Transcriptional Regulation of the Copper Transporter Mfc1 in Meiotic Cells

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Mfc1 is a meiosis-specific protein that mediates copper transport during the meiotic program in Schizosaccharomyces pombe. Although the mfc1+/H11545 gene is induced at the transcriptional level in response to copper deprivation, the molecular determinants that are required for its copper starvation-dependence induction are unknown. Promoter deletion and site-directed mutagenesis have allowed identification of a new cis-regulatory element in the promoter region of the mfc1+/H11545 gene. This cis-acting regulatory sequence containing the sequence TCGGGCG is responsible for transcriptional activation of mfc1+/H11545 under low-copper conditions. The TCGGGCG sequence contains a CGG triplet known to serve as a binding site for members of the Zn(2)Cys(6) binuclear cluster transcriptional regulator family. In agreement with this fact, one member of this group of regulators, denoted Mca1, was found to be required for maximum induction of mfc1+/H11545 gene expression. Analysis of Mca1 cellular distribution during meiosis revealed that it colocalizes with both chromosomes and sister chromatids during early, middle, and late phases of the meiotic program. Cells lacking Mca1 exhibited a meiotic arrest at metaphase I under low-copper conditions. Binding studies revealed that the N-terminal 150-residue segment of Mca1 expressed as a fusion protein in Escherichia coli specifically interacts with the TCGGGCG sequence of the mfc1+/H11545 promoter. Taken together, these results identify the cis-regulatory TCGGGCG sequence and the transcription factor Mca1 as critical components for activation of the meiotic copper transport mfc1+/H11545 gene in response to copper starvation.

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hhibited, therefore bypassing the Mei3-dependent inactivation pathway of Pat1. This temperature-sensitive mutant confers a marked advantage, since it is more synchronous than asygotitic meiosis. Nitrogen starvation response, initiation, and progression throughout meiosis are characterized by the expression of numerous genes that are modulated in four successive waves (13). The first wave of genes encodes proteins that are involved in nitrogen starvation and pheromone responses. Early-phase genes (wave 2) encode proteins that participate in premeiotic S phase and recombination. Middle-phase genes (wave 3) produce cellular components that are responsible for meiotic divisions and early steps of spore formation. Late-phase genes (wave 4) generate the cellular products required for spore maturation (13, 14).

Recent studies have shown that metal ions such as copper and zinc are required for normal progression of meiosis in *S. pombe* and mice, respectively (3, 4, 15). In *S. pombe*, copper-insufficient zygotes undergo a meiotic block at metaphase I (3). With respect to copper transport into *S. pombe* meiotic cells, low copper levels induce expression of the copper transport genes *ctr4* and *ctr5* within the first hour of meiosis, followed by their repression 3 h after meiotic induction. Consistent with *ctr4* and *ctr5* gene expression profiles, the two-component copper-transporting complex that is composed of Ctr4 and Ctr5 (16–19) is observed at the plasma membrane within 1 h and remains at the cell surface until the 3-h meiotic time point is reached (3). This step is followed by a quick increase in *mfc1* mRNA levels at the 3-h meiotic time point, with *mfc1* transcript levels remaining sustained throughout the meiotic program. *mfc1* encodes a major facilitator superfamily (MFS)-type copper transporter (3, 20). During middle to late meiosis, Mfc1 localizes at the forespore membrane, where it potentially mediates copper uptake into the forespore. Studies of the full-scale meiotic transcriptional program have revealed that *ctr4* and *mfc1* transcript levels are induced at distinct times after meiotic induction, in response to copper starvation (3). Whereas deletion of the gene encoding the copper-sensing transcription factor Cuf1 (cuf1Δ) impairs the induction of *ctr4* and *ctr5* (16), the activation of *mfc1* is unaffected, suggesting the existence of a distinct transcriptional regulator for the induction of *mfc1* in response to copper starvation (3). Among transcription factors that are known to be required for successful meiosis and sporulation, global transcripome and deletome profile analyses have shown that the *mfc1* gene is not regulated by Rep1, Rep4, Cuf2, Rsv1, Rsv2, Atf21, and Atf31 (14, 21). These observations add further support to the hypothesis that a new transcription factor is required for regulation of the *mfc1* gene.

In the present report, we show that *mfc1* transcriptional induction is exclusively detected after treatment with a copper chelator and not by iron or zinc chelators. Analysis of regions in the promoter of *mfc1* reveal that two TCGGCG regulatory elements containing CGG triplets are required for the induction of *mfc1* in response to copper starvation. We consistently find that Mca1, a putative member of the Zn(II)Cys(6) binuclear cluster class of regulators which are known to bind repeated Cys-containing elements containing CGG triplets, is necessary to mount a maximal transcriptional response of *mfc1*. Microscopic analyses reveal that a functional Mca1-Cherry protein localizes to the nucleus during the course of vegetative growth of diploid cells and colocalizes with chromosomes during the meiotic process of differentiation. Although *mca1Δ/mca1Δ* cells exhibit normal progression under basal and copper-replete conditions, these mutant cells undergo a meiotic block at metaphase I under conditions of copper starvation. Binding studies reveal that the N-terminal 150-residue segment of Mca1 expressed as a fusion protein in *Escherichia coli* specifically binds to the TCGGCG sequences of the *mfc1* promoter region. Taken together, these results have identified cis- and trans-acting elements involved in molecular control of the meiosis-specific copper transporter Mfc1.

### MATERIALS AND METHODS

**Strains and media.** The *S. pombe* strains used in this study are listed in Table 1. Standard methods were used for growth, mating, and sporulation of fission yeast cells (22). Under nonselective conditions, *S. pombe* cells

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**Table 1. S. pombe strain genotypes**

| Strain | Genotype | Source or reference |
|--------|----------|---------------------|
| FY435  | h"his7-366 leu1-32 ura4-Δ18 ade6-M210 | This study |
| FY436  | h"his7-366 leu1-32 ura4-Δ18 ade6-M216 | This study |
| JS101  | h"his7-366 leu1-32 ura4-Δ18 ade6-M210 mfc1Δ::KAN | This study |
| JS201  | h"his7-366 leu1-32 ura4-Δ18 ade6-M216 mfc1Δ::KAN | This study |
| JS106  | h"his7-366 leu1-32 ura4-Δ18 ade6-M210 mca1Δ::KAN | This study |
| JS206  | h"his7-366 leu1-32 ura4-Δ18 ade6-M216 mca1Δ::KAN | This study |
| JS107  | h"his7-366 leu1-32 ura4-Δ18 ade6-M210 mfc1Δ::loxP mca1Δ::loxP mca1Δ::KAN | This study |
| JS207  | h"his7-366 leu1-32 ura4-Δ18 ade6-M216 mfc1Δ::loxP mca1Δ::loxP mca1Δ::KAN | This study |
| JS435/436 | h"his7-366 his7-366 leu1-32/leu1-32 ura4-Δ18/ura4-Δ18 ade6-M210/ade6-M216 | This study |
| JS435/436 | h"his7-366 his7-366 leu1-32/leu1-32 ura4-Δ18/ura4-Δ18 ade6-M210/ade6-M216 mfc1Δ::KAN/mfc1Δ::KAN | This study |
| JS435/436 | h"his7-366 his7-366 leu1-32/leu1-32 ura4-Δ18/ura4-Δ18 ade6-M210/ade6-M216 mfc1Δ::loxP/mfc1Δ::loxP mca1Δ::KAN/mca1Δ::KAN | This study |
| JS435/436 | h"his7-366 his7-366 leu1-32/leu1-32 ura4-Δ18/ura4-Δ18 ade6-M210/ade6-M216 mfc1Δ::loxP/mfc1Δ::loxP mca1Δ::KAN/mca1Δ::KAN | This study |
| JS435/436 | h"his7-366 his7-366 leu1-32/leu1-32 ura4-Δ18/ura4-Δ18 ade6-M210/ade6-M216 mfc1Δ::loxP/mfc1Δ::loxP mca1Δ::KAN/mca1Δ::KAN | This study |
| JS435/436 | h"his7-366 his7-366 leu1-32/leu1-32 ura4-Δ18/ura4-Δ18 ade6-M210/ade6-M216 mfc1Δ::loxP/mfc1Δ::loxP mca1Δ::KAN/mca1Δ::KAN | This study |
| JS435/436 | h"his7-366 his7-366 leu1-32/leu1-32 ura4-Δ18/ura4-Δ18 ade6-M210/ade6-M216 mfc1Δ::loxP/mfc1Δ::loxP mca1Δ::KAN/mca1Δ::KAN | This study |

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were grown on yeast extract plus supplements (YES) containing 225 mg/liter of adenine, histidine, leucine, uracil, and lysine. When plasmid transformation was required, cells were grown in Edinburgh minimal medium (EMM) lacking specific nutrients to select and purify cells expressing the transformed plasmid. The h\(^{-}\)h\(^{-}\) diploid strains used for azygotic meiosis were isolated as follows. Haploid cells of the opposite mating types were conjugated on a solid malt extract (ME) medium, and the resulting zygotes were then returned to rich media (YES) prior to commitment to meiosis. After this step, diploid cells can undergo azygotic meiosis following a synchronized nitrogen-starvation shock. Azygotic meiosis was induced using EMM lacking nitrogen (EMM-N) and supplemented with 10 mg/liter of adenine or 10 mg/liter of adenine, histidine, leucine, uracil, and lysine. Diploid strains homozygous for the mating type (h\(^{-}\)h\(^{-}\)) were generated by protoplast fusion, as described previously (23).

To synchronize pat1-144/pat1-114 diploid cells for their entry into meiosis, cells were precultured in EMM supplemented with adenine (225 mg/liter) at 25°C. Liquid cultures were seeded to an A\(_{600}\) of 0.2 and grown to mid-log phase (A\(_{600}\) of 0.5). The cells were harvested, washed twice, and transferred to EMM-N supplemented with 10 mg/liter of adenine. After incubation for 16 hours at 25°C, NH\(_4\)Cl (0.5 mg/liter) was added and cells were separated into different lots which were treated with ammonium tetrathiomolybdate (TTM), 2,2′-dipyridyl (Dip), NN,N′,N′-tetraakis(2-pyridylmethyl)-1,2-ethanediamine (TPEN), and CuSO\(_4\) or were left untreated. At this point, the temperature was shifted to 34°C to induce meiosis. Meiosis progression was monitored with Hoescht 33342 stain (5 μg/ml) added at various times following meiotic induction.

**Plasmids.** The mfc1\(^+\) promoter containing 800, 600, 200, 109, or 79 bp of the 5′ noncoding region and the first 10 codons of the mfc1\(^+\) gene was isolated by PCR. The first set of primers were designed to generate BamHI and Apal restriction sites at the 5′ termini of the PCR products, whereas the second primer was engineered to generate Bsu36I and EcoRI restriction sites at the 3′ end of the PCR-amplified DNA fragments. Each PCR product was purified, digested with BamHI and EcoRI, and then introduced into the BamHI-EcoRI-digested Yep357R vector (24). The indicated mfc1\(^+\) promoter region was isolated from Yep537Rmfc1\(^{-}\)-800lacZ, Yep537Rmfc1\(^{-}\)-600lacZ, Yep357Rmfc1\(^{-}\)-200lacZ, Yep537Rmfc1\(^{-}\)-109lacZ, and Yep537Rmfc1\(^{-}\)-79lacZ after digestion with Apal and Bsu36I. Each of these promoter regions was then swapped for the equivalent DNA region in fragment in pBPade6str1\(-296lacZ\), creating pBPade6nfc1\(^{-}\)-800lacZ, pBPade6nfc1\(^{-}\)-600lacZ, pBPade6nfc1\(^{-}\)-200lacZ, pBPade6nfc1\(^{-}\)-109lacZ, and pBPade6nfc1\(^{-}\)-79lacZ. To generate the pBPade6str1\(-296lacZ\) vector, the pBPade6 plasmid (17) was digested with Sall, filled in using Klenow polymerase, and digested with PstI. Subsequently, a SpeI (filled in with Klenow)-PstI PCR-amplified DNA segment containing the str1\(^+\) regulatory region (up to −296 from the initiator codon) in addition to the E. coli lacZ gene was isolated from plasmid pSP1str1\(-296lacZ\) (25). This DNA fragment was filled in with Sall (in the presence of Klenow)-PstI-digested pBPade6 plasmid.

Plasmid pBPade6nfc1\(^{-}\)-109lacZ was used to introduce mutations to each or both of the TCCGGG positions (−80 to −85 and positions −99 to −104 with respect to the A of the ATG codon of mfc1\(^+\)). PCR amplification reactions of the mfc1\(^+\) promoter were carried out using primers designed to generate 5′-GATTAT-3′ instead of 5′-TCCGGG-3′ in each or both of the regulatory cis-acting elements. Each PCR product was purified, digested with Apal and Bsu36I, and then used to replace the equivalent wild-type DNA restriction fragment in pBPade6nfc1\(^{-}\)-109lacZ. The resulting plasmids were named pBPade6nfc1\(^{-}\)-109lacZmut1, pBPade6nfc1\(^{-}\)-109lacZmut2, and pBPade6nfc1\(^{-}\)-109lacZmut1-2. To generate wild-type and mutant pCF83mfc1\(^{-}\)-125I-65lacZ fusion plasmids, a series of purified oligonucleotides (with upper and lower strands that are complementary to each other) were annealed pairwise to form wild-type (TCCGGG elements) and mutant (GATTAT elements) double-stranded DNA matrices. Once annealed, each double-stranded DNA oligomer derived from the mfc1\(^−\) promoter (position −125 to −65) was ligated at the Xmal and Xhol sites of CYC1-lacZ fusion plasmid pCF83 (19).

PCR amplification of the mca1\(^+\) gene was carried out with primers designed to generate Xmal and SacII restriction sites at upstream and downstream termini of the open reading frame, respectively. The full-length gene was isolated from S. pombe strain FY435 genomic DNA. The PCR product was digested with Xmal and SacII and cloned into the corresponding sites of pBPade6 plasmid, creating plasmid pBPade6mca1\(^+\). Subsequently, the mca1\(^+\) promoter region from position −500 upstream of the start codon of the mca1\(^+\) gene was isolated by PCR amplification and was then inserted into pBPade6mca1\(^+\) at the Apal and Xmal sites. This pBPade6mca1\(^+\) derivative was named pBPade6prom-mca1\(^+\). The Cherry coding sequence derived from pBM46STT1mCherry (26) was isolated by PCR using primers designed to generate SacII and SacI sites at the 5′ and 3′ termini of the Cherry gene. The resulting DNA fragment was used to clone the Cherry gene into pBPade6prom-mca1\(^+\) plasmid to which SacII and SacI restriction sites had previously been introduced by PCR and placed immediately before the mca1\(^+\) stop codon. For this particular construct, named pBPade6prom-mca1\(^−\)-Cherry, the SacI-Sacl Cherry-encoding fragment was inserted in frame with the C-terminal region of Mca1. An identical strategy was used to clone the TAP coding sequence into pBPade6prom-mca1\(^+\) plasmid to generate pBPade6prom-mca1\(^−\)-TAP.

**RNA analysis.** Total RNA was extracted using the hot phenol method as described previously (27). RNA samples were quantified spectrophotometrically, and 15 μg of RNA per sample was used for RNA protection assays, which were performed as described previously (28). Plasmid pSKmca1\(^+\) was constructed by inserting a 214-bp BamHI-EcoRI fragment from the mca1\(^+\) gene into the same sites of pBluescript SK (Stratagene, La Jolla, CA). The antisense RNA hybridizes to the region between +1001 and +1215, downstream of the initiator codon of mca1\(^+\). Riboprobes derived from plasmids pSKmfc1\(^+\) (3) and pKSlaclz (29) were used to detect mfc1\(^+\) and laclz transcripts, respectively. The act1\(^+\) riboprobe (30) was used to detect act1\(^−\) mRNA as an internal control for normalization during quantification of RNase protection products.

**Fluorescence microscopy.** h\(^−\) mca1\(^Δ\) and h\(^−\) mca1\(^Δ\) haploid cells expressing the mca1\(^−\)-Cherry allele were grown under conditions of low nitrogen and then crossed in order to produce diploid zygotes. After mating, the cells were quickly transferred to rich YES medium to stabilize their diploid state. The azygotic meiosis of diploid cells was synchronously induced by transferring the cells to nitrogen-poor EMM, as described previously (21). After the cells had just entered meiosis, culture aliquots were sampled every hour and Hoescht 33342 stain (5 μg/ml) was added to analyze the progression of meiosis of individual cells. At the indicated meiotic phase, the cells were analyzed by microscopy using ×1,000 magnification with the following filters: 510 to 560 nm (Cherry) and 340 to 380 nm (Hoescht 33342 stain). Fluorescence and differential interference contrast images (Nomarski) were obtained using a Nikon Eclipse E800 epifluorescence microscope (Nikon, Melville, NY) equipped with a Hamamatsu ORCA-ER digital cooled camera (Hamamatsu, Bridgewater, NJ). Fields of cells shown in this study correspond to a minimum of five independent experiments. Merged images were obtained using Simple PCI software version 5.3.0.1102 (Compix, Sewickly, PA).

**Protein extraction and Western blot analysis.** Whole-cell extracts were prepared using a trichloroacetic acid extraction method (31). Equal amounts of proteins of each sample were subjected to electrophoresis on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels. Proteins were then electrophoretically onto nitrocellulose Hybond-ECL membranes (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom). Immunoblots were analyzed for the steady-state levels of Mca1-TAP and the α-tubulin protein using a polyclonal anti-mouse IgG antibody (ICN Biomedicals, Aurora, OH) and a monoclonal anti-α-tubulin antibody (clone B-5-1-2; Sigma-Aldrich Canada, Oakville, Ontario, Canada), respectively. After 1 h of incubation with the primary antibodies in 1% powdered skimmed milk–PBST (10.1 mM Na\(_2\)HPO\(_4\), 1.8 mM KH\(_2\)PO\(_4\), 138 mM NaCl, 8.1 mM KCl, 0.5% Triton X-100, 0.1% sodium azide), the membranes were washed in PBST and incubated with horseradish peroxidase-conjugated antibody (Molecular Probes, Eugene, OR) for 1 hour. Peroxidase activity was visualized using an enhanced chemiluminescence color development reagent (ECL, Amersham, Arlington Heights, IL). Signals were detected under long-wave UV light (302 nm) using a Molecular Dynamics Storm phosphorimager (Molecular Dynamics, Sunnyvale, CA).
NaCl, 2.7 mM KCl, and 0.1% Tween 20, pH 7.4), the membranes were washed three times with PBST, incubated with the appropriate horseradish peroxidase-conjugated anti-mouse secondary antibodies (GE Healthcare), and visualized by chemiluminescence detection on X-ray films.

Expression of the MBP-Mca1 fusion protein. The DNA containing mca1 codons 1 to 150 was amplified by PCR, purified, and inserted in frame in pMAL-c2X vector (New England Biolabs, Ipswich, MA) at EcoRI and SalI restriction sites. Plasmid pMAL-1mca1 was transformed in E. coli BL21(DE3). Fresh transformants of BL21 cells containing plasmid pMAL-c2X or pMAL-1mca1 were grown to an A600 of 0.5. At this growth phase, the cells were induced with 0.4 mM isopropyl-β-D-thiogalactopyranoside for 4 h at 18°C in the presence of 2% ethanol. Harvested cells were washed once with ice-cold water and resuspended in G200 buffer (20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, 10% glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 5 μM ZnCl2, pH 7.5) and a cocktail of protease inhibitors (P8340; Sigma-Aldrich). The mixture was incubated for 20 min at 4°C. Cell lysis was achieved using a FastPrep-24 instrument (MP Biomedicals, Solon, OH) for 45 s at the maximum speed setting. Insoluble material was removed by centrifugation (15,000 rpm, 1 ml column of amylase resin (New England Biolabs) that had been equilibrated with G200 buffer. Beads were washed with 10 ml of buffer G200 and then were eluted stepwise with G200 buffer containing 10 and 20 mM maltose. SDS-polyacrylamide gel electrophoresis analysis showed that the affinity-purified maltose-binding protein (MBP)-Mca1 fusion protein was recovered predominantly in the 10 mM maltose eluate fractions.

Electrophoretic mobility shift assays. Two pairs of HPLC-purified oligodeoxyoctanucleotides, one pair consisting of mca1wt-upper (5’-CCGGGGGGCCCCCTGTTTAACTCGATCGCTTATCGCGAGGAAGTTTCCAT ACGCGGAAGCAATTTGCAAC-3’) and mca1wt-lower (5’-TCGA GTTGCAATACCTGCTCGGCTTATGAACGTTCCTCGGCGATA AGCTGAGCAATTCACGAGGGCCCC-3’) and the other of mca1mut-upper (5’-CCGGGGGGCCCCCTGTTTAACTCGATCGCTTATCGCGGAAGCAATTTGCAAC-3’) and mca1mut-lower (5’-TCGA GTTGCAATACCTGCTCGGCTTATGAACGTTCCTCGGCGATA AGCTGAGCAATTCACGAGGGCCCC-3’) (underlined letters represent nucleotide substitutions that gave rise to mutations), were annealed to form double-stranded DNA. Once annealed, each pair of oligomers had 5’-CCGG and 5’-TCGA overhang ends, allowing their labeling with [α-32P]dCTP (PerkinElmer, Waltham, MA) (6,000 Ci/mmol) and the Klenow fragment. When indicated, unlabeled oligomer competitors at the concentrations specified (see Fig. 10) were added together with the radiolabeled probe. For supershifted reactions, 2 μg of anti-MBP antibody was added with the probe in the binding assays. Typically, binding reactions were carried out using binding buffer that contained 12.5 mM HEPES (pH 7.9), 75 mM NaCl, 4 mM MgCl2, 10% glycerol, 4 mM Tris-HCl (pH 7.9), 0.6 mM dithiothreitol, 1 μg of poly(dI-dC)2, 5 μM ZnCl2, and 1.0 ng of radiolabeled probe in a final reaction volume of 15 μl. Reaction mixtures were incubated at 25°C for 30 min, and protein-DNA complexes were resolved by gel electrophoresis (3 h at 40 V) on 4% polyacrylamide gels (acrylamide/bisacrylamide ratio, 37.5:1) in 0.25× Tris-borate at 4°C. Gels were then fixed, dried, and subjected to PhosphorImager analysis.

RESULTS

mfc1 mRNA levels are induced in response to low concentrations of copper but not regulated by iron or zinc deprivation. Our previous studies identified a novel meiotic copper transporter that we named Mfc1 (3). As previously observed and as shown in Fig. 1, when diploid strain pat1-114/pat1-114 mfc1+/+ was synchronized through meiosis in the presence of the copper chelator TTM (150 μM), mfc1 mRNA levels were induced ~4-fold compared to basal levels observed in untreated cells after 3 h of meiotic induction (Fig. 1). Results showed that, under low-copper conditions, the transcript levels of mfc1 remained upregulated (~4 to 12-fold) throughout the remainder of the meiotic program, being detected even after 9 h of meiotic induction (Fig. 1). To further examine whether mfc1 transcription was induced by the presence of other metal ion chelators, synchronized pat1-114/pat1-114 mfc1+/+ cells were incubated in the presence of the iron chelator Dip (150 μM) or the zinc chelator TPEN (150 μM). Results showed no induction of transcription of mfc1 mRNA in response to these chelators (Fig. 1 and data not shown). We therefore concluded that induction of mfc1 is specific to copper deprivation and not to regulation by iron or zinc deficiency.

Analysis of mfc1 promoter sequences required to induce gene expression under copper-limiting conditions. mfc1 is induced at the transcriptional level in response to copper starvation in a manner similar to that seen with the ctr4+ and ctr5+ genes that encode the high-affinity copper heteromeric transport complex located at the plasma membrane. In contrast to ctr4+ and ctr5+, for which the transcription factor Cuf1 is required for their induction under copper-limiting conditions, the inactivation of the cuf1+ gene does not affect the transcription of mfc1+ (3). This result prompted us to search for the presence of cis-acting elements within the promoter region of mfc1 that were required for its induction in response to copper starvation. A series of mfc1 promoter fragments were fused upstream of and in frame to the lacZ gene in a pBPade6lacZ derivative vector, creating pBPade6mfc1-.
Gene expression of these plasmids was analyzed by RNase protection experiments. Results showed that removal of DNA sequences between -600 and -109 of the mfc1 promoter had no significant effect on the copper starvation-dependent activation of mfc1-lacZ fusions (Fig. 2). In the presence of TTM, plasmids pBPade6mfc1 -600lacZ, pBPade6mfc1 -200lacZ, and pBPade6mfc1 -109lacZ, and pBPade6mfc1 -79lacZ. Gene expression of these plasmids was analyzed by RNase protection experiments. Results showed that removal of DNA sequences between -600 and -109 of the mfc1 promoter had no significant effect on the copper starvation-dependent activation of mfc1-lacZ fusions (Fig. 2). In the presence of TTM, plasmids pBPade6mfc1 -600lacZ, pBPade6mfc1 -200lacZ, and pBPade6mfc1 -109lacZ were still induced ~27-, 29-, and 25-fold, respectively, compared to their levels of expression in untreated (basal) or copper-treated cells (7-h time point). In the case of all three of these mfc1-lacZ promoter derivatives, basal and elevated copper concentrations resulted in a loss of mfc1-lacZ mRNA expression. When the mfc1 promoter was deleted to position -79, TTM-dependent expression was completely abolished, lowering its expression to a minimal threshold that was similar to that observed in the case of basal and copper-replete conditions (Fig. 2).

The finding that the presence of the promoter region between -109 and -79 was sufficient to drive copper deprivation-dependent expression of the mfc1-lacZ fusion gene prompted us to examine whether a mfc1 promoter segment that includes this region could contain a cis-acting element responsible for TTM-dependent expression.

**FIG 2** Mapping of mfc1 promoter sequences that are required to activate gene expression under conditions of copper starvation. A schematic representation of nested 5' deletions of mfc1 promoter sequences is shown (left side). Nucleotide numbers refer to positions relative to the initiator codon of the mfc1 gene. The black boxes indicate the location of the TCGGCG sequences within the mfc1 promoter. Cultures of *pat1-114* cells were maintained in vegetative growth at 25°C or were induced to initiate and proceed through meiosis at 34°C (right side). *pat1-114* cells were left untreated (basal) or incubated in the presence of TTM (150 μM) or CuSO4 (50 μM). Total RNA was isolated from transformants containing the indicated mfc1-lacZ promoter derivatives. Steady-state mRNA levels of lacZ and act1 were analyzed by RNase protection experiments at the indicated time points after induction of meiosis. Data are representative of the results of three independent experiments.
mediated responsiveness. Interestingly, the mfc1+ promoter region between −109 and −79 contains two copies of the sequence TCGGCG. These repeats are separated by a sequence of 13 bp and are oriented in the opposite direction with respect to the A of the ATG codon of the mfc1+ gene. CGG triplets are found within these sequences, and these triplets are quite often bound by transcription factors of the family of zinc binuclear cluster proteins (32). To determine whether these TCGGCG repeats played a role in mfc1+ gene induction in response to low copper availability, we inserted multiple point mutations in each or both of the TCGGCG elements to mimic changes known to abolish binding of the zinc cluster transcription factors to CGG triplet sequences. Mutation of the base pairs within the −99TCGGCG−104 element (GATTAT instead of TCGGCG; denoted Mut1) abolished copper starvation-dependent induction of the mfc1+−109lacZ fusion (Fig. 3). When the second TCGGCG element, −80TCGGCG−85, was mutated (GATTAT instead of TCGGCG; denoted Mut2), lacZ transcript levels were very low in response to copper-limiting conditions; the overall magnitude of the response was decreased by ∼95% (5- and 7-h time points) compared to cells containing wild-type reporter plasmid. When both TCGGCG elements were mutated, there was a complete lack of TTM responsiveness of the reporter gene (Fig. 3).

Based on the findings that the integrity of the TCGGCG elements located between positions −99 and −104 as well as −80 and −85 was essential to trigger TTM-dependent induction of the mfc1+−lacZ fusion, we examined whether these two elements could regulate a heterologous reporter gene in a TTM-dependent
A short DNA fragment derived from the mfc1/H11001 promoter (positions 65 to 125) was inserted in its natural orientation upstream of the minimal promoter of the CYC1 gene fused to lacZ in pCF83 (19). The fact that the upstream region of lacZ in pCF83 contains the CYC1 minimal promoter may explain the very weak levels of lacZ transcript that were detected in cells transformed with the plasmid alone (data not shown). However, the very weak level of lacZ mRNA from the plasmid alone was mostly observed during the early and middle phases of meiosis (after 1 to 5 ho f meiotic induction) (data not shown). When a wild-type mfc1/H11001/H11002- CYC1- lacZ fusion reporter was expressed in pat1-114/pat1-114 cells undergoing meiosis in the presence of the copper chelator TTM (150 M), lacZ mRNA expression was induced 17-fold compared to transcript levels detected in control (basal) or copper-exposed cells (Fig. 4). When the first 99TCGGCG element was mutated and the second one (80TCGGCG) left unchanged, the steady-state levels of lacZ mRNA were decreased by 92% under copper-starved (TTM) conditions compared to transcript levels of wild-type mfc1/H11001/H11002- CYC1- lacZ under the same experimental conditions (3-h time point). Furthermore, the very low levels of lacZ transcript showed an expression profile similar to that observed in the case of cells transformed with plasmid alone (data not shown). When the first 99TCGGCG element was left unaltered and the second one...
induced to undergo synchronous meiosis at 34°C under basal, copper-replete, and copper-depleted conditions. At the indicated time points, mfc1G-3 response to copper starvation.

copper limitation-dependent induction of expression of GGCG elements were mutated, a lack of TTM response of the GGCG elements in the results were consistent with the interpretation that both TC levels under basal or copper-replete conditions. Taken together, was a lack of significant down- or upregulation of 582 results of three independent experiments. Beaudoin et al.

existence of 31 genes that encode known or putative members of the Cys(6)Zn(2) binuclear cluster protein family. Among them, 30 functional domains of Mca1. The Mca1 DNA-binding domain contains one Zn(2)Cys(6) binuclear cluster motif (amino acids 24 to 51) that is followed by a linker region (first amino acid residue of the protein. Consensus amino acid sequences that represent a Zn(2)Cys(6)-type finger and a heptad repeat of leucine residues are shown.

overall majority of acidic amino acid residues. This region is predicted to act as an activation domain. Amino acid numbers refer to the position relative to the C-terminal side of the DNA-binding domain, and it is termed the middle homology region (MHR). The C-terminal 44 amino acids of Mca1 comprise an (B) pat1-114/mca1-125, CYC1-lacZ gene is required for maximal induction of the mca1-125/mca1-125 fusion and its mutant derivatives, there insertionally inactivated SPAPB1A11.04c, SPCC777.02, SPAPB24D3.01, and SPAC11D3.07c) containing coding sequences rich in Cys, Met, and His residues. Because several Cys, Met, and His residues were scattered throughout these proteins and may represent potential metal-binding ligands, we further investigated a potential role for SPAPB1A11.04c, SPCC777.02, SPAPB24D3.01, and SPAC11D3.07c in the regulation of Mfc1 expression. Using isogenic strains harboring insertionally inactivated SPAPB1A11.04cΔ, SPCC777.02Δ, SPAPB24D3.01Δ, and SPAC11D3.07cΔ genes, we assessed the potential role of the predicted SPAPB1A11.04cΔ, SPCC777.02Δ, SPAPB24D3.01Δ, and SPAC11D3.07cΔ-encoded zinc binuclear cluster transcription factors in the regulation of mfc1+. Among the four different disruption genes tested, only SPAPB1A11.04c altered the activation of Mfc1 expression. The SPAPB1A11.04c gene was found to be expressed on the same chromosome as the mfc1+ gene and was located 7,038 bp downstream of mfc1+. As is the case with most of the zinc binuclear cluster transcription factors, SPAPB1A11.04c protein contains a DNA-binding domain, a middle homology regulatory region, and a transactivation domain (Fig. 5A). When pat1-114/pat1-114 SPAPB1A11.04cΔΔ and pat1-114/pat1-114 SPAPB1A11.04cΔ/Δ diploid strains were presynchronized in the G1 phase by nitrogen starvation and then incubated at 34°C to inactivate Pat1 kinase, cells initiated and

mutated, TTM-dependent induction of lacZ mRNA was compromised in a manner similar to that observed in the −65mfc1mut1−125, CYC1-lacZ mutant (Fig. 4). When both TC GGGC elements were mutated, a lack of TTM response of the reporter gene was observed (Fig. 4). In the case of the wild-type −65mfc1−125, CYC1-lacZ fusion and its mutant derivatives, there was a lack of significant down- or upregulation of lacZ mRNA levels under basal or copper-replete conditions. Taken together, the results were consistent with the interpretation that both TC GGGC elements in the mfc1+ promoter are required to confer copper limitation-dependent induction of expression of mfc1+.

Mca1 plays a major role in activation of mfc1+ expression in response to copper starvation. We next sought to identify a trans-acting factor that recognized the cis-acting element 5′-TCGGC G-3′ DNA binding motif required for appropriate induction of mfc1+ gene expression under low-copper conditions. Analysis of genomic DNA sequences of the S. pombe database revealed the existence of 31 genes that encode known or putative members of the Cys5(6)/Zn12(2) binuclear cluster protein family. Among them, 30 of these genes are expressed during the entire course of meiosis or at a precise step during meiotic development. Transcriptional profiles of these 30 genes revealed that 26 genes are expressed during the middle meiotic phase, which corresponds to the meiotic period where Mfc1 is expressed as a function of copper availability. Of interest, among the 26 genes that were expressed during the middle meiotic phase where Mfc1 is expressed as a function of copper availability, we identified 4 genes (SPAPB1A11.04c, SPCC777.02, SPAPB24D3.01, and SPAC11D3.07c) containing

mfc1+/mca1+ mca1+ mca1Δ/mca1Δ

25°C 34°C 25°C 34°C
basal TTM basal CuSO4 TTM basal CuSO4
0 1 3 5 7 9 1 3 5 7 9 1 3 5 7 9 1 3 5 7 9 1 3 5 7 9 1 3 5 7 9 1 3 5 7 9 1 3 5 7 9 1 3 5 7 9 1 3 5 7 9 1 3 5 7 9

FIG 5 The mca1+ gene is required for maximal induction of the mfc1+ gene in response to copper starvation. (A) Schematic representation that depicts putative functional domains of Mca1. The Mca1 DNA-binding domain contains one Zn5Cys6, binuclear cluster motif (amino acids 24 to 51) that is followed by a linker region (amino acids 52 to 116) and one heptad repeat of leucine residues (amino acids 117 to 138). A regulatory domain (amino acids 336 to 412) is located on the C-terminal side of the DNA-binding domain, and it is termed the middle homology region (MHR). The C-terminal 44 amino acids of Mca1 comprise an overall majority of acidic amino acid residues. This region is predicted to act as an activation domain. Amino acid numbers refer to the position relative to the first amino acid residue of the protein. Consensus amino acid sequences that represent a Zn5Cys6-type finger and a heptad repeat of leucine residues are shown. (B) pat1-114/pat1-114 (mca1+/mca1+) and pat1-114/pat1-114 mca1Δ/mca1Δ strains were presynchronized by nitrogen starvation at 25°C (t = 0, basal) and then induced to undergo synchronous meiosis at 34°C under basal, copper-replete, and copper-depleted conditions. At the indicated time points, mfc1+ and act1+ (internal control) mRNA levels were analyzed in the control strain (mca1+/mca1+) and an isogenic strain lacking the mca1+ alleles. Data are representative of the results of three independent experiments.
proceeded through a synchronous meiosis. In the presence of TTM, inactivation of SPAPB1A11.04c resulted in strongly (~8-fold) reduced mfc1 mRNA levels in comparison to those seen with wild-type cells grown under the same conditions (Fig. 5B). Under basal and copper-replete conditions, transcript levels of mfc1 remained very low (background threshold) in wild-type and SPAPB1A11.04cΔ/Δ strains. We named the SPAPB1A11.04c gene product Mca1 (meiosis copper starvation-dependent activator) based on the fact that its presence was required to fully activate mfc1 gene expression under conditions of copper starvation.

To provide additional data to support the notion of an important role of Mca1 in mfc1 gene activation, we used the mfc1 promoter region between positions −109 and −1, which was sufficient to drive TTM-dependent induction of the mfc1-lacZ gene (Fig. 3). In the case of pat1-114/pat1-114 mca1−/− diploid cells synchronously induced into meiosis, mfc1−/−109lacZ promoter fusion strongly upregulated lacZ mRNA expression under conditions of copper deficiency (Fig. 6). In contrast, pat1-114/pat1-114 mca1Δ/Δ mutant cells containing the mfc1−/−109lacZ promoter fusion plasmid showed poor induction of lacZ mRNA which was decreased by ~85% compared to the level seen with wild-type strain (mca1+/+) under conditions of low levels of copper (Fig. 6). Furthermore, results showed that the integrity of the TCGGCG sequence was essential because a TCGGCG box mutant (with a mutation in each or both of the TCGGCG elements) abrogated TTM-dependent induction of the mfc1−/−109lacZ reporter gene (Fig. 6). Taken together, these results indicate that, under conditions of low concentrations of copper, the TCGGCG promoter sequence and the Mca1 zinc binuclear cluster protein play critical roles in the activation of mfc1 gene expression.

Mca1 is constitutively expressed during mitosis and meiosis. We next analyzed steady-state mRNA levels of mca1+ as a function of copper availability during mitosis and meiosis. Experiments using cells proliferating in mitosis showed that mca1+ steady-state mRNA levels were constitutive and unresponsive to cellular copper status (Fig. 7A). In contrast, ctr4+ copper transporter mRNA levels (assayed as a control) were induced or repressed, depending on the presence of TTM or copper, with respect to basal conditions (Fig. 7A). To further investigate the expression profile of mca1+ during meiosis, a pat1-114/pat1-114 diploid strain was synchronized to initiate and proceed through the meiotic program. Immediately prior their entry into the meiotic program, the cells were either left untreated or exposed to TTM (150 μM) or CuSO4 (50 μM). Aliquots of cultures were retrieved at distinct time intervals following meiotic induction, and steady-state levels of mca1+ mRNA were analyzed by RNase protection assays. Results showed that mca1+ transcripts were detected under basal (untreated), copper-starved, or copper-replete conditions (Fig. 7B). Interestingly, in the presence of TTM, mca1+ mRNA levels were slightly increased after 5 and 7 h of meiotic induction (~1.5-fold compared to levels observed after 3 h of

FIG 6 mfc1+ promoter TCGGCG elements are required for copper limitation-dependent induction of the −109mfc1−/−lacZ fusion gene that primarily depends on Mca1. Cultures of pat1-114/pat1-114 (mca1+/mca1−) and pat1-114/pat1-114 mca1Δ/mca1Δ diploid cells were synchronously induced into meiosis under copper-starved conditions (150 μM TTM). Total RNA was isolated from culture aliquots taken at the indicated time points. Following RNA preparation, lacZ steady-state mRNA levels were analyzed by RNase protection assays using actin (act1+) as an internal control. For each group of reactions, a schematic representation of a 109-bp mfc1+ promoter DNA fragment and its mutant derivatives is depicted. Data are representative of the results of three independent experiments.
meiotic induction). Similarly, a slight increase of mca1+ expression was observed in untreated cells after 5 h of meiotic induction (~1.4-fold compared with levels observed after 1 and 3 h of meiotic induction) (Fig. 7B). To ascertain whether the steady-state levels of Mca1 remained slightly higher 7 h and 9 h after meiotic induction (Fig. 7C). To ensure that cell incubation conditions (TTM, basal, or CuSO4) had no major negative effect on meiotic progression and sporulation, a series of microscopic analyses were performed. pat1-114/pat1-114 mca1Δ/mca1Δ diploid cells were synchronously induced into meiosis, and Hoescht 33342 stain (0.5 µg/µl) was added every hour to cell culture aliquots to visualize DNA and to monitor meiotic progression. Under basal conditions, meiosis I occurred primarily between 3 h and

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**FIG 7** Assessment of the Mca1 mRNA and protein steady-state levels during meiosis. (A) Representative expression profile of mca1+ transcripts in cells that were left untreated (basal) or treated with TTM (150 µM) or CuSO4 (50 µM) during mitosis (left panel). Under the same conditions, ctr4+ mRNA steady-state levels were monitored as a control transcript known to be induced under conditions of copper starvation (right panel). (B) Cultures of pat1-114/pat1-114 diploid cells were maintained in vegetative growth at 25°C or induced to initiate and proceed through meiosis at 34°C. Cells were either left untreated (basal) or incubated in the presence of TTM (150 µM) or CuSO4 (50 µM). Total RNA was isolated at the indicated time points after induction of meiosis. Results of representative RNase protection assays of mca1+ and act1+ (internal control) mRNA steady-state levels during meiosis are shown. (C) pat1-114/pat1-114 mca1Δ/mca1Δ diploid cells expressing Mca1-TAP were synchronously induced into meiosis under basal conditions or in the presence of TTM (150 µM) or CuSO4 (50 µM). Western blots of Mca1-TAP and α-tubulin (control loading) levels at different time points after meiotic induction are shown. (D) Meiotic progression of cells under basal conditions or incubated in the presence of TTM (150 µM) or CuSO4 (50 µM). The values shown for each condition (TTM, basal, or CuSO4) correspond to the percentage of cells with 1 nucleus, 2 nuclei, or 3 or 4 nuclei and the percentage of asci. Each determination represents the averages of the results of triplicates ± the standard deviations.
6 h after meiotic induction, meiosis II between 5 h and 8 h, and sporulation after 8 to 10 h (Fig. 7D). Although meiotic progression under conditions of copper starvation (TTM, 150 μM) was reduced by approximately 2 h compared to untreated (basal) cell results, spore formation was clearly observed at the end of meiosis (Fig. 7D). Similarly, although copper-replete (CuSO₄, 50 μM) zygotes exhibited a meiotic progression that was prolonged by 1 h compared to control cells, the resulting meiotic products were tetranucleated asci (Fig. 7D). Taken together, these results indicate that, under basal, copper-depleted, and copper-replete conditions, the mca1 gene is constantly expressed during meiosis, although the process of meiotic maturation varies slightly as a function of time.

Subcellular localization of Mca1 during meiosis. We next determined the subcellular location of Mca1 during the meiotic program. The Cherry fluorescence-coding sequence was fused in frame with the 3′-terminal end of the mca1 gene. When the Mca1-Cherry fusion protein was expressed in meiosis, it triggered the induction of mfc1 mRNA to levels similar to that of wild-type Mca1 (untagged) or TAP epitope-tagged Mca1 (data not shown). A functional mca1-Cherry allele was integrated into h mca1Δ and h mca1Δ cells to visualize the Mca1-Cherry in living zygotes and asci. Following mating, h/h mca1Δmcma1Δ-Cherry/mcma1Δ-Cherry diploid cells were cultured under conditions chosen to induce them to undergo azygotic synchronous meiosis. Following induction of meiosis under basal or low-copper conditions (TTM, 50 μM), fluorescent Mca1-Cherry was readily detected (at the 0-h time point) in the nucleus of vegetative azygotic meiotic cells (Fig. 7D). Taken together, these results indicate that, under basal, copper-depleted, and copper-replete conditions, the mca1 gene is constantly expressed during meiosis, although the process of meiotic maturation varies slightly as a function of time.

![FIG 8 Analysis of Mca1-Cherry localization during meiosis and sporulation. Mca1-Cherry fluorescence signal was observed at every stage of meiosis following azygotic meiotic induction of a h/h mca1Δmcma1Δ mca1Δ-Cherry/mcma1Δ-Cherry strain. Once induced, azygotic meiotic cells were differentiated in the presence of TTM (50 μM). At each indicated stage of meiosis, Mca1-Cherry fusion protein generated a fluorescent signal (center left) that colocalized with chromosomal material. Cells at different stages of meiosis were stained (Hoechst 33342) to visualize the DNA (center right). The merged images are shown in the far right panels. Nomarski optics were used to monitor cell morphology (far left). Data are representative of the results of five independent experiments.](attachment:fig8.png)
undergone the first meiotic division (Fig. 8). At early and late anaphase II (6- and 7-h time points), meiotic cells displayed Mca1-Cherry fluorescence as two pairs of spots per cell and this result was interpreted to correspond to chromosomal material that had undergone the second meiotic division, in which case sister chromatids segregate (Fig. 8). Cells displayed Mca1-Cherry fluorescence as four distinct spots in the zygotie during forespore membrane formation and sporulation (Fig. 8). Collectively, data from microscopic analysis of meiotic cells reveal that a functional Mca1-Cherry protein colocalizes with the chromosomes that had undergone every step of the process of meiosis. These observations add further support for the notion of a regulatory role of Mca1 at the DNA level.

**Inactivation of the mca1** gene alters the process of meiosis under copper-limited conditions. In addition to the Mca1 requirement for expression of the mfc1** gene under conditions of copper deficiency, we hypothesized that Mca1 was also important for normal progression of meiosis under copper-starved conditions. To test this hypothesis, h−/− mca1Δ/mca1Δ diploid cells were used and results compared to h+/− mca1+/+mca1+ control cell results. Diploid strains were synchronously induced by transferring the cells at the same time to nitrogen-poor medium, thus allowing cells to undergo asyngotic meiosis. Following induction of meiosis (zero time point), cells were left untreated or were treated with TTM (50 μM). In the case of wild-type cells (mca1+/+mca1+), meiosis I occurred mainly between 5 h and 7 h, meiosis II between 8 h and 10 h, and spore formation after 11 h of meiotic induction under basal (untreated) conditions (Fig. 9A). Furthermore, in the case of control cells, where meiosis was induced in the presence of TTM (50 μM), the first meiotic division was postponed by ~2 h (Fig. 9B). Despite this delay, control cells proceeded through meiosis and formed asci containing four spores after 12 h of meiotic induction. In the case of mca1Δ/mca1Δ mutant cells, where asyngotic meiosis was induced under basal conditions, the first meiotic division was delayed by ~3 h compared to the results seen with control cells (Fig. 9A). Despite this delay, mca1Δ/mca1Δ cells underwent meiosis II (12-h time point), spore maturation, and formation (14-h time point) to produce 4-spore asci similarly to the wild-type strain (Fig. 9A). In contrast, TTM (50 μM)-treated mutant cells lacking Mca1 (mca1Δ/mca1Δ) proceeded through metaphase I but then stopped their progression, exhibiting meiotic arrest (Fig. 9B). Taken together, the data suggest that, under basal conditions, Mca1 plays an important role for normal meiotic progression based on the fact that cells carrying inactivated mca1Δ/Δ alleles display delayed and prolonged meiosis. Under conditions of copper deprivation, the presence of Mca1 becomes even more critical since its absence results in arrested meiosis at metaphase I.

**Mca1 interacts with two mfc1** promoter elements containing CGG triplets. The bulk of the results were consistent with the notion that the mca1** gene was required for maximal activation of mfc1** gene expression under conditions of copper starvation. We thus investigated the possibility that Mca1 interacted directly with the sequences −99–TCGGCG−104 and −80–TCGGCG−85 located in the promoter region of mfc1** (Fig. 10A). To test this hypothesis, we made a construct where the N-terminal 150 amino acids of Mca1 were fused to MBP and expressed the plasmid in E. coli. The purified recombinant Mca1 fusion protein was used for binding studies. Results showed that a wild-type 32P-end-labeled 73-bp mfc1** promoter fragment, which contained the TCGGGCG sequences, formed a DNA-protein complex in the presence of Mca1 (Fig. 10B). The presence of Mca1 in the complex was assessed in supershift experiments using an anti-MBP antibody. Results showed the formation of a complex of slightly lower electrophoretic mobility, consistent with the interpretation of the presence of a DNA-MBP−1-Mca1−150 complex (Fig. 10B). The specificity of the DNA-protein complex was confirmed by competition assays using unlabeled oligomers containing either wild-type TCGGGCG elements or mutated elements (GATTAT instead of TCGGGCG) (Fig. 10B). Formation of the DNA-protein complexes was inhibited by incubation with excess wild-type oligomer but not by mutant competitor (Fig. 10B). These results indicate that the N-terminal 150 amino acids of Mca1 associate with TCGGGCG promoter elements of the mfc1** gene.

**DISCUSSION**

A finely tuned regulation of copper uptake is required to maintain copper homeostasis in S. pombe. On the one hand, it provides copper-dependent protein activity with sufficient amounts of copper and, on the other hand, it protects the cells against the toxic effects of copper overload. Efficient copper transport in S. pombe cells that grow mitotically requires the copper ion to be in the Cu2+ state of oxidation to be transported by a hetero-protein complex of Ctr4 and Ctr5 proteins (16, 18, 35). Transcriptional regulation of the ctr4+ and ctr5+ genes is under the control of the copper-sensing transcription factor Cuf1 which induces their expression under copper-deficient conditions (36, 37). Under high-copper conditions, Cuf1 becomes inactive and is subsequently exported outside the nucleus (38, 39).

There are relatively few data concerning the intrinsic role of copper during meiosis. It has recently been reported that S. pombe undergoes meiotic arrest at metaphase I under strong copper starvation conditions, suggesting a critical role for copper in meiotic maturation and progression (3). During early meiosis, copper uptake is most likely ensured by the heteromeric Ctr4-Ctr5 protein complex, since Ctr4 localizes to the cell surface of developing zygotie cells (3). Once middle-phase meiosis has been initiated, Ctr4 expression is abolished and Mfc1, a meiosis-specific major facilitator superfamily (MFS)-type transporter, is induced. Mfc1 is first detected in precursor vesicles and then at the forespore membrane of ascospores (3). In late-phase meiosis, Mfc1 is found at the forespore membrane, and the use of the coppersensor-1 tracker suggests that it transports copper into the forespore (3, 40). In a manner similar to that seen with the ctr4+ and ctr5+ genes, mfc1+ is induced at the level of gene transcription in response to copper starvation. However, in contrast to ctr4+ and ctr5+, for which the transcription factor Cuf1 is required for their induction under copper-limiting conditions, the inactivation of the cuf1+ gene does not affect the transcriptional activation of mfc1+ (3). This observation prompted us to look for a transcription factor that was required for copper starvation-dependent induction of mfc1+ during meiosis. One potential candidate was the meiosis-specific Cu2+ transcription factor, which exhibits high homology of its N terminus with the N terminus of Cuf1 (21). Total RNA isolated from an isogenic pair of strains, one carrying an inactivated allele of cuf2+ (cuf2Δ) and one containing a wild-type copy of the gene (cuf2+), showed no effect on mfc1+ transcript levels when the availability of copper was limited. mfc1+ was strongly induced in response to copper deficiency in both strains (data not shown).

Functional dissection of the mfc1** promoter revealed the presence of two copies of a consensus TCGGGCG element required for
Fig 9 The mca1Δ/mca1Δ mutant displays abnormal meiotic progression and defective meiotic differentiation under low-copper conditions. (A) Wild-type diploid (h+/h+ mca1+/mca1+) and h+/h+ mca1Δ/mca1Δ mutant cells expressing Sad1-Cherry were synchronously induced to undergo azygotic meiosis under basal conditions. For each strain, samples were taken every 60 min, and percentages of cells with 1 nucleus, 2 nuclei, or 3 or 4 nuclei as well as percentages of asci were calculated (graphics on left). Results are reported as the means of the results of three replicates ± standard deviations. Although the entire meiotic program occurred in both strains, meiotic maturation of a strain lacking Mca1 (mca1Δ/mca1Δ) was significantly delayed compared to that of the control. Representative microscopic images of asci 12 h and 14 h after meiotic induction in the case of the wild-type (mca1+/mca1+) and mca1Δ/mca1Δ mutant cells, respectively, are shown (right side). (B) The entire meiotic program in wild-type zygotic cells (mca1+/mca1+) incubated in the presence of TTM (50 μM) was delayed compared to that seen with untreated mca1+/mca1+ cells. TTM-treated mca1Δ/mca1Δ mutant cells underwent a meiotic block at metaphase I. The graphics (left) indicate the meiotic profiles of these cells. The numbers of cells with 1 nucleus, 2 nuclei, or 3 or 4 nuclei were determined by counting Hoechst-stained nuclei after meiotic induction. Typical images of wild-type (mca1+/mca1+) and mca1Δ/mca1Δ mutant cells at the 12-h time point are shown in the right panels. FSM, forespore membrane.
copper-starvation-induced expression of mfc1\(^+\). The two regulatory \(\text{cis}\)-acting elements contained CGG triplets that were disposed in a direct-repeat and symmetrical format, but they were oriented in the opposite direction from the A of the ATG of the mfc1\(^+\) gene. The spacing between the two CGG triplets was 16 bp, which represents a long spacer compared to other known similar binding elements containing CGG trinucleotides (41). Multiple point mutations within mfc1\(^+\) promoter TCGGCG sequences abolished copper starvation-dependent activation of mfc1\(^+\). We thus tested the possibility that a CGG-binding zinc binuclear cluster transcription factor played a role in the induction of mfc1\(^+\) gene expression. We determined that cells bearing a disruption of mca1\(^+\), which encodes a Zn\((2)\)Cys\((6)\) cluster-type protein, were unable to fully induce mfc1\(^+\) in response to copper deficiency. Consistent with a role for Mca1 as a positive regulator of mfc1\(^+\) promoter TCGGCG sequences, we found that a direct palindrome, TCGGCGN\(13\)TCGGCG, was required for copper starvation-activation of mfc1\(^+\). Given the fact that two zinc finger units are required to bind a pair of CGG triplets, this implies that Mca1 may bind the TCGGCGN\(13\)TCGGCG sequence as a homodimer. Unexpectedly, a low level of mfc1\(^+\) mRNA was still induced in a mca1\(\Delta\)/mca1\(\Delta\) mutant strain under conditions of copper starvation. One interpretation of this result is that at least one other member of the family of zinc binuclear cluster proteins may be involved in the regulation of mfc1\(^+\). Data that showed that the TCGGCGN\(13\)TCGGCG sequence was necessary for copper-starvation-mediated gene expression are consistent with this interpretation. Thus, in the absence of Mca1 (mca1\(\Delta\)/mca1\(\Delta\)), a second zinc binuclear cluster transcription factor may act as a homodimer in regulating mfc1\(^+\). In the wild-type strain (mca1\(^+\)/mca1\(^+\)), where many proteins within this class of transcription factors are found as heterodimers (32, 42, 43), the possibility exists that Mca1 may form a heterodimer complex with a second unidentified zinc binuclear cluster protein. Homodimeric or heterodimeric complexes of zinc cluster proteins may influence promoter recognition or transcriptional effects on target genes (44–46). Among the 31 putative members of the Cys\((6)\)Zn\((2)\) binuclear cluster protein family, 3 of these proteins (SPBP8B6.04c, SPAC1486.10, and SPAC11D3.07c) have a large linker region similar to that seen with Mca1. If the length of the mfc1\(^+\) promoter, the heptad repeats of Leu residues represent the predicted dimerization region of Mca1.
linker region is critical to bridging the relatively large distance between the CGG triplets in the mfc1+/− promoter, perhaps SPBPBB6.04c, SPAC1486.10, or SPAC11D3.07c might be a good candidate to form a heterodimer complex with Mcal. Zinc cluster proteins can also form heterodimeric complexes with members of other transcription factor families (47). In *Saccharomyces cerevisiae*, Arg81 (a zinc cluster protein) associates with ArgRl and Mcm1, which are two members of the MADS box proteins (47). Once assembled as a three-component protein complex, the heterotrimer binds DNA in an arginine-dependent manner. Within this heterotrimERIC complex, Arg81 serves as the arginine receptor and sensor, leading to the formation of an Arg81-ArgRl-Mcm1-DNA complex which regulates genes that encode proteins involved in arginine metabolism (47). It has been proposed that Arg81 directly binds arginine because of the presence of a region located downstream of the zinc finger unit which shares some sequence homology with the arginine-binding domain of the bacterial ArgR repressor (47).

It is not known how limiting copper concentrations are able to act as a signal for induction of mfc1+/− gene expression in a Mcal-dependent manner. Deletion of the mcal1+/− gene (mcal1Δ/mcal1Δ) leads to a phenotype linked to copper starvation. When mcal1Δ/mcal1Δ mutant cells underwent synchronous meiosis in the presence of TTM (50 μM), we observed a block in meiosis at metaphase I. This observation was reminiscent of that seen in wild-type cells in the presence of a high concentration (200 μM) of TTM. In this case, progression of meiosis was blocked at metaphase I, unless exogenous copper was added to overcome the inhibitory effect of the copper chelator (3). These observations suggested that Mca1 may play an important role in activation of other meiotic genes (besides mfc1+/−), especially those expressed in early meiosis that precedes metaphase I. Future experiments are obviously required to identify additional meiotic genes that are regulated by cellular copper availability through Mca1. Under copper-limiting conditions, a mutant strain lacking Mfc1 (mfc1Δ/mfc1Δ) exhibited meiotic progression that was delayed and prolonged by ~2 to 3 h compared to the wild-type strain (3). This observation reveals that the mcal1Δ/mcal1Δ mutant strain displays a stronger copper-deficient phenotype than mfc1Δ/mfc1Δ null cells.

The Cuf1 copper-sensing transcription factor is functionally similar to Mcal of *S. cerevisiae* (19, 29, 48, 49). Cuf1 and Mcal share a highly conserved C-terminal motif containing 5 cysteine residues and 1 histidine residue. The Cys328-X-Cys330-X3-Cys334-X-Cys336-X2-Cys339-X2-His42 motif of Cuf1 and the Cys266-X-Cys268-X4-Cys271-X-Cys273-X2-Cys276-X2-His279 sequence in Mcal constitute their copper-sensing regions (37, 50, 51). It has been proposed that the apo forms of Cuf1 and Mcal bind to copper-responsive elements to activate transcription of target genes (38, 49). In contrast, under copper-replete conditions, copper would induce intramolecular changes between the copper-sensing regions (Cys-X-Cys-X4-Cys-X-Cys-X-His) and the N-terminal DNA-binding regions of Cuf1 and Mcal, thereby activating their DNA binding activity and their ability to transactivate target gene expression. Although Mcal contains few putative metal-binding motifs throughout its amino acid sequence (e.g., Met-X-Met, Met-X2-Met, Cys-X-Cys-X-His, Cys-X2-Met-His-X2-His), there is no Cys-X-Cys-X4-Cys-X-Cys-X-His-like motif present in its sequence. This finding suggests that Mcal regulates mfc1+/− gene expression through a mechanism different from that seen with Cuf1 and Mcal. We have been unable so far to perform chromatin immunoprecipitation (ChIP) assays to determine whether Mcal was constitutively bound to its target promoter or present only under conditions of copper starvation. It has been observed that unusually high proteolytic activities are present during the meiotic program and that these interfere with the assay by inducing degradation of a large quantity of proteins, some of which may be key elements of DNA binding (data not shown). Experiments using modified protocols are under way to attempt to solve this problem. Mechanisms that regulate zinc binuclear cluster proteins include specific posttranscriptional modifications, binding of small inducer molecules to transcription factors, and interaction with effectors or partner proteins (32). Further genetic, biochemical, and molecular studies are likely to shed light on the mechanistic aspects whereby Mca1 functions to differentially activate mfc1+/− gene expression in response to copper deficiency.

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