination in the G1 phase of the cell cycle.

In the present study, production of long iss1\(^+\) antisense and cum1\(^+\) sense transcripts could result in double-stranded RNA production. This in turn could activate the RNAi pathway and then induce transient heterochromatin formation. Based on previous studies on regulation of S. pombe convergent genes in G\(_1\) cells, transient heterochromatin formation involves histone H3 lysine 9 di- tri-methylation and recruitment of proteins, including Swi6 and Cir4 (22). This mechanism could be the same for silencing cum1\(^+\) in cells undergoing G\(_1\) phase. However, it has been proposed that after S phase, cohesion is recruited by convergent gene heterochromatin to block further synthesis of long antisense transcript, thereby fostering gene-proximal transcription termination, which causes heterochromatin loss in the G\(_2\) phase. However, in the case of cum1\(^+\), its transcript was consistently undetectable throughout the cell cycle, suggesting the existence of additional mechanisms that inhibit its transcript. The forkhead transcription factor Fkh2 has been reported to be involved in vegetative repression of some meiotic genes (19). Therefore, we have examined if deletion of fkh2\(^+\) (either fkh2\(^-\) single or fkh2\(^-\) mei4\(^+\) double mutant) could foster derepression of cum1\(^+\) sense transcript during mitosis. Under our experimental conditions, we could not detect induction of cum1\(^+\) sense mRNA in any of the two mutants.

Interestingly, we observed that when cells undergo synchronous meiosis under low copper conditions, the two iss1\(^+\) antisense RNA isoforms were present as observed in vegetative cells (mitosis). After 5 h of meiotic induction, the long iss1\(^+\) isoform

3 V. Normant, J. Beaudoin, and S. Labbé, unpublished results.
As a consequence, cum1+ sense transcript exhibited only moderate levels of transcription. At the end of meiosis, however, expression of the long iss1+ isoform was markedly decreased, which led to an increase expression of cum1+ sense transcript that was not affected by the presence of the short iss1+ isoform. These findings led us to conclude that a decrease in expression of the long iss1+ isoform leads to up-regulated expression of cum1+ sense transcript, the two transcripts changing in the opposite direction relative to each other. This observation was in agreement with the fact that interference with the long iss1+ antisense transcript (by insertion of PAS) in mitosis resulted in detectable levels of vegetative cum1+ sense mRNA, especially under low copper conditions. Further studies of how antisense transcript levels are regulated and formed antisense/sense duplexes are likely to provide new insight into the mechanisms of repression between pairs of convergent genes, especially those encoding one meiosis-specific component and one ubiquitously component.

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