Identification and characterization of the mitochondrial RNA polymerase and transcription factor in the fission yeast *Schizosaccharomyces pombe*

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ABSTRACT

We have characterized the mitochondrial transcription factor (Mtf1) and RNA polymerase (Rpo41) of *Schizosaccharomyces pombe*. Deletion mutants show Mtf1 or Rpo41 to be essential for cell growth, cell morphology and mitochondrial membrane potential. Overexpression of Mtf1 and Rpo41 can induce mitochondrial transcription. Mtf1 and Rpo41 can bind and transcribe mitochondrial promoters *in vitro* and the initiating nucleotides were the same *in vivo* and *in vitro*. Mtf1 is required for efficient transcription. We discuss the functional differences between Mtf1 and Rpo41 of *S. pombe* with *Saccharomyces cerevisiae* and higher organisms. In contrast to *S. cerevisiae,* the established model for mitochondrial transcription, *S. pombe*, a petite-negative yeast, resembles higher organisms that cannot tolerate the loss of mitochondrial function. The *S. pombe* and human mitochondrial genomes are similar in size and much smaller than that of *S. cerevisiae*. This is an important first step in the development of *S. pombe* as an alternative and complementary model system for molecular genetic and biochemical studies of mitochondrial transcription and mitochondrial–nuclear interactions. This is the first systematic study of the cellular function and biochemistry of Rpo41 and Mtf1 in *S. pombe*.

INTRODUCTION

Mitochondria have their own circular genome. Mitochondrial transcription is accomplished by the mitochondrial RNA polymerase and mitochondrial transcription factors [reviewed in ref. (1–3)]. The mitochondrial RNA polymerase is a single subunit RNA polymerase that is similar to the T3 and T7 bacteriophage RNA polymerases (4). Both the budding yeast *Saccharomyces cerevisiae* Rpo41 and human (POLRMT) RNA polymerases display high sequence similarity to the C-terminal domain of the phage RNA polymerases (5–7). In contrast to the autonomous bacteriophage polymerases, the mitochondrial RNA polymerases require accessory factors.

In *S. cerevisiae,* basic mitochondrial transcription is mediated by the mitochondrial RNA polymerase Rpo41 (3,4,8) and the mitochondrial transcription factor Mtf1 (9–11). Disruption of the Mtf1 gene causes a petite phenotype, reduced mitochondrial transcription and mitochondrial DNA copy number (5,10,12). Deletion of Rpo41 also causes a petite phenotype (13). Mtf1 facilitates specific binding of the mitochondrial RNA polymerase to the promoter sequences, like the sigma factors in bacteria; combining with the core RNA polymerase to produce a functional 'holoenzyme', to ensure correct initiation of transcription in the mitochondrial genome in *S. cerevisiae* (11,14–17). Rpo41 can initiate transcription in the absence of Mtf1 under certain conditions (17–19). The crystal structure of Mtf1 showed that it consists of two domains, a large α/β N-terminal domain and a smaller

tetra-α helical C-terminal domain separated by a cleft (20). Structurally the N-terminal domain was closely related to a series of rRNA methyltransferases (21–24).

In higher organisms, the mitochondrial RNA polymerase was originally proposed to have three distinct transcription factors: transcription factor A (TFAM) and two protein paralogs of *sc*-mtTFB; mtTFB1and mtTFB2 (1,2,25–29). TFAM contains two tandem high-mobility group (HMG) boxes (30,31). Human TFAM, mtTFB2 and POLRMT have been shown to initiate transcription from promoter sequences *in vitro,* and mtTFB2 and POLRMT are sufficient to initiate transcription from promoter sequences alone (32–36). Mouse and human mtTFB1 have been shown to have rRNA methyltransferase activity and appear to play an important role in mitochondrial ribosomal subunit assembly (37–39). RNAi knock-down of *Drosophila* mtTFB2 in cell culture reduces the abundance of specific mitochondrial transcript RNA and RNAi knock-down of *Drosophila* mtTFB1 reduces mitochondrial protein synthesis (28,29).

Although the mitochondrial transcription machinery in *S. cerevisiae* and higher organisms have been extensively studied [reviewed in ref. (1–3)], the mitochondrial RNA polymerase and transcription factors in the fission yeast *Schizosaccharomyces pombe* have not been identified or characterized. *Schizosaccharomyces pombe* has been widely used for studies of cell cycle control and differentiation. The *S. pombe* mitochondrial genome is 19 431 base pairs (bp), in terms of size, it compares favorably with the human mitochondrial genome of 16 569 bp, in contrast to that of *S. cerevisiae* at 85 709 bp. It provides an alternative and complementary model to budding yeast for the investigation of the molecular mechanisms of mitochondrial transcription (40). Normal mitochondrial function relies on an orchestrated cross-talk between the nucleus and the mitochondrial genes. Identification of the mitochondrial RNA polymerase and transcription factors in *S. pombe* will also provide a new system for studying mitochondrial-nuclear interactions. We have recently shown that the predicted mitochondrial transcription factor (Mtf1) in *S. pombe* has a non-mitochondrial function as a nuclear transcription factor that regulates transcription of *srk1*, a kinase involved in the stress response and cell cycle progression (41). To fully understand this novel nuclear function of a putative mitochondrial transcription factor (Mtf1), it is therefore important to confirm and characterize the mitochondrial transcription factor activity of Mtf1 in *S. pombe*.

The mitochondrial genome of *S. pombe* includes genes that encode all of the RNA components of mitochondrial translation, eight protein-coding genes of the respiratory chain, a small ribosomal subunit protein, and three intron encoded DNA binding endonucleases (42). Mitochondrial transcription in *S. pombe* has been mapped to three promoters: Pma the major promoter where transcription of the mitochondrial chromosome is initiated at the large (*21S*) ribosomal RNA, an additional minimal promoter Pmi where transcription of the *cox3* gene is initiated and Pin which is located downstream of the *cob* group II intron 5′-splice junction (43).

In this article, we describe the cloning of the *S. pombe* mitochondrial RNA polymerase, Rpo41 and the mitochondrial transcription factor, Mtf1. We analyzed the phenotypes of Mtf1 or Rpo41 deletion mutant cells and the effect of Mtf1 or Rpo41 deletions on mitochondrial membrane potential. Deletion of Mtf1 or Rpo41 leads to reduced transcription of the mitochondrial genes. Overexpression of Mtf1 or Rpo41 can increase transcription of the mitochondrial genes. We show biochemically, that purified Mtf1 and Rpo41 together can bind to the *S. pombe* mitochondrial promoters and can support transcription from the *S. pombe* mitochondrial promoters *in vitro*. We have used 5′-RACE to map mitochondrial transcription start sites *in vivo* and *in vitro*.

MATERIALS AND METHODS

Fission yeast strains, media and techniques

The strains used in this study: *972h⁻* and *h⁻leu1-32* (gift from Jürg Bähler), strain *(mtf1Δ1 and mtf1Δ2) mtf1::kan ade6-210/ade6-216* (generated from this study) and rpo41 *Δ strain rpo41::kanMX4 ura4-D18 leu1-32* (Bioneer). Plasmid pREP3-Mtf1 and pREP3-Rpo41 were used to transform *S. pombe* strains. Plasmids pGEX-4T-Mtf1 and pET28a-Rpo41 were generated for the purification of Mtf1 and Rpo41 in *E. coli BL21* strain.

PCR primer sequences and oligonucleotide sequences used in this study

Real-time PCR or PCR primer sequences for cloning and sequences of synthetic DNA promoter regions are listed in Supplementary Table S1.

Plasmid construction

For plasmid transformation into *S. pombe h⁻leu1-32* strain, pREP3-Mtf1 was created by inserting PCR fragments of the Mtf1 coding sequence into the SmaI site in pREP3 (44). pREP3-Rpo41 was generated by insertion of a PCR fragment of Rpo41 into the SmaI site of pREP3.

For overexpression and purification of Mtf1 and Rpo41 in *E. coli*, the Mtf1 coding sequence was ligated into pGEX-4T-1 using the XhoI and EcoRI sites to construct the plasmid pGEX-4T-1-Mtf1 (N-terminal GST tagged Mtf1). The Rpo41 coding sequence was ligated into vector pET28a using the EcoRI and XhoI sites to create pET28a-Rpo41 (N terminal His tagged Rpo41). Clones were confirmed by DNA sequencing.

Disruption of *mtf1⁺*

Mtf1 was deleted in diploid cells by using a PCR-based approach using plasmid pFA6a-kanMX6 as the PCR template to generate strain *mtf1Δ1 or mtf1Δ2 mtf1::kan ade6-210/ade6-216* [described in ref. (45)]. The primer sequence can be found in Supplementary Table S1. The disruption was confirmed by real-time PCR of the mRNA expression level of the Mtf1 gene beyond the insertion point and by PCR of the genomic DNA using carefully designed primer pairs. The diploid cells were mated and

sporulated to generate haploid cells for phenotype analysis.

Cell staining and confocal microscopy

Cells were stained and visualized as described previously (41). Briefly, cells were grown to an OD 600 of 0.5, fixed and stained with 4′,6′-diamidino-2-phenylindole (DAPI) or calcofluor. Unfixed cells were stained directly with DiOC6 (3). Cells were stained with 50 µg/ml of calcofluor in buffer containing 50% glycerol and 0.3 mg/ml p-phenylenediamine. Cells were visualized by confocal microscopy with a Leika TCS-SP5 microscope.

Real-time PCR analysis

Real-time PCR was performed as described previously (41). In brief, reverse transcripts were generated from total cellular RNA, followed by quantitative real-time PCR using an iQ5 Continuous Fluorescence Detector System (Bio-Rad). At least three independent biological repeats and four technical repeats were done for all experiments. Error bars were generated from the standard deviation (calculated by the instrument software). Transcripts were detected for the mitochondrial large (21S) and small (15S) ribosomal RNAs (*rns* and *rnl*, respectively) cytochrome oxidase subunits 1, 2 and 3 (*cox*1, *cox*2 and *cox*3), apocytochrome *b* (*cob*1), ATP-synthase subunits 6 and 9 (*atp*6 and *atp*9), the mitochondrial small subunit ribosomal protein 3 (*rps*3) and the group I (SPMIT.03) and group II (SPMIT.06) introns that encode DNA binding endonucleases. The housekeeping gene β actin was used as a reference, and α tubulin as an internal control. N.B. Two of the mitochondrial genes (the group I intron encoded SPMIT.02 and *atp*8) were not detected by real-time PCR presumably because transcripts were too short to detect.

Electrophoretic mobility shift assay

Electrophoretic mobility shift assay (EMSA) experiments were carried out as described previously (41), in each reaction, pure GST tagged Mtf1 protein and/or His tagged Rpo41 was incubated with 5′ biotinylated oligonucleotide probes in the presence of competitor DNA (polydI/dC) in binding buffer, and then electrophoresed through a 6% polyacrylamide native gel at 4°C. DNA/protein complexes were transferred to nylon membrane (Hybond-N+) (GE) (46) and detected by chemiluminescence.

Expression and purification of recombinant Mtf1 and Rpo41

GST-tagged Mtf1 was expressed in *E. coli* BL21 cells from pGEX4T-1-Mtf1. Cells were grown to an OD of 0.5 and induced with 0.1 mM IPTG at 30°C for 6 h. Cell pellets were lysed in PBS buffer (pH 8.0), GST-tagged Mtf1 was purified using Glutathione–Sepharose 4B (GE 17-1969-01). His-tagged Rpo41 was expressed in *E. coli* BL21 cells from pET28a-Rpo41. Cells were grown to an OD of 0.5 and induced with 0.1 mM IPTG at 16°C for 20 h. His-tagged Rpo41 cell pellets were lysed in binding buffer (20 mM imidazole, 20 mM Na Phosphate, 0.5 M NaCl, pH 7.4, 1 mM phenylmethylsulfonyl fluoride) and purified using Ni²⁺-NTA–agarose and, after washing with binding buffer with added 20 mM imidazole, was eluted with 500 mM imidazole, 20 mM Na Phosphate, 0.5 M NaCl, pH 7.4. Both Mtf1 and Rpo41 were further purified using a cation exchange (SP) column. The eluate from the GST-column or nickel column was buffer-exchanged on a gel filtration column (HiTrap, GE) with SP binding buffer (20 mM Na Phosphate, 0.1 M NaCl, 1mM DTT, pH 7.4), and then loaded onto the SP column. After a 10 column volume wash with SP-binding buffer, the protein peak was eluted with 20 mM Na Phosphate, 0.5 M NaCl, 1 mM DTT, pH 7.4. The eluate was concentrated by centricon (Millipore), and then desalted by dialysis against SP-binding buffer.

5′-RACE to determine the mitochondrial transcription start sites *in vivo*

The wild-type cells were grown to an OD of 0.5, 10 ml of culture was used for extraction of total RNA (41). DNA was removed from the RNA sample by treatment with DNase I (Fermentas). Reverse transcription and PCR reactions were carried out using a SMART™ RACE cDNA Amplification Kit (Clontech). The sequence of PCR primers can be found in Supplementary Table S1. The transcription start site was determined from the DNA sequence.

In vitro transcription and 5′-RACE analysis of RNA transcripts

DNA templates containing the promoters [Pma (738bp), Pmi (597bp) and Pin (431bp)] for *in vitro* transcription reactions were generated by PCR. A fourth predicted promoter (43) within *rps*3 was not present in the genome database at GeneDB (http://genedb.org/genedb/pombe/). His-tagged Rpo41 and GST-tagged Mtf1 proteins were expressed and purified in *E. coli* for *in vitro* transcription. Transcription reactions were performed using 150 nM of DNA template and 450 nM of Rpo41 in a reaction buffer containing 40 mM Tris (pH 7.9), 20 mM MgCl₂, 10 mM DTT, 1 mM nucleotide triphosphates, 2 mM spermidine, 0.01% Triton X-100 and 40 U of RNase inhibitor in a total volume of 50 µl, 450 nM Mtf1 was added to reactions that included Mtf1. Reactions were incubated at 37°C for 3 h, and transcription was terminated by heating at 70°C for 10 min. The mixtures were extracted with phenol/chloroform followed by ethanol precipitation. The DNA template was digested with DNase I at 37°C for 1 h, DNase I was then inactivated at 70°C for 10 min followed by a further phenol/chloroform extraction. Reverse transcription and PCR reactions were carried out as described in the previous section. The sequences of PCR primers are shown in Supplementary Table S1. The transcription start sites were determined from the DNA sequence. This experiment was repeated three times with three different batches of purified Mtf1 and Rpo41.

In vitro transcription and semi-quantitative PCR

In vitro transcriptions were performed as described in the preceding section, in the presence and absence of Mtf1. Control RNA was added to 2 μg of each RNA transcript from *in vitro* transcription. The mixture of control and transcribed RNA was then reverse transcribed and then subjected to 28 cycles of PCR amplification. The sequence of the control RNA and primer sequences for the Pma, Pmi and Pin promoters can be found in Supplementary Table S1. The PCR products for each lane of the gel were then quantified relative to the control RNA which allowed the relative abundance of transcripts to be compared between transcription reactions and compensates for possible PCR or loading artifacts.

RESULTS

Cloning of *mtf1* and *rpo41* in fission yeast

A search of the Welcome Trust Sanger Institute genome database GeneDB (http://genedb.org/genedb/pombe/) identified two putative proteins SPAC26H5.12 and SPAC1002.08c that are predicted by homology to be the mitochondrial RNA polymerase (Rpo41) and the mitochondrial transcription factor (Mtf1) in fission yeast (47). In a genome-wide protein localization study, Mtf1 was reported to localize to the mitochondrion, Rpo41 however, was localized as cytoplasmic dots (48). *Mtf1* and *rpo41* are encoded on chromosome I of the fission yeast genome. Mtf1 has ∼23% amino acid identity and ∼61% sequence similarity to the Mtf1 of *S. cerevisiae* and Rpo41 was found to have ∼36% amino acid identity and ∼74% sequence similarity to the Rpo41 of *S. cerevisiae* (Figure 1A and B) (49). We next cloned *mtf1* and *rpo41* into protein expression vectors to create plasmid pGEX-4T-Mtf1 (N-terminal GST tagged Mtf1) and pET28a-Rpo41 (N-terminal His tagged Rpo41), respectively (50).

Mtf1 or Rpo41 are essential in fission yeast

To investigate Mtf1 deletion phenotypes, we generated two *mtf1* deletion mutants (*mtf1Δ1* and *mtf1Δ2*) by insertion of a Kanamycin resistance gene into the C- or the N-terminal domain of the Mtf1 coding sequence on the chromosome in a diploid strain. An Rpo41 diploid deletion mutant was supplied by Bioneer. Mtf1 or Rpo41 diploid deletion cells were mated and sporulated, and haploid segregates were visualized by microscopy after germination. *S. pombe* Mtf1 and Rpo41 deletion mutants appear to share the same phenotypes. Cells lacking Mtf1 (*mtf1Δ1* and *mtf1Δ2*) or Rpo41 (*rpo41Δ*) can not form colonies or grow in liquid medium and only undergo a few cell divisions after germination on plates. *Mtf1Δ1* and *mtf1Δ2* or *rpo41Δ* cells have abnormal cell morphology (Figure 2); they became elongated or enlarged and 'egg' or 'bottle' shaped compared to wild-type cells. DAPI staining showed some visible nuclear defects in *mtf1Δ1* and *mtf1Δ2* or *rpo41Δ* cells (Figure 2A). Calcofluor staining revealed Mtf1 or Rpo41 deletion cells to have abnormal septation including multiple septa (Figure 2B). These phenotypes together suggest that Mtf1 and Rpo41

are essential for cell growth and cell morphology in fission yeast. The *mtf1Δ1* and *mtf1Δ2* or *rpo41Δ* deletion phenotypes could be rescued by transformation of plasmids that overexpress Mtf1 or Rpo41 (plasmid pREP3-Mtf1 or pREP3-Rpo41, described in the following section) into the diploid strain. After germination, the haploid cells with Mtf1 or Rpo41 chromosomal deletions were rescued on G418 plates by plasmids that overexpress Mtf1 or Rpo41 (data not shown).

To examine the effect of Mtf1 or Rpo41 deletion on the mitochondrion, we performed mitochondrial staining with a fluorescent dye DiOC6 (3) which has been used to detect mitochondrial membrane potential in live cells (51). This dye is a green fluorescent lipophilic cation that can pass through the plasma membrane by the virtue of its lipophilicity and then accumulates in the mitochondrion through the attraction of the positive charge on the molecule to the high negative mitochondrial membrane potential. Mitochondrial staining with DiOC6 (3) showed that wild-type cells have intense green staining with long filamentous structures but the *mtf1Δ1, mtf1Δ2* or *rpo41Δ* cells stain dimly showing dispersed green signals with no structure, suggesting that Mtf1 or Rpo41 deletion resulted in a low mitochondrial membrane potential (Figure 2C).

Mtf1 and Rpo41 are both associated with the transcription of mitochondrial genes in fission yeast

To investigate the role of Mtf1 in the transcription of mitochondrial genes, we performed real-time PCR experiments in the two types of Mtf1 deletion cells to analyze the transcription of nine of the mitochondrial genes (that encode 21S and 12S rRNAs, *cox1*, *cox3*, *cob1*, *atp6*, *rps3*, *atp9* and *cox2*) and two of the genes encoded by the group I (SPMIT.03) and group II (SPMIT.06) introns that encode DNA binding endonucleases. We first confirmed that transcript abundance of the *mtf1* gene in *mtf1Δ1* and *mtf1Δ2* cells was reduced to background levels compared to the wild-type cells (Figure 3A). We next showed that transcription of each of the mitochondrial genes was nearly abolished in the *mtf1Δ1* and *mtf1Δ2* cells compared to wild-type cells, in comparison, transcription of α tubulin is relatively unchanged in wild type and both *mtf1* deletion strains (Figure 3B).

We next tested the effects of Mtf1 over expression on the transcript abundance of mitochondrial genes in *S. pombe* by real-time PCR. The Mtf1 coding sequence was cloned into the pREP3 vector under the control of the thiamine repressible *nmt1* promoter to generate plasmid pREP3-Mtf1. This plasmid was transformed into the *h⁻ leu1-32* strain (44) and cells were grown in the absence or in the presence of 60 μM thiamine. Initially we confirmed that *mtf1* mRNA abundance in cells over expressing *mtf1* was higher than for cells transformed with the pREP3 vector alone. The presence of 60 μM thiamine can repress transcription of *mtf1* to minimal levels although this suppression is not complete (Figure 3C). Under these conditions, Mtf1 protein levels were shown to be induced in the absence of thiamine and repressed in the presence of thiamine as described previously (41). This was consistent with the
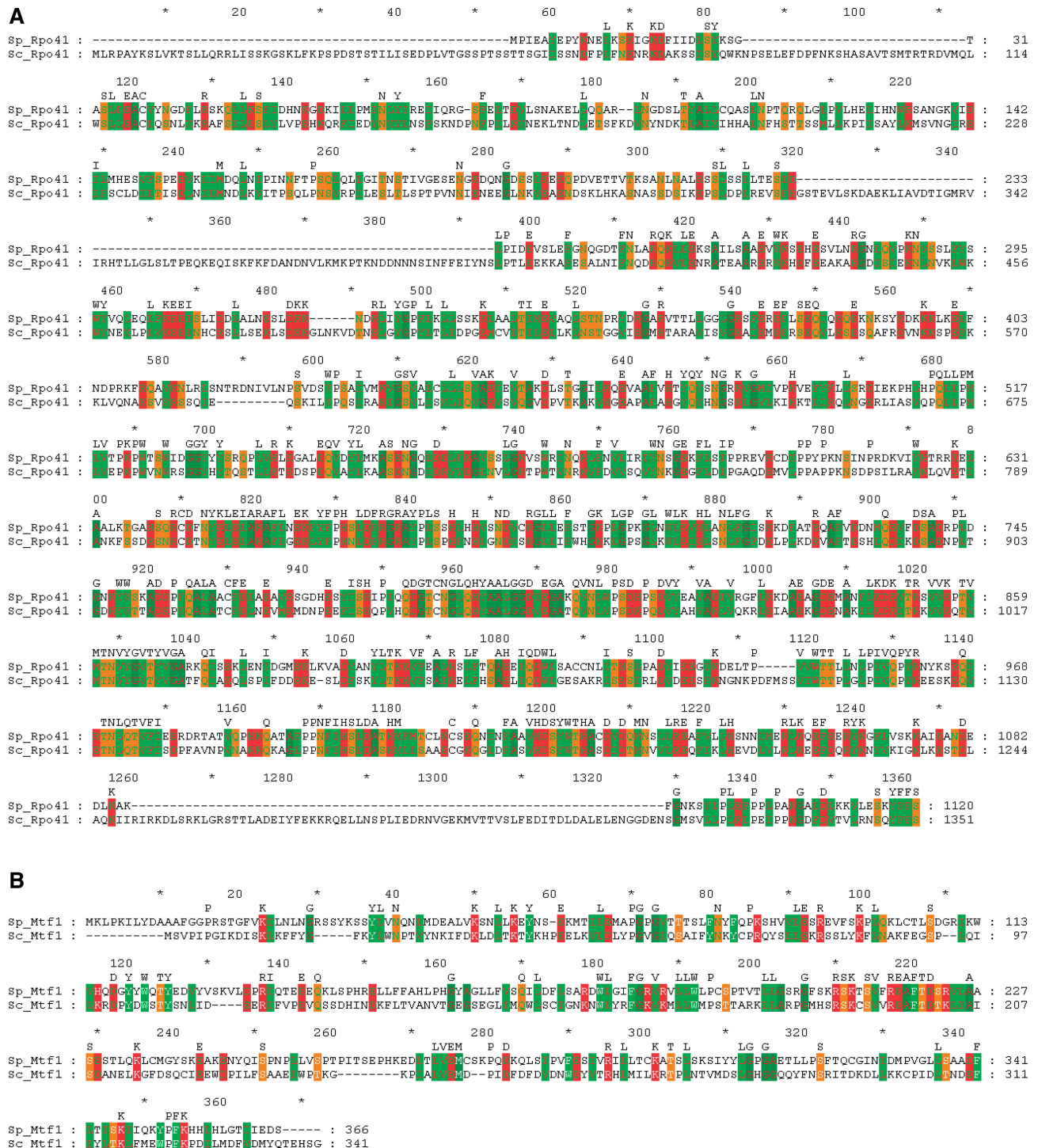
Figure 1. Alignment of *S. pombe* Mtf1 and Rpo41 with their *S. cerevisiae* homologs. *Schizosaccharomyces pombe* Mtf1 and Rpo41 were aligned with their *S. cerevisiae* homologs using Clustal W and Clustal X (49). Conserved residues are displayed above the alignment. Similar residues in *S. pombe* Mtf1 or Rpo41 are highlighted by the amino acid properties such that 1: green on red (DEHKR), 2: green on orange (NQST), 3: red on green (LIVMFYW) and 4: red on dark green (AG). ClustalW alignments were refined using GeneDoc (www.psc.edu/biomed/genedoc).

measurement of *mtf1* mRNA abundance in cells over expressing *mtf1*. We next showed that transcription of the mitochondrial genes was elevated in cells over expressing Mtf1 but remained unchanged in cells transformed with the vector only. In contrast, transcription of the nuclear gene α

tubulin was unaffected in cells over expressing Mtf1 (Figure 3D). The effect of Mtf1 deletion and over expression on the transcription of mitochondrial genes suggests that Mtf1 does indeed associate with the transcription of mitochondrial genes.
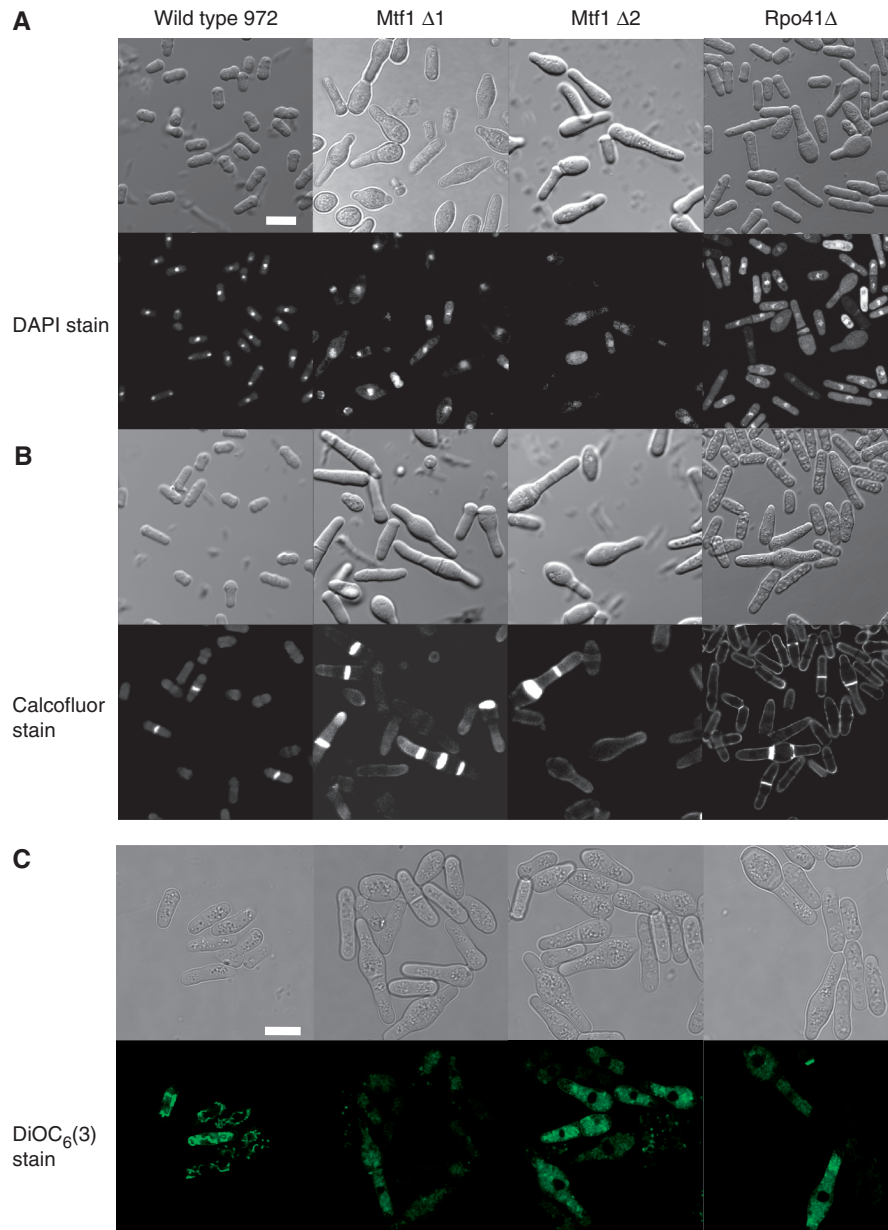
Figure 2. The phenotype of *mtf1* or *rpo41* deletions. (**A**) Wild-type 972 cells and *mtf1Δ1* and *mtf1Δ2* or *rpo41Δ* cells grown on YES/YES + G418 plates and cells were fixed and stained with DAPI. The top row shows phase contrast images of unstained cells, the bottom row shows DAPI stained cells viewed by confocal microscope. Bar is 10 μm. (**B**) Wild-type 972 cells and *mtf1Δ1* and *mtf1Δ2* or *rpo41Δ* cells were grown on plates and cells were fixed and stained with Calcofluor. The top row shows phase contrast images of unstained cells, the bottom row shows Calcofluor stained cells viewed by confocal microscope. (**C**) Wild-type 972 cells, *mtf1Δ1*, *mtf1Δ2* and *rpo41Δ* cells were stained with the green fluorescent mitochondrial stain DiOC6(3) and cells were observed by confocal microscope. Bar is 10 μm.

To associate Rpo41 with the transcription of the mitochondrial genome, we performed real-time PCR to detect transcript abundance of the mitochondrial genes in *rpo41Δ* cells. We first showed that transcription of the *rpo41* gene was reduced in the *rpo41Δ* cells (Figure 3E). Transcription of the mitochondrial genes was also reduced in the *rpo41Δ* cells compared to wild-type cells, but transcription of the nuclear gene α tubulin remained at similar levels in *rpo41Δ* and wild-type cells (Figure 3F). We next investigated the effect of Rpo41 over expression on the transcription of the mitochondrial genes. We constructed

the plasmid pREP3-Rpo41 by cloning the Rpo41 coding sequence under the control of the thiamine repressible *nmt1* promoter into the pREP3 vector, and transformed this plasmid into the *h⁻leu1-32* strain. We tested the transcription of the mitochondrial genes by real-time PCR in cells in which Rpo41 is over expressed in the absence of thiamine. We first confirmed that transcription of *rpo41* in cells over expressing *rpo41* was higher than for cells transformed with the pREP3 vector alone in the absence of thiamine (Figure 3G). We next showed that transcription of the mitochondrial genes was induced when Rpo41 was
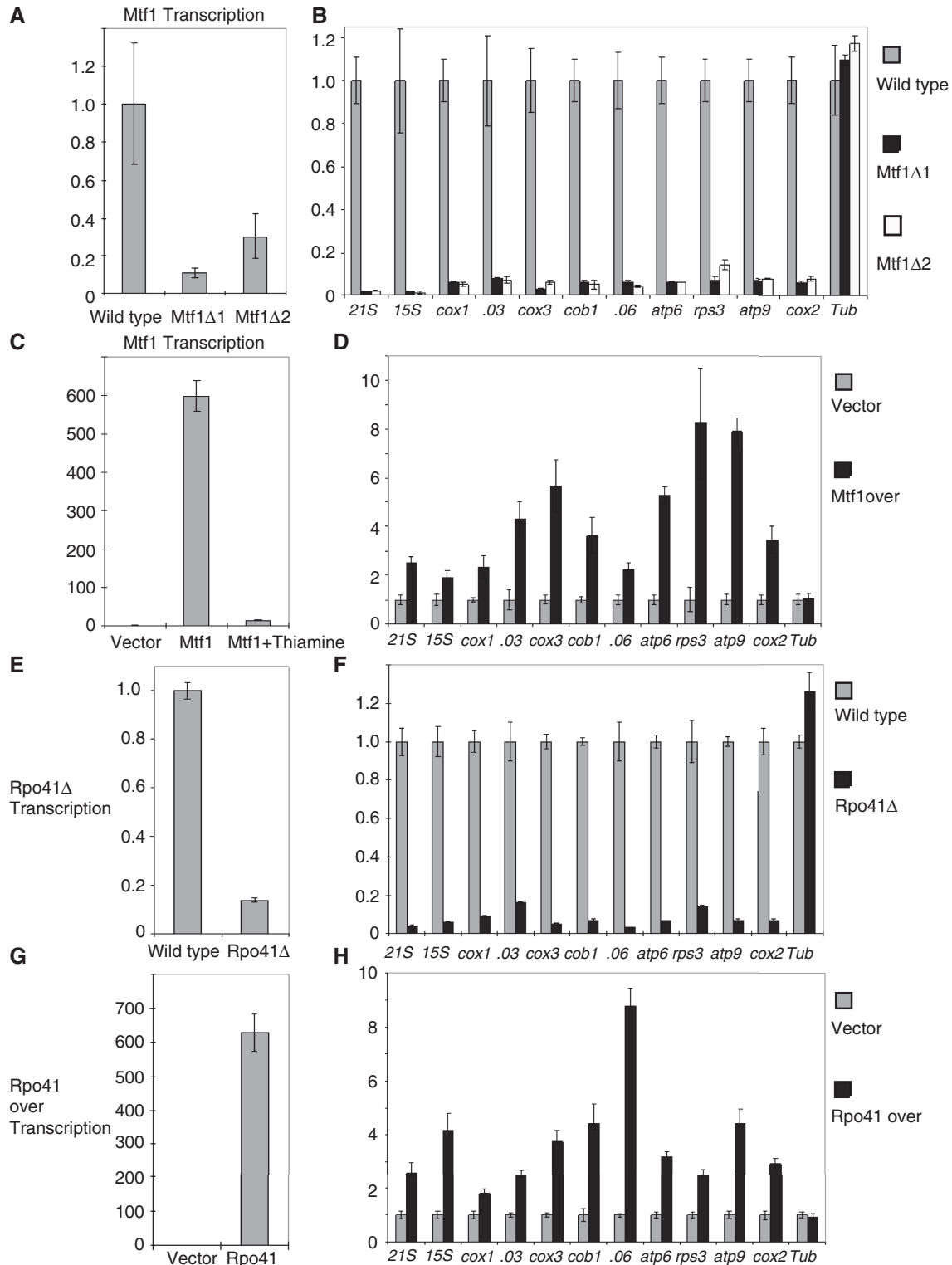
Figure 3. The transcription of mitochondrial genes is associated with Mtf1 and Rpo41. (**A**) Real-time PCR of *mtf1* mRNA abundance in wild-type cells and *mtf1Δ1* and *mtf1Δ2* cells, showing that *mtf1* mRNA abundance is reduced in *mtf1Δ1* and *mtf1Δ2* cells. (**B**) Real-time PCR of transcript abundance of the mitochondrial genes in wild-type cells and *mtf1Δ1* and *mtf1Δ2* cells. NB the intronic genes SPMIT.03 and SPMIT.06 are labeled .03 and .06 for convenience. (**C**) Real-time PCR of *mtf1* mRNA abundance in cells transformed with vector only and cells transformed with pREP3-Mtf1 overexpressing Mtf1 without thiamine and with 60 μM thiamine. (**D**) Real-time PCR of transcription abundance of the mitochondrial genes in cells transformed with vector only and cells transformed with pREP3- Mtf1 overexpressing Mtf1, showing that transcription of all of the mitochondrial genes is elevated when Mtf1 is overexpressed. (**E**) Real-time PCR of *rpo41* mRNA abundance in wild-type cells and *rpo41Δ*cells. We note that *rpo41* mRNA abundance is reduced to background level in *rpo41Δ*cells. (**F**) Real-time PCR of transcription abundance of the mitochondrial genes in wild-type cells and *rpo41Δ*cells, showing that transcription is reduced in *rpo41Δ*cells. (**G**) Real-time PCR of *rpo41* mRNA abundance in cells transformed with vector only and cells transformed with pREP3-Rpo41 overexpressing Rpo41. (**H**) Real-time PCR of transcription abundance

(continued)

over expressed, while the transcription of the nuclear gene α tubulin was not effected by over expression of Rpo41 (Figure 3H). To confirm that the transcriptional induction of the mitochondrial genes was due to the induction of Rpo41 expression we performed the analogous experiment under conditions where Rpo41 was not expressed (in the presence of 60 μM thiamine); we found that transcription of all of the mitochondrial genes remained unchanged (data not shown).

Mtf1 and Rpo41 together bind to the mitochondrial promoters in fission yeast

We have shown that Mtf1 and Rpo41 are associated with the transcription of the mitochondrial genes. To further explore the potential interactions between Mtf1, Rpo41 and the mitochondrial promoters biochemically, we performed EMSA using biotin and streptavidin-based detection (52). We over-expressed and purified Mtf1 and Rpo41 using plasmid pGEX-4T-Mtf1 (N-terminal GST tagged Mtf1) and pET28a-Rpo41 (N-terminal His tagged Rpo41) in *E. coli* BL21 cells (Supplementary Figure S1). Pma, Pmi and Pin promoter sequences (5′ biotinylated oligonucleotide DNA) and purified Mtf1 and Rpo41 were used in EMSA. Mtf1 alone did not cause a detectable shift of the Pma, Pmi and Pin promoter DNA but Rpo41 could generate a weak retarded species in the gel on its own with Pma, Pmi and Pin promoter DNA (for Pin the band shift is too weak to be seen). Mtf1 and Rpo41 together caused a strong super-shift of the probes (Figure 4). However, upon prolonged exposure we have also observed a weak retarded species for Pma, Pmi and Pin promoter DNAs with Mtf1 alone under different conditions (41). We noted that in the presence of the Pma, Pmi and Pin and Rpo41, addition of Mtf1 eliminated the band-shift by Rpo41 alone and caused a strong super-shift, suggesting that Mtf1 and Rpo41 together can bind to the promoters much more efficiently whereas Rpo41 or Mtf1 can only bind weakly to the promoters. To investigate the specificity of the binding that we observed, we generated mutated Pma, Pmi and Pin promoters that contained three-base mutations within the core sequence of the promoters, and performed EMSA with the mutated promoters. Mtf1 and Rpo41 with the mutant promoters exhibited a significantly reduced band shift compared to the wild-type promoters (Figure 4). This suggests that Mtf1 and Rpo41 bind to the promoters specifically and that the core sequences of the promoters are required for recognition by Mtf1 and Rpo41.

In vivo mapping of mitochondrial promoters in fission yeast by 5′-RACE

The mitochondrial transcription initiation sites in fission yeast were previously mapped to Pma, Pmi by *in vitro*

capping of RNA with guanylyl-transferase but Pin could not be detected by this method. Pin was detected by primer extension analysis (43). We performed 5′-RACE to examine the mitochondrial transcription initiation sites for Pma, Pmi and Pin *in vivo*. Total RNA was extracted from cells and treated with DNase I to remove DNA from the RNA sample. RNA was reverse transcribed and the cDNA amplified by 5′-RACE PCR. The transcription start sites were determined by DNA sequencing. Supplementary Figure S2A shows that the 5′-RACE products for Pma, Pmi and Pin are the correct size (153, 250 and 141 bases, respectively). Comparison of the *in vivo* 5′-RACE products shows that Pma generates the most intense product band, and that Pin generates the least intense product band, which is consistent with previous observations that Pma is the major mitochondrial promoter, Pmi a minor promoter and Pin a weak promoter (43). 5′-RACE products for Pmi, Pma and Pin were gel purified for DNA sequencing. Supplementary Figure S2B–D shows the DNA sequencing results that unambiguously indicate the transcription initiation sites for Pmi, Pma and Pin. The transcription initiation sites for Pmi and Pma are the same as those previously identified through *in vitro* capping of the RNA. However, for Pin the initial nucleotide of the RNA transcript is a G (Supplementary Figure S2D), and not U as previously described (43). The 5′-RACE technique appears to be more accurate than primer extension analysis for the transcription initiation site of Pin.

In vitro transcription of the mitochondrial promoters by Rpo41/Mtf1 and analysis of RNA transcripts by 5′-RACE

To examine the transcriptional activity of Mtf1 and Rpo41, we performed *in vitro* transcription with purified Rpo41 in the presence and absence of Mtf1 (Supplementary Figure S1) using the Pma, Pmi and Pin promoters. The DNA templates for Pma, Pmi and Pin promoters were generated by PCR to give template sizes of 738, 597 and 431 base pairs, which in turn gave rise to transcript sizes of 598, 426 and 377 nts, respectively. The RNA transcripts were then subjected to 5′-RACE analysis. The RNA transcripts were reverse transcribed and the cDNA was amplified by PCR using a forward linker primer and a sequence specific reverse primer. 5′-RACE products were then gel purified for DNA sequencing. Figure 5A shows the 5′-RACE products of RNA transcripts generated from the *in vitro* transcription with and without Mtf1 for Pma, Pmi and Pin promoters. The corresponding cellular RNAs, extracted from cells for 5′-RACE analysis of Pma, Pmi and Pin promoters were used as a control to indicate the size of the *in vivo* transcripts. The 5′-RACE products of the RNA transcripts synthesized by Rpo41 *in vitro* are the same size as the control *in vivo* transcripts in the presence or absence of

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Figure 3. Continued

of the mitochondrial genes in cells transformed with vector only and cells transformed with pREP3-Rpo41 that over expresses Rpo41, showing that transcription of the mitochondrial genes is elevated. *Act*1 was used as a reference for all of the Real-time PCR experiments and each experiment was biologically repeated at least three times. Error bars were generated from the standard deviation (calculated by the software on the Bio-Rad iQ5 Continuous Fluorescence Detector System) of four technical repeats.
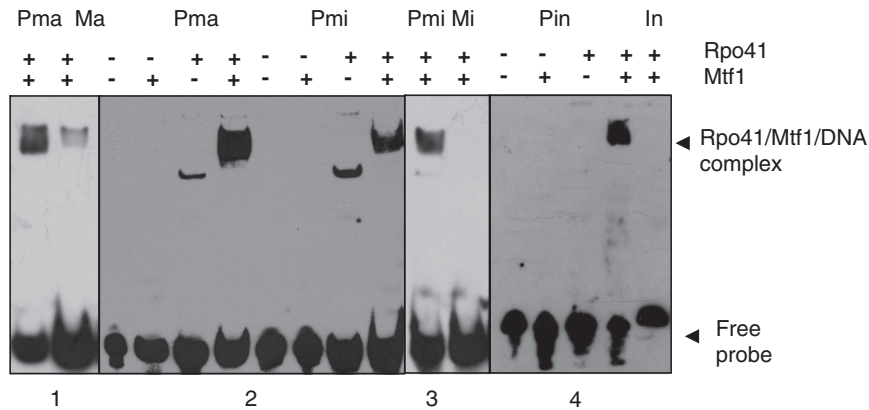
Figure 4. Promoter recognition by Mtf1 and Rpo41. Six percent of polyacrylamide native gels showing EMSA with the mitochondrial promoters Pmi, Pma and Pin in the present of purified Mtf1 or Rpo41 or both proteins together. Note that Mtf1 and Rpo41 together can bind to the promoters more efficiently. The wild-type Pma contains the core promoter sequence ATATATGT, which is mutated to ATCGCTGT (Ma). Similarly, the wild-type Pmi contains the core promoter sequence TTATATGT and Pin contains ATATATGTG, which is mutated ATCGCTGT (Mi or In). Ma, Mi and In all show a reduction in affinity compared the wild-type promoter sequence, indeed for Mi or In, binding is completely abolished.
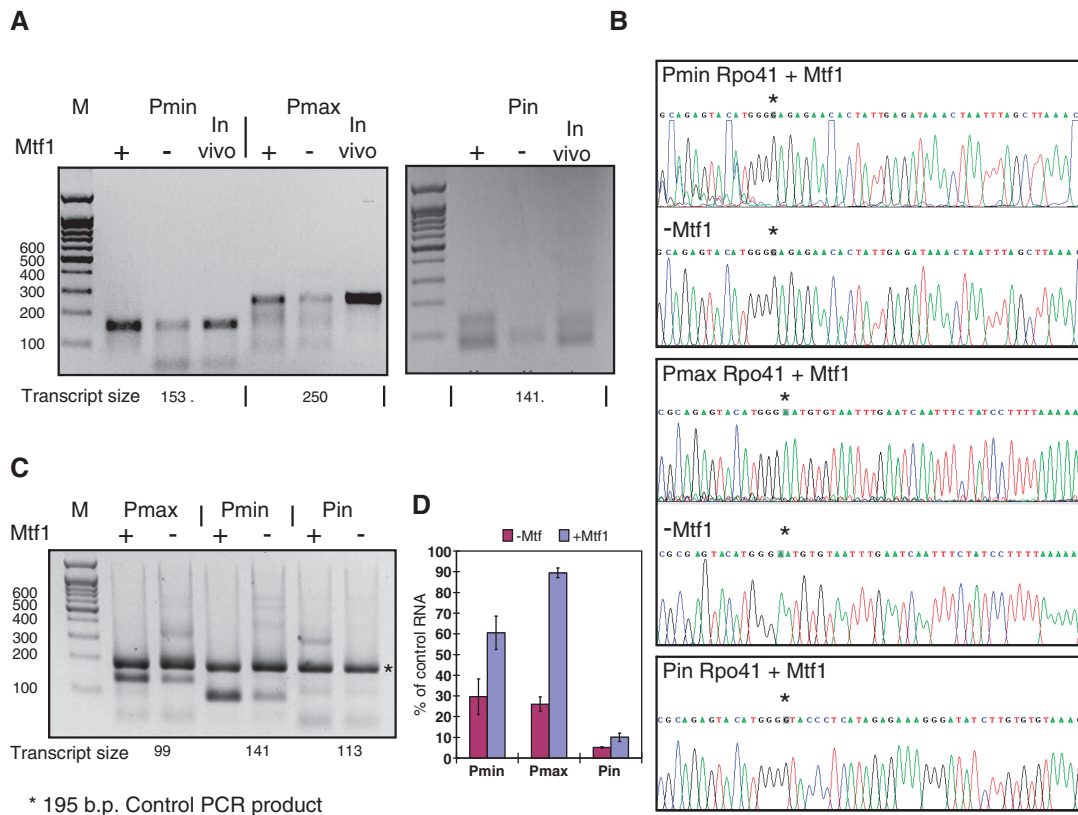

Figure 5. *In vitro* transcription from the mitochondrial promoters and 5′-RACE analysis of the transcripts. (**A**) *In vitro* transcription of Pmi, Pma and Pin promoters by purified Rpo41 was performed with and without Mtf1. RNA transcripts were then used for 5′-RACE analysis. Extracted cellular RNA was used as a control. The 5′-RACE products were run on a 2% agrose gel. The transcript sizes corresponding to the 5′RACE products are labeled at the bottom of the gel. (**B**) DNA sequence results of 5′-RACE products showing the *in vitro* transcription initiation site for Pmi, Pma and Pin promoters with and without Mtf1. The position corresponding to the initial nucleotide is marked with an asterisk. (**C**) Semi quantitative PCR of the RNA transcripts from *in vitro* transcription of Pmi, Pma and Pin promoters. The PCR products are run on 2% agarose gel. The size of the control PCR product and the transcript sizes for Pmi, Pma and Pin are labeled on the gel. (**D**) Quantitation of (C) showing that Mtf1 increases Rpo41 transcriptional efficiency.

added Mtf1. Figure 5B shows the DNA sequencing results and indicates that Rpo41/Mtf1 or Rpo41 alone can initiate transcription *in vitro*. We note that the *in vitro* start sites for all three promoters are exactly the same as

the *in vivo* mapped start sites for Pma, Pmi and Pin, confirming the transcriptional activity of Mtf1 and Rpo41 *in vitro* (Figure 5B and Supplementary Figure 2B–D). The 5′-RACE products of Pin transcripts in the absence

of Mtf1 were insufficient for DNA sequencing. Although the Rpo41 from both fission and budding yeast resembles the single polypeptide bacteriophage RNA polymerases, we note that both yeast enzymes can tolerate the inclusion of an N-terminal His tag, and remain active in *in vitro* transcription reactions, in contrast to T7 RNA polymerase (50,53). Compared to Pma and Pmi, *in vitro*, the Pin promoter is much weaker (Figure 5A), indeed the relative promoter strengths that are observed *in vitro* are remarkably similar to those that are observed *in vivo* (Supplementary Figure S2A and Figure 5A). Figure 5A shows that for all three promoter sequences, inclusion of Mtf1 in transcription reactions gave rise to significantly more 5′-RACE PCR product. Although *in vitro* Rpo41 alone can also initiate transcription in the absence of Mtf1, the presence of Mtf1 significantly improves the efficiency of transcription. To further investigate this, we performed semi quantitative PCR with *in vitro* RNA transcripts prepared with and without Mtf1. An equal amount of a control RNA was spiked into each RNA sample before the reverse transcription and PCR amplification was performed. The control RNA produces a PCR product of 195 nts. The PCR products corresponding to the RNA transcripts from Pma, Pmi and Pin are 99, 141, 113 nts, respectively (Figure 5C). Inclusion of the control RNA allows the intensity of the PCR products from Pma, Pmi and Pin to be normalized relative to the control RNA, and eliminates possible PCR and loading artifacts, so that relative promoter efficiencies can be compared. Figure 5D shows that Mtf1 increases transcription efficiency for all three promoters *in vitro* significantly.

DISCUSSION

In fission yeast the product of SPAC1002.08c is predicted to be Mtf1. Here we confirm the predicted mitochondrial transcription factor activity of this protein. We have recently shown that Mtf1 has an extra-mitochondrial function as a nuclear transcription factor. As a nuclear transcription factor Mtf1 regulates transcription of *srk1*, a kinase involved in the stress response and cell cycle progression. Mtf1 localizes to the nucleus, and interacts specifically with the *srk1* promoter sequence *in vivo* and *in vitro*. *Srk1* is induced in cells where Mtf1 is over-expressed and abolished in cells where Mtf1 is deleted. Over expression of Mtf1 causes cell elongation (41). Thus Mtf1 functions as a transcription factor in both the nucleus and the mitochondrion. It was therefore important to confirm the mitochondrial transcription factor activity of Mtf1 and not simply rely on predicted protein functions; and in this context it was also important to characterize its putative mitochondrial partner the RNA polymerase Rpo41.

In *S. cerevisiae* cells lacking Mtf1 or Rpo41 exhibit a slow-growing or petite-colony phenotype and cannot grow on non-fermentable carbon sources and are respiratory deficient (10,11). We found in this study that *S. pombe* cells lacking Mtf1 or Rpo41 can not form colonies or grow in liquid medium; they can only undergo several cell divisions on YES plates and display abnormal cell morphology and disturbed mitochondrial membrane potential (Figure 2). Thus the phenotypes that we observe for Mtf1 or Rpo41 deletion mutant cells in *S. pombe* are different from those reported for *S. cerevisiae* and reflect the contrasting tolerances for mitochondrial loss between the two organisms. *Schizosaccharomyces pombe* is a petite-negative yeast, and resembles higher organisms in that it cannot tolerate the loss of mitochondrial function. Disruption of the nuclear encoded mitochondrial DNA polymerase (pog1) is sufficient to deplete mtDNA in *S. pombe* and pog1Δ cells are defective in mitochondrial function (54), pog1Δ cells have a slow growing phenotype and show similar cell morphology as Mtf1Δ or Rpo41Δ cells. Deletion of Mtf1 or Rpo41 causes a reduction in mitochondrial membrane potential in *S. pombe*. Thus the phenotypes for Mtf1 and Rpo41 deletion mutants that we observe in this study are consistent with the idea that Mtf1 and Rpo41 have a role in *S. pombe* mitochondrial function. These data also suggest a link between the role of Mtf1 and Rpo41 in mitochondrial transcription and the aberrant phenotypes observed in the deletion cells. Deletion of either Mtf1 or Rpo41 caused a reduction in transcription of mitochondrial genes to background levels (Figure 3B and F), suggesting that both Mtf1 and Rpo41 are required for the transcription of mitochondrial genes. Significantly, cells in which Mtf1 has been deleted contain normal levels of Rpo41, but mitochondrial transcription remains low, indicating that in the absence of Mtf1, Rpo41 retains no residual RNA polymerase activity *in vivo* although we observed that Rpo41 alone caused a band shift to the mitochondrial promoters in EMSA and that Rpo41 alone can transcribe the Pma, Pmi and Pin promoters at low levels *in vitro* (Figure 5A and B). We also showed that over-expression of Mtf1 leads to an increase in mitochondrial transcription (Figure 3D) although in cells that over express Mtf1, Rpo41 levels were limiting. From this we infer that an increase in Mtf1 levels led to an increase in transcriptional efficiency by Rpo41. In *S. cerevisiae* Mtf1 and Rpo41 combine as a holoenzyme to initiate transcription at the promoter sequence, before Mtf1 is released from the transcription complex (55). In this model, Mtf1 is a component of the Mtf1/Rpo41 holoenzyme for transcriptional initiation and also exists in a free form. An excess of Mtf1 would be predicted to increase the concentration of the 'holoenzyme' complex and therefore increase transcriptional initiation at mitochondrial promoters. This was shown to be the case with Mtf1 homologs mtTFB2 in *Drosophila* (28) and human mtTFB1/mtTFB2 (26). When Mtf1 and Rpo41 are present together, the complex (55) melts the promoter from −4 to +2 without requiring initiating NTPs (19). Direct interactions between *S. cerevisiae* Mtf1 and its human homolog mtTFB2 and the non-template strand have recently been observed (56–58). The influence of Mtf1 deletion or over expression on mitochondrial transcription in cells is consistent with the notion that Mtf1 is a mitochondrial transcription factor. We next provide biochemical evidence through EMSA and *in vitro* transcription experiments to show that Mtf1 is a mitochondrial

transcription factor and Rpo41 the mitochondrial RNA polymerase (Figures 4, 5A and B). The transcriptional start sites identified by 5′-RACE *in vitro* map to the same positions as the mitochondrial promoters *in vivo*, demonstrating the transcriptional activity of Mtf1 and Rpo41 (Supplementary Figure 2B–D and Figure 5B).

A band shift of the mitochondrial promoter DNA by *S. pombe* Mtf1 could only be detected upon prolonged exposure of the gel in the EMSA experiments. We note that direct interactions between promoter DNA and *S. cerevisiae* Mtf1 and human mtTFB2 have also been detected (9,19,56–58). *Schizosaccharomyces pombe* Rpo41 on its own however, can cause a band shift to Pma, Pmi and Pin (Figure 4), we observed that Rpo41 on its own can also initiate low levels of transcription from all three promoters *in vitro* (Figure 5A). This is in contrast to the situation in *S. cerevisiae*. *Saccharomyces cerevisiae* Rpo41 can not recognize and melt the mitochondrial promoter on its own, or initiate RNA synthesis unless the promoter is pre-melted around the transcription start site (18,19). We also note that both Mtf1 and Rpo41 together produce a more intense supershifted band, suggesting that the Mtf1/Rpo41 holoenzyme binds to all three promoter sequences more efficiently. This is also consistent with the observation that Mtf1 overexpression can induce mitochondrial transcription *in vivo* and *in vitro* (Figures 3D, 5A and B).

As the first systematic study of the cellular function and biochemistry of Rpo41 and Mtf1 in *S. pombe,* these studies represent a further significant step in the development of *S. pombe* as an alternative and complementary model system for molecular genetic and biochemical study of mitochondrial transcription. We anticipate that in the future the convenience and malleability of *S. pombe* will also establish it as a valuable model system for the study of mitochondrial-nuclear interactions.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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