

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

Received for publication, June 25, 2015, and in revised form, July 23, 2015 Published, JBC Papers in Press, July 30, 2015, DOI 10.1074/jbc.M115.674556

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**Background:** Convergent gene pairs produce sense and antisense transcripts that could interfere with one another.

**Results:** RNAi, sense, and antisense transcripts play a causal role in *cum1*<sup>+</sup> 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*<sup>+</sup> gene produces two transcript isoforms, including a long antisense mRNA that is complementary to the meiotic *cum1*<sup>+</sup> sense transcript, inhibiting *cum1*<sup>+</sup> expression in vegetative cells. Inhibition of *cum1*<sup>+</sup> transcription was not at the level of its initiation because fusion of the *cum1*<sup>+</sup> 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 Cuf1 and conserved copper-signaling elements (CuSEs) are required for induction of *cum1*<sup>+</sup>-*lacZ* transcription under copper deficiency. Insertion of a multipartite polyadenylation signal immediately downstream of *iss1*<sup>+</sup> led to the exclusive production of a shorter *iss1*<sup>+</sup> mRNA isoform, thereby allowing accumulation of *cum1*<sup>+</sup> sense mRNA in copper-limited vegetative cells. This finding suggested that the long *iss1*<sup>+</sup> antisense mRNA could pair with *cum1*<sup>+</sup> sense mRNA, thereby producing double-stranded RNA molecules that could induce RNAi. We consistently found that mutant strains for RNAi (*dcrl1Δ*, *ago1Δ*, *rdp1Δ*, and *clr4Δ*) are defective in selectively eliminating *cum1*<sup>+</sup> sense transcript in the G<sub>1</sub> 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 G<sub>1</sub> 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

\* This work was supported by Canadian Institutes of Health Research Grant MOP-114986 (to S. L.). The authors declare that they have no conflicts of interest with the contents of this article.

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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 G<sub>1</sub> phase of the cell cycle, especially in regions where convergent genes are present (22). In G<sub>1</sub>, 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 G<sub>2</sub> 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/A)DDHGCTG-3' (D = A, G, or T; H = A, C, or T), which are denoted CuSEs<sup>2</sup> (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*<sup>+</sup>, whose transcript is induced in copper-starved meiotic cells (28). *cum1*<sup>+</sup> encodes a small polypeptide of 140 amino acid residues with a predicted molec-

ular 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*<sup>+</sup> is expressed exclusively during meiosis. However, results showed that when a reporter gene is expressed under the control of the wild-type *cum1*<sup>+</sup> promoter, its copper-dependent expression is detected in mitotically growing cells. These findings supported the notion that an elimination of *cum1*<sup>+</sup> mRNA occurs after its transcriptional initiation in mitotic cells. Northern blot analyses revealed that the *iss1*<sup>+</sup> gene in proximity to *cum1*<sup>+</sup> produces two antisense transcripts, including a longer one that is complementary to the sense *cum1*<sup>+</sup> mRNA. We showed that the insertion of a multipartite polyadenylation signal (PAS) immediately downstream of *iss1*<sup>+</sup> allows *cum1*<sup>+</sup> sense mRNA expression during vegetative growth, markedly under low copper conditions. Using G<sub>1</sub>-synchronized wild-type and *dcr1Δ*, *ago1Δ*, *rpm1Δ*, and *clr4Δ* mutant cells, we determined that components of the RNAi pathway are involved in antisense-mediated repression of *cum1*<sup>+</sup> transcription, especially during the G<sub>1</sub> 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 A<sub>600</sub> of 0.5, grown to exponential phase (A<sub>600</sub> of ~1.0), and left untreated or were treated with ammonium tetrathiomolybdate (TTM) (250 μM) or CuSO<sub>4</sub> (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 CuSO<sub>4</sub> (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*<sup>+</sup> 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-

<sup>2</sup> The abbreviations used are: CuSE, copper-signaling element; PAS, multipartite polyadenylation signal; TTM, ammonium tetrathiomolybdate; YES, yeast extract plus supplements; qPCR, quantitative PCR.

TABLE 1

*S. pombe* strains genotypes

Strain	Genotype	Source
FY435	<i>h</i> <sup>+</sup> <i>his7-366 leu1-32 ura4-Δ18 ade6-M210</i>	(49)
JSY484	<i>h</i> <sup>+</sup> <i>pat1-114 ade6-M210</i>	(50)
JSY3	<i>h</i> <sup>+</sup> <i>pat1-114 ade6-M210 cuf1Δ::KAN</i> <sup>r</sup>	(28)
<i>cuf1Δ cuf1</i> <sup>+</sup>	<i>h</i> <sup>+</sup> <i>his7-366 leu1-32 ura4-Δ18 ade6-M210 cuf1Δ::loxP cuf1</i> <sup>+</sup> <i>ura4</i> <sup>+</sup>	This study
<i>cuf1Δ</i>	<i>h</i> <sup>+</sup> <i>his7-366 leu1-32 ura4-Δ18 ade6-M210 cuf1Δ::loxP</i>	This study
<i>cuf1</i> <sup>+</sup> - <i>Myc</i> <sub>12</sub>	<i>cuf1</i> <sup>+</sup> - <i>Myc</i> <sub>12</sub> :: <i>ura4</i> <sup>+</sup>	
JSY17	<i>h</i> <sup>+</sup> <i>his7-366 leu1-32 ura4-Δ18 ade6-M210 cuf1Δ::ura4</i> <sup>+</sup>	(27)
AMY52	<i>h</i> <sup>+</sup> <i>his7-366 leu1-32 ura4-Δ18 ade6-M210 dcr1Δ::KAN</i> <sup>r</sup>	This study
VNY3	<i>h</i> <sup>+</sup> <i>his7-366 leu1-32 ura4-Δ18 ade6-M210 dcr1Δ::loxP cuf1Δ::KAN</i> <sup>r</sup>	This study
VNY4	<i>h</i> <sup>+</sup> <i>his7-366 leu1-32 ura4-Δ18 ade6-M210 ago1Δ::KAN</i> <sup>r</sup>	This study
<i>dcr1Δ cuf1Δ</i>	<i>h</i> <sup>+</sup> <i>his7-366 leu1-32 ura4-Δ18 ade6-M210 dcr1Δ::loxP cuf1Δ::KAN</i> <sup>r</sup> <i>cuf1</i> <sup>+</sup> <i>ura4</i> <sup>+</sup>	This study
VNY6	<i>h</i> <sup>+</sup> <i>his7-366 leu1-32 ura4-Δ18 ade6-M210 iss1</i> <sup>+</sup> - <i>ura4PAS::KAN</i> <sup>r</sup>	This study
VNY7	<i>h</i> <sup>+</sup> <i>his7-366 leu1-32 ura4-Δ18 ade6-M210 cuf1Δ::loxP iss1</i> <sup>+</sup> - <i>ura4PAS::KAN</i> <sup>r</sup>	This study
VNY8	<i>h</i> <sup>+</sup> <i>his7-366 leu1-32 ura4-Δ18 ade6-M210 rpd1Δ::KAN</i> <sup>r</sup>	This study
VNY9	<i>h</i> <sup>+</sup> <i>his7-366 leu1-32 ura4-Δ18 ade6-M210 clr4Δ::KAN</i> <sup>r</sup>	This study

neered 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*<sup>+</sup> promoter region was isolated from Yep357R*cum1*<sup>+</sup>-1608*lacZ*, Yep357R*cum1*<sup>+</sup>-376*lacZ*, and Yep357R*cum1*<sup>+</sup>-174*lacZ* after digestion with ApaI and Bsu36I (a unique restriction site into *lacZ*). Each of these promoter regions was then swapped for the equivalent DNA restriction fragment in pBPade6*mfc1*<sup>+</sup>-109*lacZ* (30), creating pBPade6*cum1*<sup>+</sup>-1608*lacZ*, pBPade6*cum1*<sup>+</sup>-376*lacZ*, and pBPade6*cum1*<sup>+</sup>-174*lacZ*. Using the *ctr4*<sup>+</sup> promoter (up to -737 from the initiator codon) and the first 10 codons of the *ctr4*<sup>+</sup> gene, an identical molecular cloning strategy (as described above) was applied to create Yep357R*ctr4*<sup>+</sup>-737*lacZ* and pBPade6*ctr4*<sup>+</sup>-737*lacZ*, respectively.

A *cum1*<sup>+</sup> promoter region encompassing positions -100 to -351 (with respect to A of the ATG codon of *cum1*<sup>+</sup>) was amplified by PCR. Primers were designed to generate XmaI and ApaI sites at the 5' end of the PCR fragment, whereas a XhoI site was introduced at the 3' end of that fragment. After purification, the DNA fragment was digested with XmaI and XhoI and cloned into the corresponding sites of *CYC1-lacZ* fusion plasmid pCF83 (25), creating plasmid pCF83*cum1*<sup>+</sup>-351/-100*CYC1-lacZ*. Subsequently, pCF83*cum1*<sup>+</sup>-351/-100*CYC1-lacZ* was digested with ApaI and Bsu36I, allowing purification of the *cum1*<sup>+</sup>-351/-100*CYC1-lacZ* DNA fragment that was swapped for a DNA restriction fragment in pBPade6*cum1*<sup>+</sup>-174*lacZ*, producing plasmid pBPade6*cum1*<sup>+</sup>-351/-100*CYC1-lacZ*. In the case of pBPade6*cum1*<sup>+</sup>-351/-100*CYC1-lacZ* containing mutated CuSE elements, it was created by a PCR overlap extension method as described previously (33).

We generated strains in which a PAS derived from *ura4*<sup>+</sup> (34) was integrated at the 3' end of *iss1*<sup>+</sup>. The *ura4*<sup>+</sup> PAS located downstream of the stop codon of *ura4*<sup>+</sup> was isolated by PCR and then inserted into pKSloxp-KAN-loxP at the BamHI and EcoRV sites. This plasmid was designated pKSura4PASloxp-KAN-loxP. A 513-bp SacII-BamHI fragment from the *iss1*<sup>+</sup> gene (starting at +614 after the start codon up to +1127) was amplified and cloned into the same sites of pKSura4PASloxp-KAN-loxP. The resulting plasmid was named pKSiss1<sup>614-1127</sup>-ura4PASloxp-KAN-loxP. A 846-bp XhoI-Asp718 fragment from the *iss1*<sup>+</sup> 3' untranslated region

was amplified and cloned into the corresponding sites of pKSiss1<sup>614-1127</sup>-ura4PASloxp-KAN-loxP, creating plasmid pKSiss1<sup>614-1127</sup>-ura4PASloxp-KAN-loxP-iss1<sup>1128-1974</sup>. The plasmid was subsequently digested with XmaI and ClaI (for which the restriction sites are unique and found within DNA regions 614-1127 and 1128-1974, respectively) to produce a DNA fragment that allowed homologous integration of *ura4*<sup>+</sup> PAS at the chromosomal locus of *iss1*<sup>+</sup>, thereby replacing its wild-type terminator by the *ura4*<sup>+</sup> terminator.

**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 pKSlacZ, pSK*ctr4*<sup>+</sup>, and pSK*act1*<sup>+</sup> were used to produce antisense RNA probes that served to determine *lacZ*, *ctr4*<sup>+</sup>, and *act1*<sup>+</sup> mRNA levels, respectively (25, 37). pSK*cum1*<sup>+</sup> was constructed by inserting a 199-bp BamHI-EcoRI fragment of the *cum1*<sup>+</sup> 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*<sup>+</sup>. <sup>32</sup>P-Labeled antisense RNA probes were generated using either HindIII (pKSlacZ)- or BamHI-linearized plasmids (pSK*ctr4*<sup>+</sup>, pSK*cum1*<sup>+</sup>, and pSK*act1*<sup>+</sup>), [ $\alpha$ -<sup>32</sup>P]UTP, and T7 RNA polymerase as described previously (36). In the case of Northern blot analysis, total RNA (25  $\mu$ g per sample) was resolved on 1.25% agarose-formaldehyde gels and transferred to, and immobilized on nylon membranes. Prehybridation and hybridization were carried out in Church's buffer (1% bovine serum albumin, 1 mM EDTA, 0.5 M NaHPO<sub>4</sub>, pH 7.2, 7% SDS). In the case of prehybridation experiments, freshly denatured herring sperm DNA (50  $\mu$ g/ml) was added to Church's buffer in which membranes were soaked. After the hybridization step with <sup>32</sup>P-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*<sup>+</sup>. 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*<sup>+</sup>. The *act1*<sup>+</sup> probe (internal control) hybridized to the coding region



between positions +472 and +702 downstream from A of the ATG codon of *act1*<sup>+</sup>.

**ChIP Experiments**—*cuf1*Δ cells expressing untagged or Myc<sub>12</sub>-tagged *cuf1*<sup>+</sup> 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 CuSO<sub>4</sub> (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 Myc<sub>12</sub>-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 *cum1*<sup>+</sup> promoter region, which includes three elements that match the CuSE consensus motif (25). As positive controls, primers that encompassed a *ctr4*<sup>+</sup> promoter region that contains functional CuSEs (25) were also used for qPCR analysis. Myc<sub>12</sub>-tagged Cuf1 density at the *cum1*<sup>+</sup> or *ctr4*<sup>+</sup> promoter was calculated as the enrichment of the specific genomic *cum1*<sup>+</sup> or *ctr4*<sup>+</sup> promoter region relative to a 18S 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' ends relative to that of the translational initiation codon: *cum1*–344 (5'-GCATCGATAGAGCTGCTAGTTTC-3'), *cum1*–165 (5'-TTTCCATCCCATTTCAGCATTTTC-3'), *ctr4*–737 (5'-GAAATGTCACCTGTTTGTGATGCTG-3'), and *ctr4*–584 (5'-CAGCTCTTTTACACGGCAATAATAC-3'). Two primers derived from a 18S ribosomal DNA coding region were used as internal background controls: 18S-1 (5'-GCCCTATCAACTTTCGATGGTAG-3') and 18S-2 (5'-GATGTGGTAGCCGTTTCTCAG-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 (*A*<sub>600</sub> of ~1.0), cells were harvested, washed twice, and transferred to Edinburgh nitrogen-deficient medium to arrest cells at the G<sub>1</sub> 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), CuSO<sub>4</sub> (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).

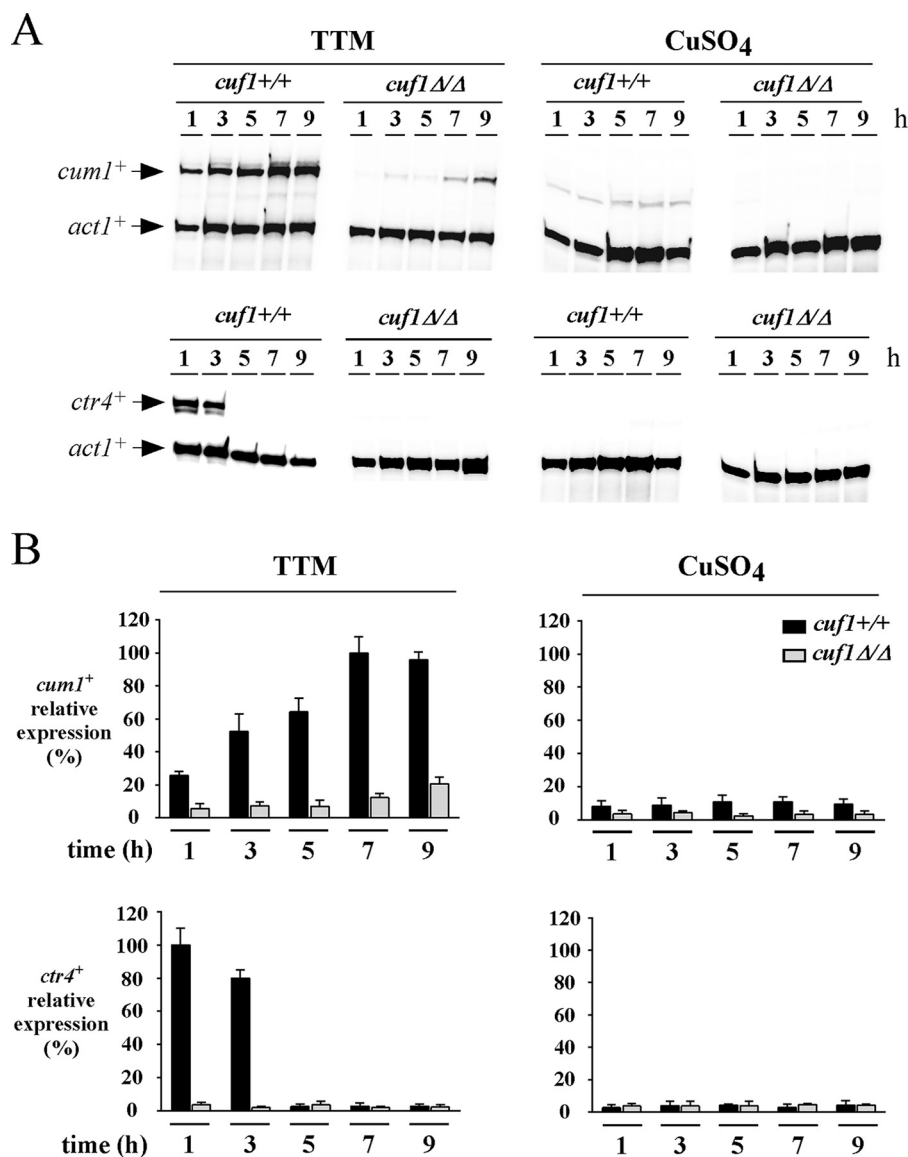
## Results

***cum1*<sup>+</sup> Is Regulated in a Copper- and Cuf1-dependent Manner in Meiotic Cells**—Genome profiling studies have revealed that *cum1*<sup>+</sup> exhibited higher levels of expression in copper-starved meiotic cells (28). To further characterize the expres-

sion profile of *cum1*<sup>+</sup> during meiosis in relationship with copper and Cuf1 availability, *pat1*–114/*pat1*–114 (*cuf1*<sup>+</sup>/*cuf1*<sup>+</sup>) and *pat1*–114/*pat1*–114 *cuf1*Δ/*cuf1*Δ diploid strains were pre-synchronized in G<sub>1</sub> mitotic 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 CuSO<sub>4</sub> (50 μM). Aliquots of cell cultures were taken 1, 3, 5, 7, and 9 h after meiotic induction, and steady-state levels of *cum1*<sup>+</sup> mRNA were analyzed by RNase protection assays. Under low-copper conditions, transcript levels of *cum1*<sup>+</sup> were induced in *cuf1*<sup>+</sup>/*cuf1*<sup>+</sup> cells (Fig. 1). The magnitude of *cum1*<sup>+</sup> gene expression increased throughout the meiotic program and was more robust under TTM conditions (Fig. 1). In contrast, mRNA levels of *cum1*<sup>+</sup> remained very low under copper-replete conditions. RNA samples prepared from a *pat1*–114/*pat1*–114 *cuf1*Δ/*cuf1*Δ deletion strain showed very weak *cum1*<sup>+</sup> mRNA levels in TTM-treated cells. Although in *cuf1*Δ/*cuf1*Δ cells *cum1*<sup>+</sup> 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*<sup>+</sup> mRNA compared with levels observed with *cuf1*<sup>+</sup>/*cuf1*<sup>+</sup> cells that had been exposed to TTM. Furthermore, *cuf1*Δ/*cuf1*Δ cells showed loss of *cum1*<sup>+</sup> expression under copper-replete conditions (Fig. 1). As a control and known copper- and Cuf1-regulated gene, *ctr4*<sup>+</sup> expression was measured in the same strains under the conditions described above. Results showed that under low copper conditions, *ctr4*<sup>+</sup> 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 *ctr4*<sup>+</sup> mRNA under copper-replete conditions. Furthermore, *ctr4*<sup>+</sup> transcripts were absent in mutant cells lacking Cuf1 (*cuf1*Δ/*cuf1*Δ) (Fig. 1) (28, 42). Collectively, results showed that under low copper conditions Cuf1 is required to robustly activate *cum1*<sup>+</sup> gene expression during meiosis, especially in mid and late phases of the program.

***cum1*<sup>+</sup> Transcripts Are Detected Exclusively during Meiosis**—We next analyzed mRNA levels of *cum1*<sup>+</sup> in mitotically growing cells as a function of copper and Cuf1 availability. Logarithmic phase cultures of wild type (*cuf1*<sup>+</sup>) and *cuf1*Δ strains were grown in the absence or presence of TTM (250 μM) or CuSO<sub>4</sub> (100 μM) for 90 min. RNA analysis experiments failed to detect *cum1*<sup>+</sup> transcripts regardless of cellular copper or Cuf1 status (Fig. 2). In the case of the expression of *ctr4*<sup>+</sup> (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 (*cuf1*Δ), *ctr4*<sup>+</sup> mRNA was not detected because its expression is dependent of Cuf1 (28, 42). Together, the results revealed that *cum1*<sup>+</sup> transcripts are found exclusively during meiosis.

**Copper-responsiveness of *cum1*<sup>+</sup> Promoter**—Although *cum1*<sup>+</sup> 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



**FIGURE 1. *cum1*<sup>+</sup> is required for maximal expression of *cum1*<sup>+</sup> in meiotic cells under copper-deficient conditions.** A, representative expression profiles of *cum1*<sup>+</sup> and *ctr4*<sup>+</sup> transcripts in *pat1-114/pat1-114 cum1*<sup>+/+</sup> and *pat1-114/pat1-114 cum1*<sup>Δ/Δ</sup> cells that were induced to undergo synchronous meiosis. Cells were incubated in the presence of TTM (150 μM) or CuSO<sub>4</sub> (50 μM). Total RNA was isolated from culture aliquots taken at the indicated time points. *cum1*<sup>+</sup>, *ctr4*<sup>+</sup>, and *act1*<sup>+</sup> steady-state mRNA levels were analyzed by RNase protection assays. *ctr4*<sup>+</sup> 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 *cum1*<sup>+</sup> and *ctr4*<sup>+</sup> transcript levels relative to *act1*<sup>+</sup> mRNA levels. The histogram values represent the averages ± S.D.

translational initiator codon of *cum1*<sup>+</sup> (Fig. 3A). A fourth element was also orientated in the same direction relative to the transcription of *cum1*<sup>+</sup>, 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 *cum1*<sup>+</sup> promoter segments were fused upstream of and in-frame with the *lacZ* gene in a reporter plasmid. Surprisingly, results showed that wild-type cells harboring <sup>−1608</sup>*cum1*<sup>+</sup>-*lacZ* and <sup>−376</sup>*cum1*<sup>+</sup>-*lacZ* reporter plasmids were able to induce *lacZ* mRNA expression under conditions of copper starvation (Fig. 3, B and C). In fact, *lacZ* mRNA expression was induced ~6.3 (<sup>−1608</sup>*cum1*<sup>+</sup>-*lacZ*)- and ~4.3 (<sup>−376</sup>*cum1*<sup>+</sup>-*lacZ*)-fold compared with transcript levels detected in untreated cells (Fig. 3, B and C). In contrast, *lacZ* mRNA was poorly expressed in wild-

type cells treated with copper. When <sup>−174</sup>*cum1*<sup>+</sup>-*lacZ* fusion plasmid was transformed in wild-type cells, there was a lack of significant up- or down-regulation of *lacZ* mRNA levels as a function of copper availability (Fig. 3, B and C). Intriguingly, we observed that the overall magnitude of *lacZ* constitutive expression was higher for this transformant as compared with other transformants (containing other *lacZ* fusion derivatives). When <sup>−1608</sup>*cum1*<sup>+</sup>-*lacZ* plasmid was expressed in *cuf1*<sup>Δ</sup> cells, *lacZ* mRNA expression was absent, revealing that Cuf1 was required for induction of <sup>−1608</sup>*cum1*<sup>+</sup>-*lacZ* transcription in response to copper starvation. In the case of <sup>−376</sup>*cum1*<sup>+</sup>-*lacZ* plasmid, its transformation in *cuf1*<sup>Δ</sup> cells resulted in constitutive *lacZ* gene expression without any significant changes in response to the presence of TTM or copper (Fig. 3, B and C). The fact that *lacZ* mRNA was constitutively expressed when it

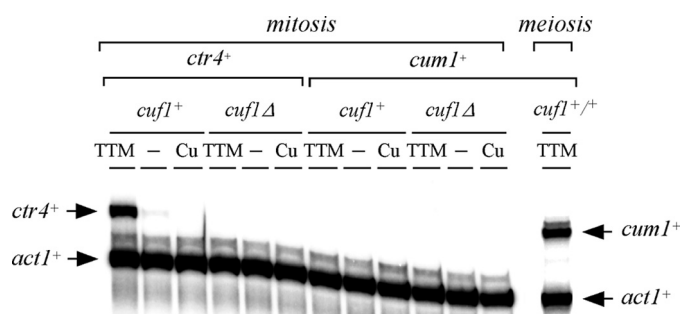


FIGURE 2. *cum1*<sup>+</sup> is expressed exclusively during meiosis. Vegetatively logarithmic growing cells expressing or lacking *cuf1*<sup>+</sup> were incubated in the absence (–) or presence of TTM (250  $\mu$ M) or CuSO<sub>4</sub> (Cu, 100  $\mu$ M) for 90 min. Shown are representative RNase protection assays of *ctr4*<sup>+</sup>, *cum1*<sup>+</sup>, and *act1*<sup>+</sup> mRNA steady-state levels during mitosis. As a control for *cum1*<sup>+</sup> mRNA signal, azygotic meiotic *cuf1*<sup>+/+</sup> cells were induced to undergo meiosis under low copper conditions. Five hours after meiotic induction, *cum1*<sup>+</sup> steady-state mRNA levels were analyzed by RNase protection assays (far right, meiosis).

was under control of the <sup>–376</sup>*cum*<sup>+</sup> promoter may be due to the loss of negative *cis*-acting regulatory elements located between positions –1608 and –377 within the promoter. Similarly to <sup>–376</sup>*cum*<sup>+</sup>–*lacZ* fusion reporter, *lacZ* transcript levels from *cuf1* $\Delta$  cells harboring <sup>–174</sup>*cum1*<sup>+</sup>–*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 <sup>–376</sup>*cum*<sup>+</sup>–*lacZ* and <sup>–174</sup>*cum1*<sup>+</sup>–*lacZ* fusion plasmids suggested that regulation of *cum1*<sup>+</sup> transcription is complicated and may involve additional *trans*-acting regulators.

To further characterize the ability of the proximal <sup>–376</sup>*cum*<sup>+</sup> 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*<sup>+</sup> promoter (positions –100 to –351) was inserted upstream of the minimal promoter of the *CYC1* gene fused to *lacZ* (Fig. 4A). This <sup>–351</sup>*cum1*<sup>–100</sup>–*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 the three CuSEs were mutated in the fusion reporter, very weak expression of *lacZ* mRNA was observed in *cuf1*<sup>+</sup> cells irrespective of cellular copper status (Fig. 4, B and C). In the case of *cuf1* $\Delta$  mutant cells harboring the <sup>–351</sup>*cum1*<sup>–100</sup>–*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*<sup>+</sup> are functional and could be recognized and used by the transcription factor Cuf1.

**Cuf1 Interacts with the *cum1*<sup>+</sup> Promoter in Vivo under Low Copper Conditions**—Despite the fact that there was no detection of *cum1*<sup>+</sup> transcripts in cells proliferating in mitosis under low copper conditions, we tested whether Cuf1 could possibly bind to *cum1*<sup>+</sup> promoter *in vivo*. *cuf1* $\Delta$  cells expressing an integrated untagged or Myc<sub>12</sub>-tagged *cuf1*<sup>+</sup> allele were precultured

until they reached the logarithmic phase. Cells were harvested, washed, and resuspended in media containing TTM (250  $\mu$ M) or CuSO<sub>4</sub> (100  $\mu$ M) for 90 min. Results from ChIP analysis showed that in the case of cells treated with TTM, Cuf1-Myc<sub>12</sub> occupied *cum1*<sup>+</sup> and *ctr4*<sup>+</sup> 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*<sup>+</sup> and *ctr4*<sup>+</sup> promoter fragments were immunoprecipitated. These low levels of immunoprecipitated chromatin were similar to the background signals observed when ChIP assays were performed in a *cuf1* $\Delta$  strain expressing an untagged *cuf1*<sup>+</sup> allele, which had been re-integrated (Fig. 4B).

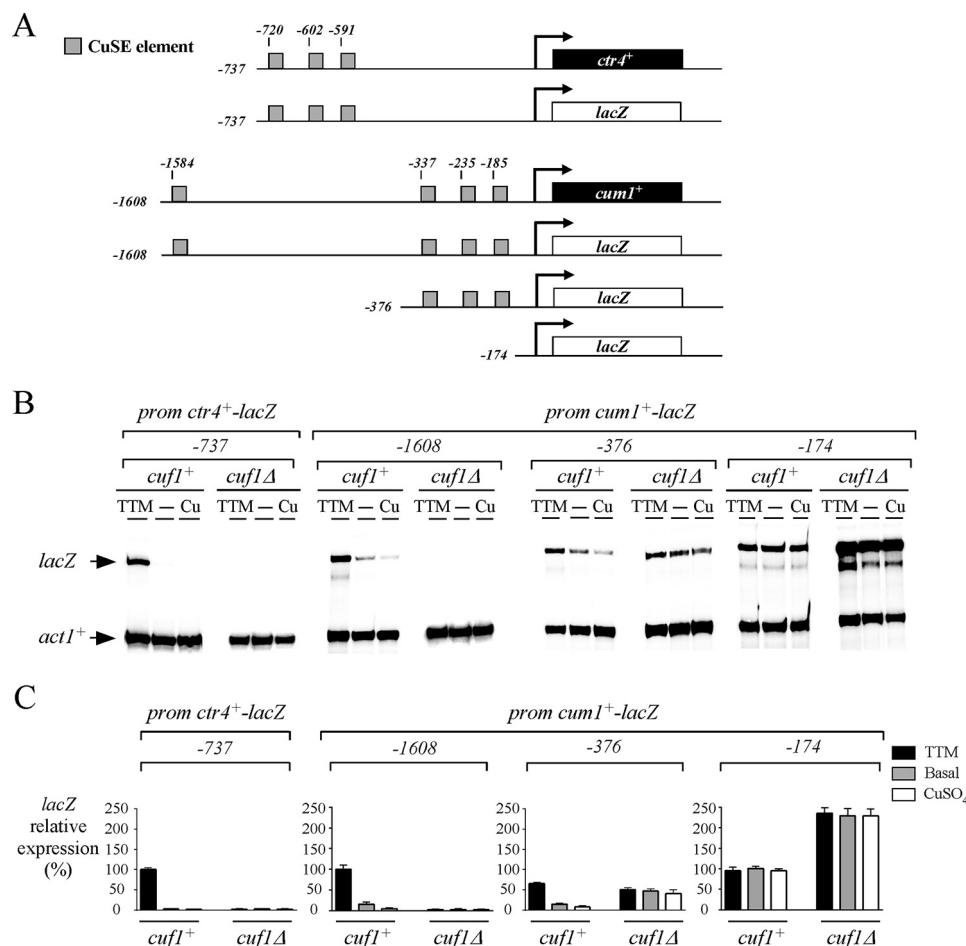
To test whether insertion of the epitope Myc<sub>12</sub> tag interfered with Cuf1 function, the untagged (*cuf1*<sup>+</sup>) and tagged (*cuf1*<sup>+</sup>–Myc<sub>12</sub>) alleles were separately expressed in *cuf1* $\Delta$  cells. *S. pombe* cells harboring disrupted *cuf1*<sup>+</sup> gene (*cuf1* $\Delta$ ) 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 *cuf1* $\Delta$  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 *cuf1* $\Delta$  mutant cells were transformed with wild-type or with Myc<sub>12</sub> epitope-tagged *cuf1*<sup>+</sup> allele (Fig. 5C). These results indicated that expression of Cuf1 or Cuf1-Myc<sub>12</sub> complemented the *cuf1* $\Delta$  growth defect in glycerol and ethanol as sources of respiratory carbon.

To further ensure that fusion of Myc<sub>12</sub> to the C terminus of Cuf1 did not interfere with its function, we analyzed if copper starvation-dependent induction of *ctr4*<sup>+</sup> gene expression was restored in *cuf1* $\Delta$  cells carrying a wild-type or Myc<sub>12</sub> epitope-tagged *cuf1*<sup>+</sup> allele. Results showed that cells expressing Cuf1-Myc<sub>12</sub> fostered induction of *ctr4*<sup>+</sup> mRNA levels under low copper conditions in a manner similar to that of cells expressing an untagged Cuf1 (Fig. 5D). As expected, *ctr4*<sup>+</sup> expression was down-regulated in cells harboring the same alleles under untreated (basal) and copper-replete conditions. Analysis of Cuf1-Myc<sub>12</sub> 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*<sup>+</sup> transcripts in copper-starved mitotically growing cells, the *cum1*<sup>+</sup> promoter was occupied by Cuf1 under conditions of low copper levels.

***iss1*<sup>+</sup> Produces Two Transcripts Including One That Is Partially Complementary with the *cum1*<sup>+</sup> Transcript**—Based on genome-wide transcriptome and genome sequence data, *iss1*<sup>+</sup> and *cum1*<sup>+</sup> genes are arranged with their transcription oriented convergently (43, 44). Furthermore, a long 3' untranslated region (UTR) of *iss1*<sup>+</sup> was predicted to overlap the *cum1*<sup>+</sup> open reading frame found in the opposite DNA strand (Fig. 6A). To validate the existence of the long *iss1*<sup>+</sup> transcript isoform, we used two distinct probes. The first probe (denoted AS1) hybridized in the coding sequence of *iss1*<sup>+</sup> (positions +292 to +525



## Elimination of *cum1*<sup>+</sup> Meiotic Transcripts in Mitotic Cells

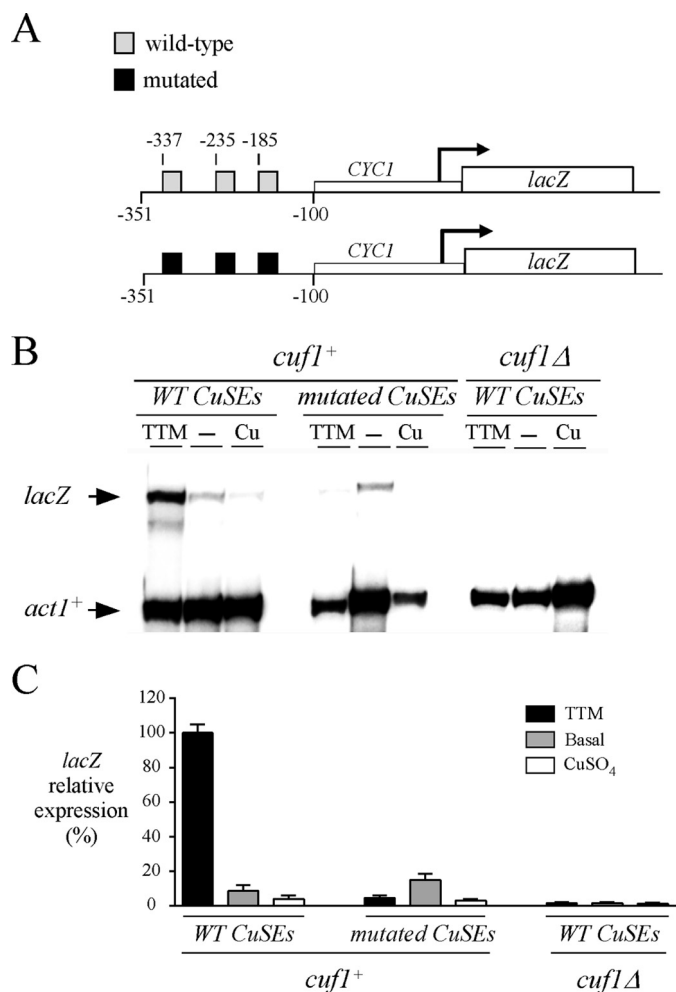


**FIGURE 3. Fusion of *cum1*<sup>+</sup> promoter regions to a reporter gene resulted in its regulated expression by Cuf1 in a CuSE-dependent manner.** *A*, schematic representation of *ctr4*<sup>+</sup> and *cum1*<sup>+</sup> promoter regions. Nucleotide numbers refer to positions relative to initiator codons of *ctr4*<sup>+</sup> and *cum1*<sup>+</sup> genes. Gray boxes represent CuSE elements. The empty box indicates the *lacZ* gene, whereas the filled box refers to *ctr4*<sup>+</sup> or *cum1*<sup>+</sup> gene. *B*, Logarithmic-phase cultures of wild-type (WT) and *cuf1* $\Delta$  cells harboring the indicated reporter plasmid that were left untreated (–) or treated with TTM (250  $\mu$ M) or CuSO<sub>4</sub> (Cu, 100  $\mu$ M) for 90 min. Total RNA was isolated, and steady-state mRNA levels of *lacZ* and *act1*<sup>+</sup> were analyzed by RNase protection assays. *C*, RNase protection assays were quantified. Values indicate the normalized *lacZ* transcript levels relative to *act1*<sup>+</sup> mRNA levels. Data are the averages of triplicate determinations of three independent experiments  $\pm$  S.D.

from the first base of the initiator codon), whereas the second probe (termed AS3) paired in the 3' UTR of *iss1*<sup>+</sup> (positions +1529 to +1790) (Fig. 6A). Cells proliferating in mitosis were exposed to TTM (250  $\mu$ M) or CuSO<sub>4</sub> (100  $\mu$ M) for 90 min. Total RNA was then isolated and analyzed for quantitation of steady-state levels of *iss1*<sup>+</sup>, *cum1*<sup>+</sup>, and *act1*<sup>+</sup> transcripts. RNA preparations probed with AS1 revealed two forms of *iss1*<sup>+</sup> transcripts (Fig. 6B). A long  $\sim$ 2.0-kb transcript matched the predicted size of *iss1*<sup>+</sup> transcript from genome-wide mapping studies (44). The 3' UTR of this antisense transcript overlapped with *cum1*<sup>+</sup> sense mRNA. In addition, a shorter  $\sim$ 1.4-kb transcript was detected, revealing the presence of an alternative polyadenylation site that led to the production of a second transcript isoform (Fig. 6B). According to its size, the second transcript isoform did not appear to overlap with *cum1*<sup>+</sup> sense mRNA. RNA preparations hybridized with AS3 probe revealed that only the long *iss1*<sup>+</sup> transcript ( $\sim$ 2.0 kb) was detected (Fig. 6B). Experiments using RNA preparations from cells grown under copper-deficient or copper-replete conditions showed that steady-state levels of both isoforms of *iss1*<sup>+</sup> mRNA were not significantly affected by either TTM or exogenous copper

(Fig. 6B). We were unable to detect the *cum1*<sup>+</sup> transcript ( $\sim$ 0.7 kb) (S1 probe) in cells grown under standard conditions (low or high copper) in which two *iss1*<sup>+</sup> mRNA isoforms ( $\sim$ 2.0 and  $\sim$ 1.4 kb) were produced (Fig. 6B). Based on apparent absence of *cum1*<sup>+</sup> transcript detection, we predicted that long *iss1*<sup>+</sup> mRNAs ( $\sim$ 2.0 kb) could form antisense transcripts with *cum1*<sup>+</sup> 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*<sup>+</sup> transcript, (ii) that the antisense transcript could result in early transcriptional termination of the *cum1*<sup>+</sup> transcript, or (iii) RNAi-mediated heterochromatin formation could decreased *cum1*<sup>+</sup> transcription.

***cum1*<sup>+</sup> Transcripts Are Detected in Copper-starved Mitotic Cells When the Long *iss1*<sup>+</sup> Antisense Transcript Is Absent**—To obtain further evidence that the long transcript isoform produced by *iss1*<sup>+</sup> overlapped with *cum1*<sup>+</sup> sense transcript, we generated mutant cells containing a PAS integrated immediately at the 3' end of the chromosomal locus of *iss1*<sup>+</sup> (Fig. 7A). Results showed that this approach led to exclusive production of a short *iss1*<sup>+</sup> mRNA isoform ( $\sim$ 1.4 kb) (Fig. 7B). When these



**FIGURE 4. CuSE elements of *cum1*<sup>+</sup> promoter are required for induction of gene expression in a Cuf1-dependent manner under copper-limiting conditions.** A, scheme of a 251-bp *cum1*<sup>+</sup> promoter DNA fragment and its mutant derivative that was inserted into the minimal promoter of the *CYC1* gene fused to *lacZ*. Gray boxes indicate wild-type CuSE elements (5'-(T/A)D-DHGCTG-3', D = A, G, or T; H = A, C, or T), whereas filled boxes depict mutated versions (5'-(C/G)DDHTCAT-3'). Nucleotide numbers refer to the positions relative to the translational initiator codon of *cum1*<sup>+</sup>. B, wild-type (*cuf1*<sup>+</sup>) and *cuf1*Δ cells were transformed with the indicated *cum1*<sup>+</sup>-*CYC1*-*lacZ* heterologous reporter plasmid. Total RNA was isolated from control (–), TTM (250 μM), or CuSO<sub>4</sub> (Cu, 100 μM)-treated cultures. Shown is a representative RNase protection assay of *lacZ* and *act1*<sup>+</sup> mRNA steady-state levels. C, the histogram shows quantification of *lacZ* mRNA levels relative to *act1*<sup>+</sup> transcript levels after treatments shown in panel B. Values are the averages of triplicate determinations ± S.D.

cells (+PAS) in which Cuf1 (*cuf1*<sup>+</sup>) was expressed were assayed, results showed that *cum1*<sup>+</sup> mRNAs were highly induced under conditions of copper starvation (~8-fold) (Fig. 7B). In contrast, *cum1*<sup>+</sup> transcripts were repressed in *cuf1*<sup>+</sup> cells grown under copper-replete conditions. To test whether Cuf1 was required for induction of *cum1*<sup>+</sup> expression in copper-starved cells harboring PAS, we deleted the *cuf1*<sup>+</sup> gene (*cuf1*Δ). Inactivation of *cuf1*<sup>+</sup> resulted in strongly reduced (~7.5-fold) *cum1*<sup>+</sup> mRNA levels in comparison with those seen with *cuf1*<sup>+</sup> (*iss1*<sup>+</sup>PAS) cells grown under the same copper-limited conditions (Fig. 7B). When *cuf1*Δ cells containing PAS were incubated in the presence of copper, they exhibited very weak levels of *cum1*<sup>+</sup> transcript that were similar to those observed with the same cells (*cuf1*Δ *iss1*<sup>+</sup>PAS) grown under

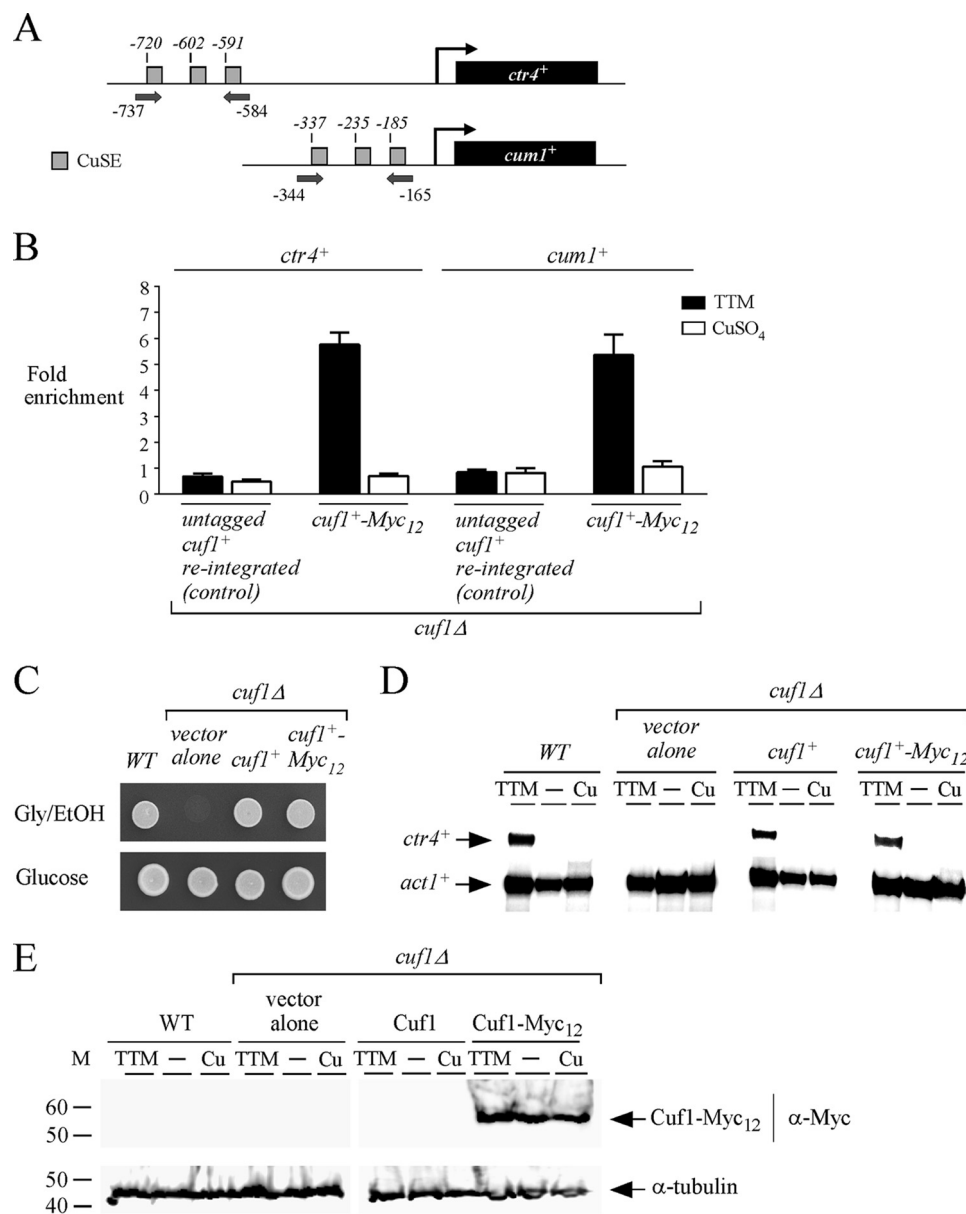
copper starvation. Wild-type (*cuf1*<sup>+</sup>) and *cuf1*Δ mutant strains in which the long *iss1*<sup>+</sup> transcript isoform was produced (no PAS) failed to generate significant amounts of *cum1*<sup>+</sup> mRNAs when incubated in the presence of TTM and CuSO<sub>4</sub> (Fig. 7B).

To validate the effect of the presence of PAS integrated at the 3' end region of *iss1*<sup>+</sup>, we probed total RNA isolated from copper-deficient and copper-replete cells with AS1 probe. Cells bearing the *iss1*<sup>+</sup>-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*<sup>+</sup>, two forms of *iss1*<sup>+</sup> 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*<sup>+</sup>-PAS allele failed to produce the long transcript isoform (Fig. 7B). Taken together, these results revealed that production of the long *iss1*<sup>+</sup> antisense transcript precludes detection of *cum1*<sup>+</sup> sense mRNA in vegetative cells.

**A Role for the RNAi Machinery in Silencing *cum1*<sup>+</sup> Transcription in the G<sub>1</sub> 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 G<sub>1</sub> phase of the *S. pombe* cell cycle (20, 22). To assess involvement of the RNAi machinery in silencing *cum1*<sup>+</sup> transcription, we probed *cum1*<sup>+</sup> mRNA levels in four mutants that affect the RNAi pathway. Cells were blocked in G<sub>1</sub> using nitrogen starvation, and then they underwent synchronized mitosis in the presence of TTM or copper or were left untreated. These mutants were *dcr1*Δ (dicer), *ago1*Δ (argonaute), *rdp1*Δ (RNA-dependent RNA polymerase), and *clr4*Δ (histone H3 lysine methyltransferase). DNA content was determined by flow cytometry (FACS) analysis to assess that cell populations were largely restricted to G<sub>1</sub> (1N) at the zero-point time and after 30 min of mitotic induction, whereas cells were primarily in G<sub>2</sub> phase at 6 h (Fig. 8). Experiments using wild-type cells, either in the G<sub>1</sub> or G<sub>2</sub> phase, failed to detect *cum1*<sup>+</sup> mRNA as a function of copper availability (Fig. 9). When *dcr1*Δ, *ago1*Δ, *rdp1*Δ, and *clr4*Δ mutant strains were blocked in G<sub>1</sub> and then synchronously cultured in nitrogen-replenished medium under low copper conditions, sense *cum1*<sup>+</sup> mRNA levels were detected after 30 min of mitotic entry, with an induction of ~2.9 (*dcr1*Δ)-, ~6.3 (*ago1*Δ)-, and ~5.1-fold (*rdp1*Δ) compared with that observed in the case of the wild-type strain (background threshold) (Fig. 9). In the case of *clr4*Δ mutant, *cum1*<sup>+</sup> expression was induced ~2.4-fold after 15 min of mitotic induction. Sense *cum1*<sup>+</sup> 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 G<sub>2</sub> cells at the 6-h time point, there was no significant increase of sense *cum1*<sup>+</sup>



# Elimination of *cum1*<sup>+</sup> Meiotic Transcripts in Mitotic Cells



**FIGURE 5. Cuf1 binds to the *cum1*<sup>+</sup> promoter in vegetative cells under copper-limiting conditions.** *A*, schematic representation of *ctr4*<sup>+</sup> and *cum1*<sup>+</sup> promoter regions. Gray boxes represent CuSEs that are numbered relative to the initiator codon of each gene (*ctr4*<sup>+</sup> or *cum1*<sup>+</sup>). Arrows indicate locations of the primers for qPCR analysis. *B*, ChIP analysis of *ctr4*<sup>+</sup> and *cum1*<sup>+</sup> promoters in a *cuf1*Δ mutant strain expressing an integrated untagged or Myc<sub>12</sub>-tagged *cuf1*<sup>+</sup> allele. Cells were treated with TTM (250 μM) or CuSO<sub>4</sub> (100 μM) for 90 min. Chromatin was immunoprecipitated using anti-*c-myc* antibodies. Specific regions of *ctr4*<sup>+</sup> and *cum1*<sup>+</sup> promoters were analyzed by qPCR to determine Cuf1 occupancy. Binding of Cuf1-Myc<sub>12</sub> to *ctr4*<sup>+</sup> and *cum1*<sup>+</sup> promoters was calculated as the enrichment of specific *ctr4*<sup>+</sup> and *cum1*<sup>+</sup> 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 *cuf1*Δ allele were transformed with an empty vector (vector alone), *cuf1*<sup>+</sup>, and *cuf1*<sup>+</sup>-Myc<sub>12</sub>. Cultures were spotted onto YES media containing glucose or glycerol/ethanol (Gly/EtOH). *WT*, isogenic parental strain FY435 (*cuf1*<sup>+</sup>). *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 CuSO<sub>4</sub> (Cu, 100 μM) for 90 min. Total RNA was isolated, and steady-state *ctr4*<sup>+</sup> and *act1*<sup>+</sup> mRNA levels were analyzed by RNase 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.

transcript in *dcr1*Δ, *rdp1*Δ, and *clr4*Δ mutants under all three experimental conditions (TTM, CuSO<sub>4</sub>, and untreated) (Fig. 9). With respect to *ago1*Δ mutant, only a very weak level of sense *cum1*<sup>+</sup> expression was observed after 6 h of mitotic induction. Using unsynchronized cells, *cum1*<sup>+</sup> mRNA was also detected in *ago1*Δ and *dcr1*Δ mutant strains (Fig. 10). However, derepression of *cum1*<sup>+</sup> 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, Rdp1, and Clr4 are involved in the antisense repression of *cum1*<sup>+</sup> transcription in mitotic cells, especially cells undergoing G<sub>1</sub> cell-cycle phase.

Due to the fact that *cum1*<sup>+</sup> mRNA levels were induced in copper-starved RNAi mutant strains that were synchronously cultured in G<sub>1</sub> phase, we sought to ascertain if this induction was Cuf1-dependent. To obtain evidence of the requirement of

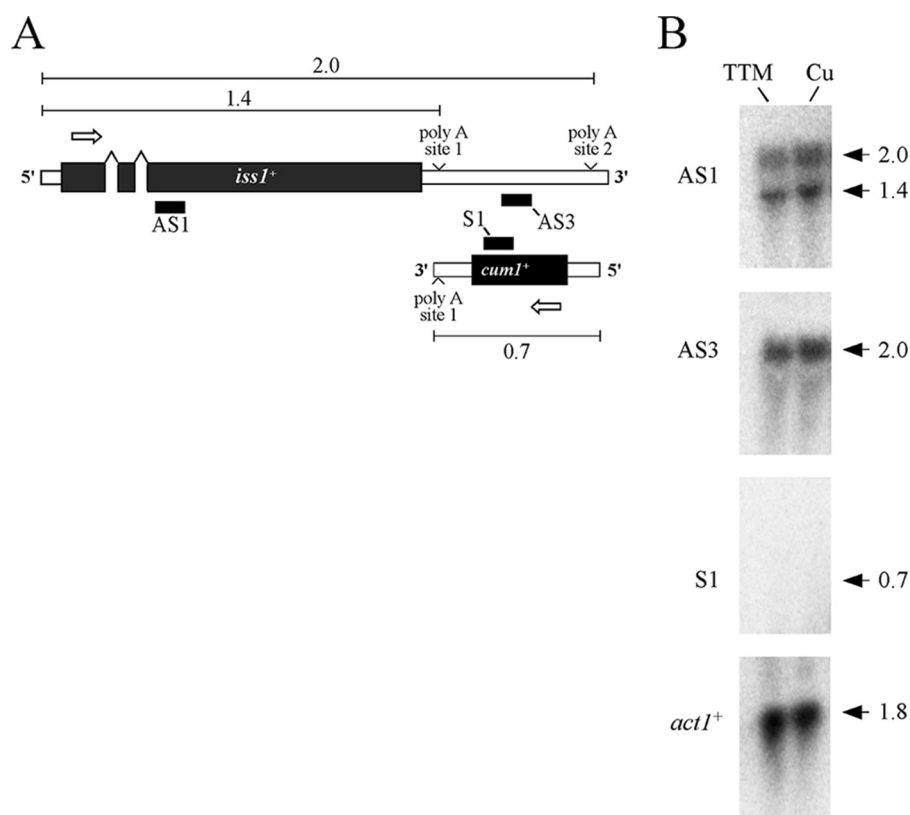
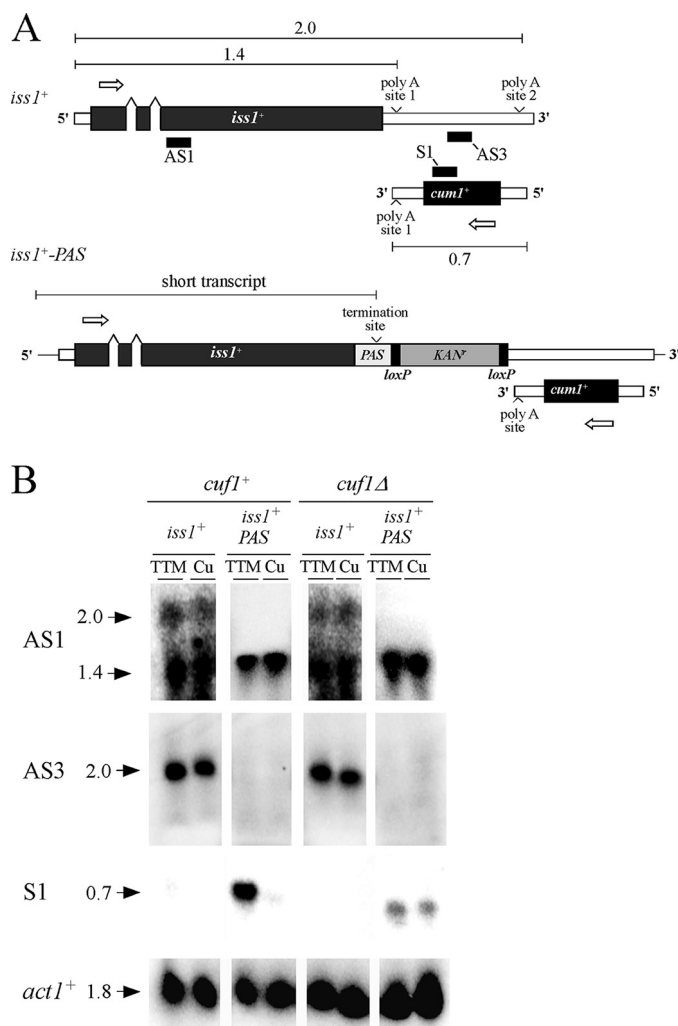


FIGURE 6. **Expression of *iss1*<sup>+</sup> produces two different sizes of transcripts.** A, diagram of the arrangement of *iss1*<sup>+</sup> and *cum1*<sup>+</sup> genes. Putative polyadenylation sites are indicated with arrowheads. White arrows indicate the direction of transcription. Filled rectangles show the positions of radiolabeled strand-specific probes (AS1, AS3, and S1) that were used to detect *iss1*<sup>+</sup> and *cum1*<sup>+</sup> mRNA steady-state levels. Numbers refer to the predicted sizes of transcripts. B, total RNA was isolated from cultures treated with TTM (250  $\mu$ M) or CuSO<sub>4</sub> (Cu, 100  $\mu$ M) and then analyzed by Northern blot assays. AS1, AS3, S1, and *act1*<sup>+</sup> probes are indicated on the left, whereas approximate transcript sizes (in kilobases) are shown on the right.

Cuf1, the *cuf1*<sup>+</sup> gene was deleted in a *dcr1* $\Delta$  mutant strain, creating a *dcr1* $\Delta$  *cuf1* $\Delta$  double mutant strain. In another series of experiments, a functional *cuf1*<sup>+</sup> allele was reintegrated into *dcr1* $\Delta$  *cuf1* $\Delta$  cells. Wild-type (*cuf1*<sup>+</sup> *dcr1*<sup>+</sup>), *cuf1*<sup>+</sup> *dcr1* $\Delta$ , *cuf1* $\Delta$  *dcr1* $\Delta$ , and *cuf1* $\Delta$  *dcr1* $\Delta$  + *cuf1*<sup>+</sup> strains were cultured in a way that they underwent synchronous mitosis. After mitosis had occurred, cells were incubated in the presence of TTM or CuSO<sub>4</sub> for 1 h. Results showed that inactivation of *cuf1*<sup>+</sup> (*cuf1* $\Delta$ ) in a *dcr1* $\Delta$  strain resulted in a loss of TTM-dependent activation of *cum1*<sup>+</sup> expression in response to low copper conditions (Fig. 11A). When RNA samples were prepared from *dcr1* $\Delta$  *cuf1* $\Delta$  cells in which a wild-type copy of *cuf1*<sup>+</sup> allele was returned by integration, cells regained the capacity to induce *cum1*<sup>+</sup> gene expression in response to copper starvation (Fig. 11A). Indeed, the up-regulated levels of *cum1*<sup>+</sup> in the *cuf1* $\Delta$  *dcr1* $\Delta$  + *cuf1*<sup>+</sup> strain were similar to that of *dcr1* $\Delta$  mutant cells (containing an endogenous *cuf1*<sup>+</sup> gene). These observations confirmed a role of Cuf1 in TTM-mediated activation of *cum1*<sup>+</sup> expression when the RNAi system was defective in cells undergoing G<sub>1</sub> cell-cycle phase. As expected, *ctr4*<sup>+</sup> mRNA levels (assayed as a control) were up- and down-regulated after treatment with TTM and copper, respectively (Fig. 11B). Furthermore, Cuf1 was required for induction of *ctr4*<sup>+</sup> mRNA levels under low copper conditions. Conversely, no TTM-dependent induction of *ctr4*<sup>+</sup> transcription was observed in *cuf1* $\Delta$  *dcr1* $\Delta$  double mutant cells.

*cum1*<sup>+</sup> and the Long *iss1*<sup>+</sup> RNA Isoform Are Expressed in the Opposite Direction Relative to Each Other during Meiosis—Because *cum1*<sup>+</sup> is induced in copper-starved meiotic cells, we investigated its profile of expression compared with profiles of the two *iss1*<sup>+</sup> RNA isoforms during meiosis. *pat1*–114/*pat1*–114 diploid cells were synchronized through meiosis in the absence or the presence of either TTM or CuSO<sub>4</sub>. Aliquots of cultures were picked up at representative time points after meiotic induction, and steady-state levels of *cum1*<sup>+</sup> and *iss1*<sup>+</sup> mRNAs were analyzed by Northern blot assays. *cum1*<sup>+</sup> 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*<sup>+</sup> mRNA under copper-replete conditions. When copper levels were low, the long *iss1*<sup>+</sup> 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*<sup>+</sup> antisense transcript was anti-correlated with the abundance of *cum1*<sup>+</sup> sense mRNA. Consistent with this observation, under copper-replete conditions, results showed that steady-state levels of the long *iss1*<sup>+</sup> isoform remained high through the meiotic program (even after 9 h) (Fig. 12). In the case of the short *iss1*<sup>+</sup> isoform (~1.4 kb), results showed that its expression changed in the opposite direction relative to the long *iss1*<sup>+</sup> isoform under copper-starved and copper-replete conditions (Fig. 12). Taken

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**FIGURE 7. Elimination of the long *iss1*<sup>+</sup> transcript leads to derepression of *cum1*<sup>+</sup> mRNA in a TTM- and Cuf1-dependent manner.** **A**, schematic arrangement of *iss1*<sup>+</sup>, *iss1*<sup>+</sup>-PAS, and *cum1*<sup>+</sup> 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*<sup>+</sup> and *cum1*<sup>+</sup> transcripts. Numbers refer to the predicted sizes of transcripts. The PAS sequence derived from the *ura4*<sup>+</sup> 3' UTR was integrated at the chromosomal locus of *iss1*<sup>+</sup> to trigger its immediate transcription termination at a proximal site downstream of the gene. **B**, logarithmic-phase cultures of wild-type (*cuf1*<sup>+</sup>) and *cuf1*Δ strains expressing the indicated *iss1*<sup>+</sup> or *iss1*<sup>+</sup>-PAS allele were exposed to TTM (250 μM) or CuSO<sub>4</sub> (Cu, 100 μM) for 90 min. After RNA isolation, *iss1*<sup>+</sup> and *cum1*<sup>+</sup> steady-state mRNA levels were analyzed by Northern blot assays. AS1, AS3, S1, and *act1*<sup>+</sup> probes as well as approximate transcript sizes (in kilobases) are indicated on the left.

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

### Discussion

*cum1*<sup>+</sup> is a copper-regulated meiotic gene that is under the control of copper-dependent transcription factor Cuf1. Although *cum1*<sup>+</sup> 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*<sup>+</sup> 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*<sup>+</sup> and *iss1*<sup>+</sup> genes are arranged in convergent orientation and are transcribed from opposing DNA strands. Furthermore, genome-wide transcriptome data suggested that *iss1*<sup>+</sup> produces a long antisense transcript (~2.0 kb), which can hybridize with *cum1*<sup>+</sup> sense mRNA. In *S. pombe*, *iss1*<sup>+</sup> 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*<sup>+</sup> gene. Results showed that the relative abundance of *iss1*<sup>+</sup> mRNA isoforms is not significantly affected in response to changes in copper levels. This observation was different than that of the *adh1*<sup>+</sup> antisense transcript (*adh1AS*) in *S. pombe* (11, 46). The *adh1AS* is generated at the *adh1*<sup>+</sup> locus in zinc-limited cells. It serves to suppress sense RNA transcription of *adh1*<sup>+</sup>, which encodes an abundant zinc-binding protein that is required for the conversion of acetaldehyde to ethanol. As a result, *adh1AS* down-regulates *adh1*<sup>+</sup> 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*<sup>+</sup> sense mRNA (which is abundant under high zinc conditions). Although increased expression of the *adh1AS* transcript leads to reduced levels of the *adh1*<sup>+</sup> sense transcript, the mechanism to eliminate this paired antisense/sense transcripts remains unclear. Another distinction between the two gene pairs, *cum1*<sup>+</sup>/*iss1*<sup>+</sup> and *adh1*<sup>+</sup>/*adhAS*, is the nature of the biological process that is involved. In the case of *adh1*<sup>+</sup>/*adhAS*, the transcriptional interference mechanism is important for maintaining zinc homeostasis during vegetative growth (mitosis). In the case of *cum1*<sup>+</sup>/*iss1*<sup>+</sup>, action of the long *iss1*<sup>+</sup> antisense transcript is important for maintaining tight vegetative (mitotic) repression of the meiosis-specific *cum1*<sup>+</sup> 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*<sup>+</sup>/*SPBC1778.05c* and *mug28*<sup>+</sup>/*mrp17*<sup>+</sup>. In a manner similar to *cum1*<sup>+</sup>/*iss1*<sup>+</sup>, insertion of a transcription terminator into *SPBC1778.05c* (the source of antisense RNA for *spo6*<sup>+</sup>) led to a corresponding increase in *spo6*<sup>+</sup> sense mRNA levels in vegetative cells (19). However, the levels of *spo6*<sup>+</sup> expression (in mitosis) remained low compared with levels of the *spo6*<sup>+</sup> sense transcripts in meiotic cells. In the case of *spo6*<sup>+</sup>, 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*<sup>+</sup> expression.

To eliminate *cum1*<sup>+</sup> sense transcripts in vegetative cells, the mechanism of antisense-mediated transcriptional repression should be efficient, especially under low copper conditions. In this situation, *cum1*<sup>+</sup> 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*<sup>+</sup> antisense tran-



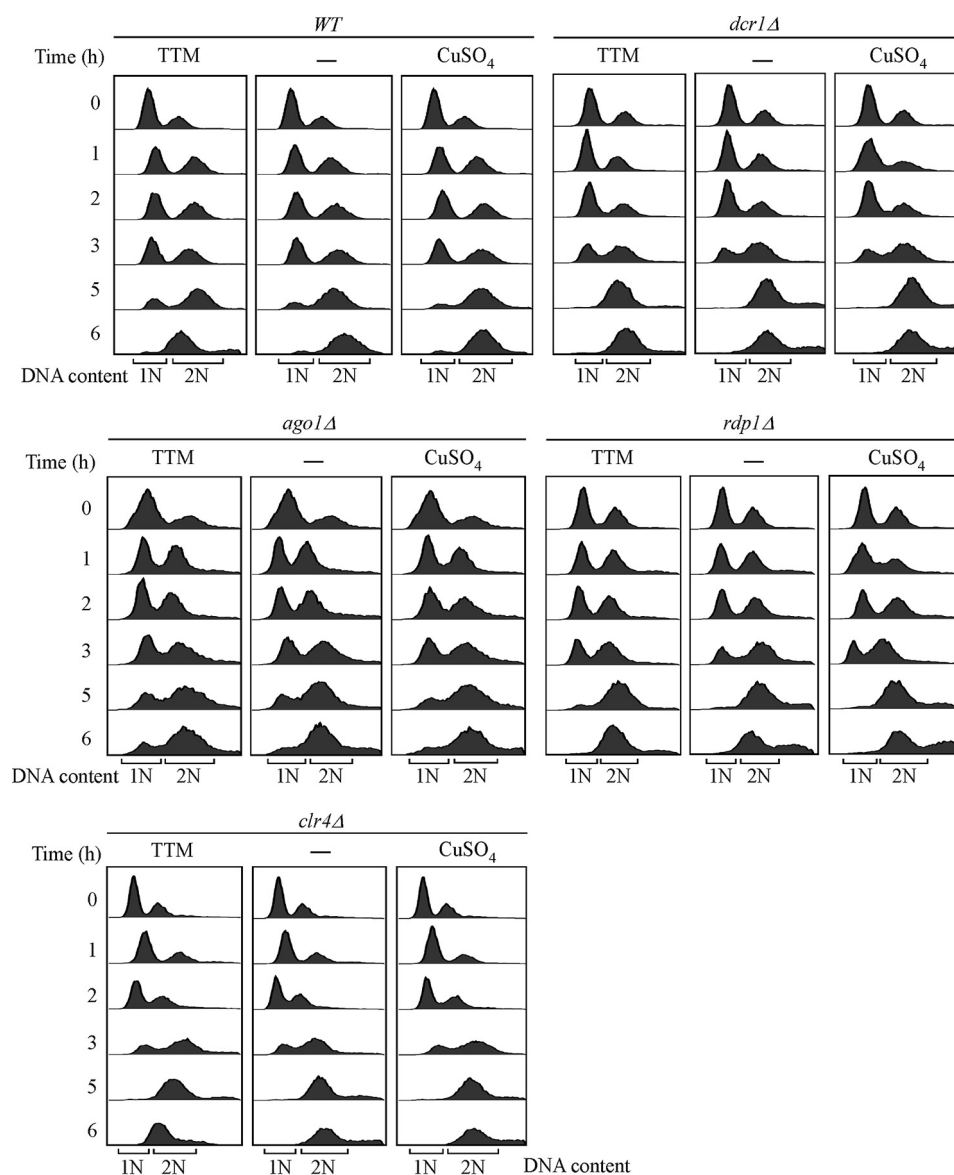
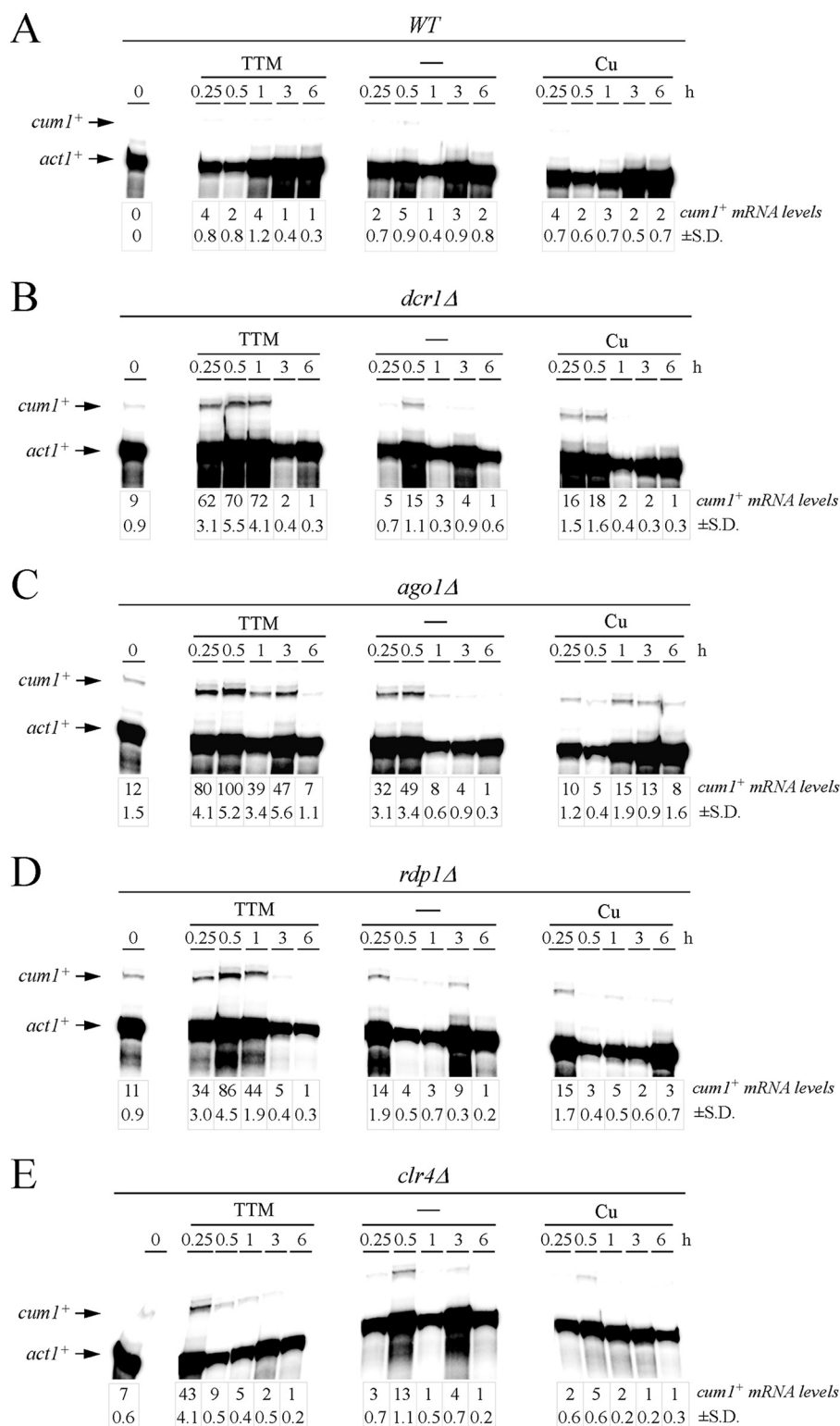


FIGURE 8. **Synchronization of wild type and mutant strains using nitrogen starvation.** The indicated isogenic strains were arrested at the G<sub>1</sub> phase using growth conditions lacking nitrogen. Strains were maintained in nitrogen-deficient medium for 24 h and then shifted to a nitrogen-replete medium. At this step strains were treated with TTM (250  $\mu$ M) or CuSO<sub>4</sub> (100  $\mu$ M), or were left untreated (–) for the indicated times. FACS analysis showed mitotic progression of cell populations after their transferred in nitrogen-replenished medium as a function of time. G<sub>1</sub> (1N) cells were detected shortly after switching medium during the first 60 min, whereas G<sub>2</sub> (2N) cells were predominantly found after ~5–6 h of mitotic induction.

script is synthesized in sufficiently high concentrations to hybridize with all *cum1*<sup>+</sup> 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*<sup>+</sup> sense and *iss1*<sup>+</sup> 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*<sup>+</sup> antisense RNA to inhibit *cum1*<sup>+</sup> sense mRNA in the G<sub>1</sub> phase of the cell cycle. Synchronization in the G<sub>1</sub> phase revealed an increase of *cum1*<sup>+</sup> sense transcripts in four mutants (*dcr1* $\Delta$ , *ago1* $\Delta$ , *rpd1* $\Delta$ , and *clr4* $\Delta$ ) 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*<sup>+</sup>/*SPBC1778.05c* and *mug28*<sup>+</sup>/*mrp17*<sup>+</sup>), 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*<sup>+</sup>/*SPBC1778.05c* and *mug28*<sup>+</sup>/*mrp17*<sup>+</sup> 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 G<sub>1</sub> phase of the *S. pombe* cell cycle hinders detection of small RNAs derived from convergent genes. Furthermore, these

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**FIGURE 9. Elimination of *cum1*<sup>+</sup> mRNA in G<sub>1</sub> cells requires components of the RNAi machinery.** WT (panel A) and isogenic *dcr1Δ* (panel B), *ago1Δ* (panel C), *rdp1Δ* (panel D), and *clr4Δ* (panel E) mutant strains were blocked at G<sub>1</sub> phase by nitrogen deprivation and then (at time point 0) induced to undergo synchronous mitosis. Cells underwent mitosis in the absence (—) or presence of TTM (250 μM) or CuSO<sub>4</sub> (Cu, 100 μM). Total RNA was isolated from culture aliquots taken at the indicated time points. *cum1*<sup>+</sup> and *act1*<sup>+</sup> steady-state mRNA levels were analyzed by RNase protection 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*<sup>+</sup> levels relative to *act1*<sup>+</sup>. The highest value was assigned to 100%.

experiments were not carried out under low copper conditions, which represent an optimal context to produce *cum1*<sup>+</sup> sense mRNA that would hybridize with long *iss1*<sup>+</sup> antisense tran-

script. 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

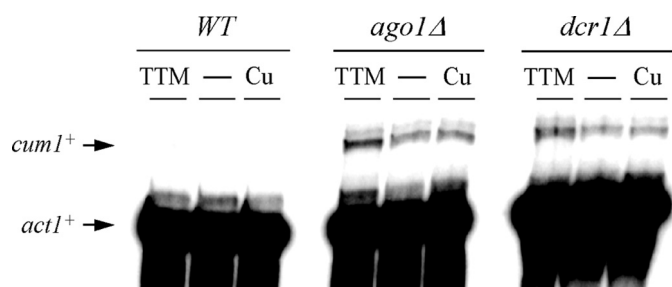


FIGURE 10. Silencing *cum1*<sup>+</sup> expression in unsynchronized vegetative cells requires RNAi components. The indicated isogenic strains were grown to logarithmic phase. Cultures were incubated in the absence (–) or presence of TTM (250 μM) or CuSO<sub>4</sub> (Cu, 100 μM) for 90 min. Total RNA was isolated and steady-state mRNA levels of *cum1*<sup>+</sup> and *act1*<sup>+</sup> were analyzed by RNase protection assays. Results shown are representative of three independent experiments.

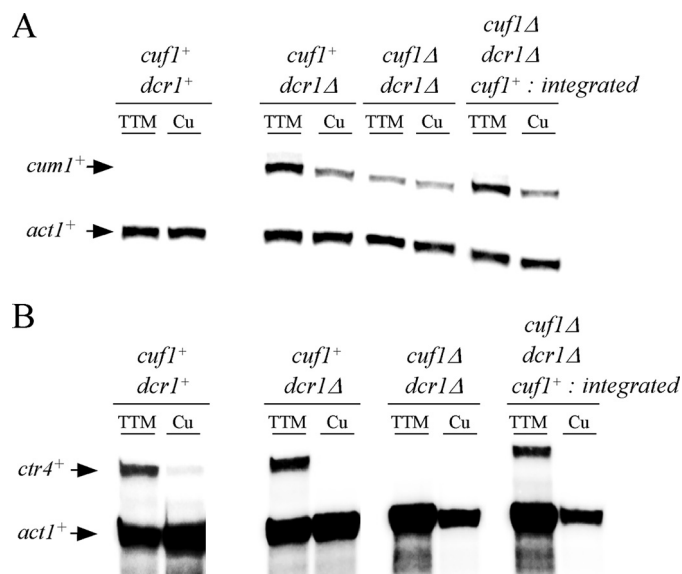


FIGURE 11. *cuf1*<sup>+</sup> is required for TTM-dependent induction of *cum1*<sup>+</sup> mRNA in a *dcr1*Δ mutant strain undergoing G<sub>1</sub> cell-cycle phase. A, cultures of *dcr1*<sup>+</sup> *cuf1*<sup>+</sup>, *dcr1*Δ *cuf1*<sup>+</sup>, *dcr1*Δ *cuf1*Δ, and *dcr1*Δ *cuf1*Δ + *cuf1*<sup>+</sup> cells were arrested in G<sub>1</sub> using nitrogen starvation. Once blocked, cultures were simultaneously switched to nitrogen-replete conditions, allowing their synchronous entry into mitosis. Cells were incubated in the presence of TTM (250 μM) or CuSO<sub>4</sub> (Cu, 100 μM). Cultures were taken after 1 h of the mitotic program, and total RNA was extracted and analyzed for steady-state levels of *cum1*<sup>+</sup> and *act1*<sup>+</sup> mRNAs. Results shown are representative of three independent experiments. B, the indicated isogenic strains were cultured as described in panel A. *ctr4*<sup>+</sup> (used as a control gene known to be regulated by Cuf1 as a function of copper availability) and *act1*<sup>+</sup> mRNA levels were determined by RNase protection assays.

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*<sup>+</sup> and *mug28*<sup>+</sup>, 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*<sup>+</sup> sense transcription was highly induced in vegetative copper-starved cells. Under this latter condition, accumulation of *cum1*<sup>+</sup> mRNA generated sufficient amounts of sense transcripts for their hybridization with *iss1*<sup>+</sup> 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

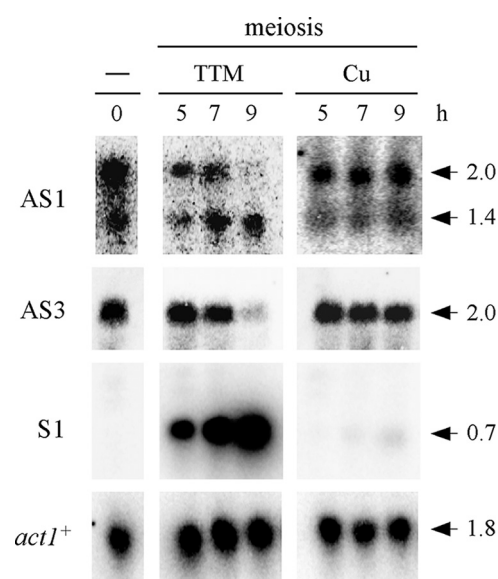


FIGURE 12. Copper starvation represses the long *iss1*<sup>+</sup> RNA isoform at the end of meiosis. *pat1-114/pat1-114* cells underwent synchronous meiosis under copper-depleted (TTM, 150 μM) and copper-replete (Cu, 50 μM) conditions. At the indicated time points, *iss1*<sup>+</sup> (AS1 and AS3 probes), *cum1*<sup>+</sup> (S1 probe), and *act1*<sup>+</sup> steady-state mRNA levels were analyzed by Northern blot assays. Approximate transcript sizes (in kilobases) are indicated. As a control, an untreated (–) culture was maintained in mitosis at 25 °C.

ensure high levels of *cum1*<sup>+</sup> transcription in vegetative cells under low copper conditions, therefore, fostering *cum1*<sup>+</sup> mRNA elimination in the G<sub>1</sub> phase of the cell cycle.

In the present study, production of long *iss1*<sup>+</sup> antisense and *cum1*<sup>+</sup> 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<sub>1</sub> cells, transient heterochromatin formation involves histone H3 lysine 9 di- tri-methylation and recruitment of proteins, including Swi6 and Clr4 (22). This mechanism could be the same for silencing *cum1*<sup>+</sup> in cells undergoing G<sub>1</sub> phase. However, it has been proposed that after S phase, cohesin 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<sub>2</sub> phase. However, in the case of *cum1*<sup>+</sup>, 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*<sup>+</sup> (either *fkh2*Δ single or *fkh2*Δ *mei4*Δ double mutant) could foster derepression of *cum1*<sup>+</sup> sense transcript during mitosis. Under our experimental conditions, we could not detect induction of *cum1*<sup>+</sup> sense mRNA in any of the two mutants.<sup>3</sup>

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

<sup>3</sup> V. Normant, J. Beaudoin, and S. Labbé, unpublished results.



(~2.0 kb) was more abundant than the short isoform (~1.4 kb). As a consequence, *cum1*<sup>+</sup> sense transcript exhibited only moderate levels of transcription. At the end of meiosis, however, expression of the long *iss1*<sup>+</sup> isoform was markedly decreased, which led to an increase expression of *cum1*<sup>+</sup> sense transcript that was not affected by the presence of the short *iss1*<sup>+</sup> isoform. These findings led us to conclude that a decrease in expression of the long *iss1*<sup>+</sup> isoform leads to up-regulated expression of *cum1*<sup>+</sup> 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*<sup>+</sup> antisense transcript (by insertion of PAS) in mitosis resulted in detectable levels of vegetative *cum1*<sup>+</sup> 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 ubiquitous component.

**Author Contributions**—V. N., J. B., and S. L. conceived, designed, performed, and analyzed the experiments. V. N. and S. L. wrote the paper. All authors reviewed the results and approved the final version of the manuscript.

**Acknowledgments**—We are grateful to Dr. Gilles Dupuis for critical reading of the manuscript and for valuable comments. We gratefully acknowledge Dr. Léonid Volkov for excellent assistance in flow cytometry experiments.

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*J. Biol. Chem.* 2015, 290:22622-22637.

doi: 10.1074/jbc.M115.674556 originally published online July 30, 2015

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Access the most updated version of this article at doi: [10.1074/jbc.M115.674556](https://doi.org/10.1074/jbc.M115.674556)

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