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A novel function of the mitochondrial transcription factor Mtf1 in fission yeast; Mtf1 regulates the nuclear transcription of *srk1*

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ABSTRACT

In eukaryotic cells, Mtf1 and its homologues function as mitochondrial transcription factors for the mitochondrial RNA polymerase in the mitochondrion. Here we show that in fission yeast Mtf1 exerts a non-mitochondrial function as a nuclear factor that regulates transcription of *srk1*, which is a kinase involved in the stress response and cell cycle progression. We first found Mtf1 expression in the nucleus. A ChIP-chip approach identified *srk1* as a putative Mtf1 target gene. Over expression of Mtf1 induced transcription of the *srk1* gene and Mtf1 deletion led to a reduction in transcription of the *srk1* gene *in vivo*. Mtf1 overexpression causes cell elongation in a *srk1* dependent manner. Mtf1 overexpression can cause cytoplasmic accumulation of Cdc25. We also provide biochemical evidence that Mtf1 binds to the upstream sequence of *srk1*. This is the first evidence that a mitochondrial transcription factor Mtf1 can regulate a nuclear gene. Mtf1 may also have a role in cell cycle progression.

INTRODUCTION

Mitochondrial transcription is independent of nuclear transcription and is relatively simple. Although the mitochondrion has its own genome, which encodes the mitochondrial rRNAs, tRNAs and some proteins in the respiratory chain, the majority of mitochondrial proteins are encoded by nuclear genes, synthesized in the cytoplasm and then imported into the mitochondrion to exert their mitochondrial activity. These proteins include the mitochondrial RNA polymerase and mitochondrial transcription factors that are responsible for mitochondrial transcription. Mtf1 in *Saccharomyces cerevisiae* and its homologues in higher organisms have been extensively characterized as mitochondrial transcription factors for the mitochondrial RNA polymerase [reviewed in (1–3)]. We have recently cloned and characterized Mtf1 in the fission yeast *Schizosaccharomyces pombe* as a mitochondrial transcription factor. Analysis of the phenotypes of Mtf1 deletion cells suggested that *S. pombe* Mtf1 was essential for cell growth and cell morphology. We found that Mtf1 deletion caused a reduction in mitochondrial membrane potential. Deletion of Mtf1 led to a decrease in transcription of the mitochondrial genes. Correspondingly, over expression of Mtf1 increased transcription of the mitochondrial genes. Purified Mtf1 and Rpo41 (the mitochondrial RNA polymerase) together were shown to bind to the *S. pombe* mitochondrial promoters. They also can support transcription from the *S. pombe* mitochondrial promoters *in vitro* (H. Jiang, W. Sun, Z. Wang, J. Zhang, D. Chen and A.I.H. Murchie submitted for publication). To date, all of the biological activities associated with Mtf1 or its homologues have been limited to the mitochondrion.

Genomic studies of transcription and its regulation in proliferating cells has shed light on the cell cycle regulated transcriptional program and the underlying transcriptional networks of cell cycle control (4–12). There are several hundred genes that are periodically expressed throughout the cell cycle (13). Although some transcription factors which regulate each of the four main phases of cell cycle have been characterized in *S. pombe* [reviewed in (14)], for

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The authors wish it to be known that, in their opinion, the third and fourth authors should be regarded as joint Second Authors.

many periodically expressed genes, the mechanisms responsible for their cell-cycle regulation are still not known and not all of the transcription factors involved in cell cycle control have been identified.

The stress response kinase *srk1* is a nuclear encoded gene and has no known mitochondrial function. *srk1* was initially identified from global transcriptional responses to environmental stress in *S. pombe*. The transcription of the *srk1* gene is up regulated upon exposure to different environmental stresses (15,16). Srk1 can form a complex with the stress activated Sty1/Spc1 MAP kinase and is directly phorsphorylated by Sty1 (15). In fission yeast Cdc25, a universally conserved protein phosphatase promotes mitotic entry by activating the cyclin-dependent kinase Cdc2 (Cdk1) through dephosphorylation (17–19). Cdc25 builds up in the nucleus during the cell cycle peaking in late G2 (20,21). Srk1 regulates the onset of mitosis by inhibiting the Cdc25 phosphatase. Overexpression of Srk1 causes cell cycle arrest in late G2 phase, and cells that lack Srk1 enter into mitosis pre-maturely. Srk1 phosphorylates Cdc25 and this phosphorylation is necessary for Srk1 to delay mitotic entry and leads to an accumulation of Cdc25 in the cytoplasm (22). These studies demonstrated that Srk1 controls mitotic entry by directly phosphorylating Cdc25 during the cell cycle.

In this study, we report that the transcription of the *srk1* gene is regulated by the mitochondrial transcription factor Mtf1 at the transcriptional level and that Mtf1 may have a role in cell cycle control.

MATERIALS AND METHODS

Fission yeast strains, media and techniques

The strains used in this study are listed in Table 1. Media and genetic methods for studying *S. pombe* were as described in (20).

Oligonucleotide DNA 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.

Table 1. Strains and plasmids used in this study

Strain/ plasmid	Genotype	Origin
AM01	*972h⁻*	J. Bähler
AM02	*h⁻leu1-32*	J. Bähler
AM101	*h⁻mtf1-tap::kan*	This study
AM09	*h⁻cdc25-GFP:kan ura4-D18 ade6-M210 leu1-32*	(R. Aligué/ K. Gould)
RA112	*h⁻srk1::kanMX6leu1-32ura4-D18*	R. Aligué
AM03	*h⁻srk1-tap::kan*	This study
AM103	*h⁻leu1-32* pREP3-Mtf1	This study
AM107	*h⁻leu1-32* pREP3-Mtf1GFP	This study
AM108	*h⁻leu1-32* pREP3-Mtf1TAP	This study
AM109	*h⁻leu1-32* pREP3-GFP	This study
AM210	*E. coli BL21-* pGEX-4T-mtf1	This study
AM213	pFA6a2PA	J. Bähler

Plasmid construction

pREP3-Mtf1GFP was generated by insertion of PCR fragment of GFP (Green Fluorescent Protein) in the SmalI site of pREP3-Mtf1 as described (H. Jiang, W. Sun, Z. Wang, J. Zhang, D. Chen and A.I.H. Murchie submitted for publication).

ChIP-chip analysis

Mtf1 tandem protein-A (TAP) tagged cells were grown to OD_{600} 0.5 and fixed in 1% formaldehyde for 15 min at room temperature and then in 125 mM glycine for 5 min. After being washed twice with TBS and resuspended in 0.4 ml of lysis buffer [50 mM HEPES-KOH (pH 7.4), 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin], the cells were disrupted with glass beads in a bead beater for 1 min followed by sonication four times for 15 s each. Supernatant was then used for binding the IgG Sepharose 6 fast flow beads (GE 17-0969-01). DNA samples (2 μg) were labelled with Cye-3 or Cye-5 (Klenow fragment, Invitrogen) and followed by ethanol percepertation and resuspention in 10 μl of hybridization buffer (5×SSC, 6 Denhardt's, 60 mM Tris–HCl pH 7.6, 0.12% sarkosyl, 48% formamide; filter sterilized). Samples were hybridized to microarrays at 49°C in a Grant Boekel hybridization oven for 16 h. Slides were washed as described (23). The microarray used was an intergenic array covering all non-coding regions as described (23). The microarray do not cover the mitochondrial chromosome. Data acquisition, processing and normalization were as described (23).

Real-time PCR analysis

Total RNA was extracted from cells using TRIzol Reagent (Invitrogen). Reverse transcription was performed using TaKaRa PrimeScipt™ 1st Strand cDNA Synthesis kit followed by quantitative real-time PCR on iQ5 Continuous Fluorescence Detector System (Bio-Rad). The PCR reactions contained 250 nM of primers, 1 μl cDNA (5 ng), 10 μl 2X SYBR-green Realtime PCR Master Mix (SYBR Premix Ex Taq ™, TaKaRa) in a total volume of 20 μl with at least two independent biological repeats and four technical repeats. Error bars were generated from the standard deviation (calculated by the Bio-Rad iQ5 Continuous Fluorescence Detector System software).

Chromosomal TAP tagging of *srk1* or *mtf1*

The chromosomal *srk1* or *mtf1* TAP tagged strain (*h⁻srk11-tap::kan or h⁻mtf11-tap::kan*) was constructed in haploid cells by using PCR based approach as described previously (4). Plasmid pFA6a2PA containing TAP sequence was used as PCR template. The primer sequence can be found in Supplementary Table S1. Chromosomal tagging was confirmed by colony PCR and detection of the TAP tag by western blot using IgG peroxidase antibody (Sigma 127K4847).

Western blot analysis

The *srk1* TAP tagged strain was grown in 50 ml EMM medium with or without 60 μM thiamine at 30°C to an OD_{600} of 0.5 as previously described (H. Jiang, W. Sun, Z. Wang, J. Zhang, D. Chen and A.I.H. Murchie submitted for publication).

Cell staining and confocal microscopy

Cells were fixed in 70% ethanol followed by staining with 4′,6′-diamidino-2- phenylindole (DAPI) or 100 nM of Rhodamine B in buffer (10 mM HEPES buffer, pH 7.4, containing 5% glucose) or mitochondrial dye DiOC6(3) before visualization under a confocal Leica TCS-SP5 microscope.

5′ RACE to determine the transcription start site of *srk1*

The wild-type cells were grown to OD = 0.5, 10ml of culture was used for extraction of total RNA (16). DNA was removed by DNase I (Fermentas) from the RNA sample. Reverse transcription and PCR reaction was carried out using SMART™RACE cDNA Amplification Kit (Clontech). The sequence of the gene specific primer can be found in Supplementary Table S1. The transcription start site was determined from the DNA sequence.

Electrophoretic Mobility Shift Assay

Biotinylated probes are made by PCR with 5′-end biotin-labelled forward primer. About 200 ng of purified Mtf1 protein was incubated with 30–50 fMol of labelled probes in the presence of 5 ng/μl of polydI/dC unless stated otherwise in binding buffer (10 mM Tris–HCl pH 7.5, 50 mM KCl, 0.1 mM EDTA, 1 mM DTT) for 30 min on ice. The DNA/protein mixture was then used to run a 6% polyacrylamide native gel at 4°C followed by transferring to a Hybond-N+ membrane (Amersham) and detected with a LightShift Chemiluminescent EMSA (Electrophoretic Mobility Shift Assay) kit (Thermo Scientific) and Mtf1 was expressed and purified as previously described (H. Jiang, W. Sun, Z. Wang, J. Zhang, D. Chen and A.I.H. Murchie submitted for publication).

RESULTS

The mitochondrial transcription factor Mtf1 also localizes to the nucleus

We have characterized the *S. pombe* gene SPAC1002.08c as the mitochondrial RNA polymerase specificity factor Mtf1 in *S. pombe*. Mtf1 has been localized to the mitochondrion of *S. pombe* in a genome-wide protein localization study (24). To further investigate Mtf1 localization in *S. pombe* we constructed the plasmids pREP3-Mtf1GFP and pREP3-GFP that express Mtf1 and GFP fusion and GFP only as a control (25). Expression of Mtf1-GFP or GFP was under the control of the thiamine repressible *nmt1* promoter (26). After transformation we observed cells in the presence and absence of 60 μM thiamine by confocal microscope. Surprisingly, in the absence of thiamine some cells expressing Mtf1-GFP have an intense green fluorescence signal in the position of the nucleus together with scattered fluorescent spots in the cell cytoplasm (Figure 1A). Figure 1A shows a series of focal plane images of two cells expressing Mtf1-GFP scanned at 0.5 μm intervals by confocal microscope. Examination of the location of the intense fluorescence by comparison with DAPI (nuclear stain) and DiOC6 (mitochondrial stain) stained cells (Figure 1B) suggested that it could originate in the nucleus. In contrast, in the presence of thiamine, no green fluorescence signal was observed in cells containing pREP3-Mtf1GFP (data not shown), and cells transformed with the control plasmid pREP3-GFP showed an even distribution of the GFP signal in the absence of thiamine (Figure 1B). As a control we initially stained cells transformed with pREP3-Mtf1GFP in the absence of thiamine with the mitochondrial dye Rhodamine B and then observed Mtf1GFP fluorescence in these cells. The overlaid image from the Rhodamine B staining (red) and the Mtf1-GFP signal (green) shows that the Mtf1-GFP signal is mostly mitochondrial and that some cells have an additional intense source of green fluorescence that may originate in the nucleus (Figure 1C). To further investigate the possibility of nuclear localization of Mtf1-GFP, we co-stained Mtf1-GFP expressing cells with DAPI and overlaid the Mtf1-GFP image (Figure 1D). Figure 1D shows that the Mtf1-GFP signal superimposes on the nuclear signal from DAPI confirming that Mtf1-GFP does indeed localize to the nucleus. In addition to the strong fluorescence from the nucleus, mitochondrial Mtf1-GFP signals are visible as scattered spots in the cytoplasm as has been previously described in a genome-wide protein localization study (24). The different intensities and distribution of the Mtf1-GFP signal that were observed in cells carrying pREP3-Mtf1GFP, are presumably due to differences in plasmid copy number within the population of transformed cells. To investigate whether endogenous levels of Mtf1 could also be detected in the nucleus we used the strain Mtf1-TAP (described in the following section) in which the *mtf1* gene is fused to a TAP tag on the chromosome, the presence of Mtf1-TAP in the nucleus was confirmed by western blotting of nuclear extracts (from isolated nuclei) (Supplementary Figure S1).

A ChIP-chip approach to identify putative Mtf1 target genes in the nucleus

We have characterized Mtf1 as a mitochondrial transcription factor in *S. pombe*. The localization of Mtf1 in the nucleus led us to speculate that Mtf1 might also have a novel nuclear transcription factor activity. To search for the nuclear target genes of Mtf1 we employed a chromatin-immuno-precipitation microarray strategy (ChIP-chip). First we created a strain with a TAP tag to the *mtf1* gene on *S. pombe* chromosome by targeted recombination (4). This strain was then used to isolate Mtf1-binding DNA for hybridization to microarrays. Mtf1-TAP tagged cells display the same phenotype and

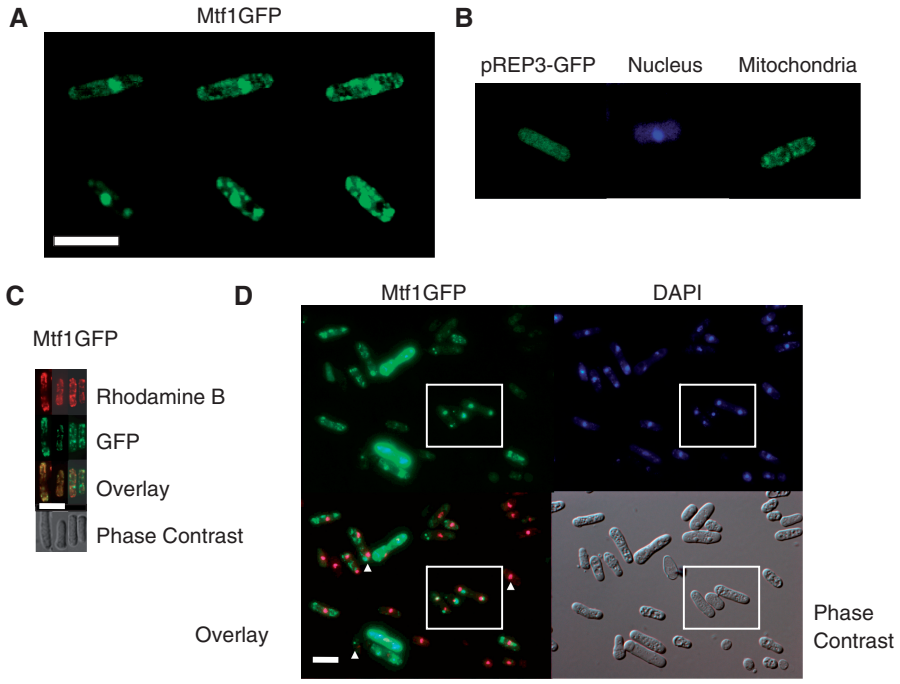

Figure 1. Mtf1 localizes to the nucleus. (**A**) Plasmid pREP3-Mtf1GFP encoding an Mtf1GFP fusion protein was transformed into *S. pombe* strain *h⁻leu1-32*. A series of focal plane images of cells generated by scanning cells at 0.5 µm intervals by confocal microscope. Bar is 10 µm. (**B**) Controls showing even distribution of GFP in cells transformed with pREP3-GFP and location of nucleus (stained with DAPI) and mitochondrion [stained with the mitochondrial dye DiOC6(3)] in fission yeast. (**C**) Cells transformed with plasmid pREP3-Mtf1GFP were stained with the mitochondrial dye rhodamine B as shown in red in the top panel; Mtf1 GFP is shown in green in the second panel; the top and second panels were merged in the third panel. The fourth panel shows phase contrast images of the cells. The bar corresponds to 10 µm. (**D**) Cells transformed with the plasmid pREP3-Mtf1GFP were stained with DAPI and photographed with a filter specific for GFP fluorescence and DAPI. The merged image is shown in the overlay with DAPI changed from blue to red color for easy visualization. The white box and white arrow indicate the cells with the red (DAPI) and green color (GFP) shown in the same location. Bar is 10 µm.

viability as wild-type cells, while, in contrast, cells in which Mtf1 function was impaired had poor viability and an abnormal phenotype. We therefore reasoned that in the TAP-tagged *mtf1* strain the function of Mtf1 was probably unaffected by the presence of the TAP tag. Cells containing TAP-tagged *mtf1* were incubated in formaldehyde (1%) to cross-link proteins to nucleic acids, and potential Mtf1 binding target sequences were pulled down from lysed cells by binding to IgG Sepharose beads (27). The cross-links were reversed and Mtf1 binding DNA fragments were labelled with a fluorescent dye (Cy3 or Cy5), and hybridized to the microarray, which contained probes covering all of the intergenic regions of the *S. pombe* genome (23). The microarray contained only nuclear encoded genes. This method reveals intergenic nuclear DNA fragments that are bound to Mtf1 *in vivo* over the entire *S. pombe* genome. Analysis of these DNA fragments may therefore identify intergenic DNA sequences for which Mtf1 plays a role in the transcriptional regulation of neighbouring genes. Typically the tile DNA sequence is positioned between the coding sequence of two genes. To validate the ChIP-chip data, we designed PCR primers for the two genes on each side of the tiles and performed real-time PCR to detect the transcription of the genes in cells where Mtf1 was overexpressed or deleted. The details of the real-time PCR experiments to detect the transcription of the genes in cells where Mtf1 was overexpressed or deleted are described in the next section. Our analysis focused on the top 22 tiles (intergenic regions of DNA) that had the highest ratio in the ChIP-chip experiment, we analysed 41 neighbouring genes. The ratios of the 22 tiles are shown in Table 2, together with real-time PCR data of the transcription profile of the 41 neighbouring genes in the Mtf1 deletion and overexpression strains and their relative orientation. The genes adjacent to the tile are organized in three possible orientations; they can be divergent (D), convergent (C) or in tandem (T), thus, for divergent genes the tile sequence on the microarray is 5′ to both genes and for convergent genes the tile sequence on the microarray is 3′ to both genes. Transcription of 15 of the 41 genes was found to be induced when Mtf1 was overexpressed and reduced in the Mtf1 deletion strain (Table 2). These 15 genes are therefore potentially transcriptionally regulated by Mtf1. One DNA fragment in particular gave a consistently high ratio on the microarray (Table 2), this was the upstream region of *srk1*, a well characterized kinase involved in the stress response and in cell cycle control (15,22,28). Additionally, transcription of 6 genes was induced in cells where Mtf1 was overexpressed but unchanged in the Mtf1 deletion strain. Transcription of a further two convergent genes was induced in the Mtf1

Table 2. Analysis of the intergenic regions that had the highest ratios in the ChIP-chip experiment

Tile	Ratio ChIP-chip	Name	RT-PCR Deletion	RT-PCR Over	Product	Orientation	Name	RT-PCR Deletion	RT-PCR Over	Product
chr2.insertionoverlap:	6.1	SPCC1322.07c	1.8	3.3	Mug150	D	SPCC1322.08	0.7	18.5	Srk1
chr2.mini.tile157:	4.99	SPCC1322.12c	0.3	0.4	Bub1	D	SPCC1322.13	0.4	2.8	Ade6
chr1.2362.1	5.05	SPAC212.11	0.9	0.3	RecQ	D	SPAC212.09c	1.4	0.9	pseudogene
chr1.15s.1	5.92	SPAC1805.09c	0.9	2.4	Fmt1	D	SPAC1805.10	N/A	N/A	orphan
chr2.mini.tile179:	5.03	SPBCPT2R1.08c	1	0.4	RecQ	–	–	–	–	–
chr2.1850.1	5.38	SPBC1289.11	1.2	5.6	Spf38	T	SPBC1289.12	N/A	N/A	usp109
chr3.291.3	4.67	SPCC1020.10	1.9	5.3	Oca2	T	SPCC1020.09	1.1	1.9	Gnr1
chr1.1035.1	4.05	SPAC6B12.03c	0.8	2.6	HbrB	T	SPAC6B12.02c	1.2	1.1	Mus7/Mms22
chr1.9s.1	5.24	SPAPB17E12.06	0.4	2	pseudogene	T	SPAPB17E12.05	0.5	0.8	L37
chr3.715.1	6.82	SPCC162.06c	0.7	2.8	Vps60	C	SPCC162.05	0.7	3.7	Coq3
chr1.103.1	4.54	SPAC806.05	5.1	2.8	DUF1763	C	SPAC806.06c	0.6	1.8	NMN
chr1.444.1	4.72	SPAC23C4.19	0.6	2.5	Spt5	C	SPAC1A6.01c	0.9	1.9	HTR
chr1.1112.1	6.39	SPAC823.04	0.5	2.4	DUF947	C	SPAC823.05c	0.5	2.5	Tlg2
chr2.1500.1	4.68	SPBC16D10.03	1	2.1	Pgp2	C	SPBC16D10.04c	1.6	1.8	Dna2
chr2.119.1	4.67	SPBC106.01	1	1.9	Mph1	C	SPBC106.02c	28.6	4.5	Srx1
chr3.405.1	4.6	SPCC1393.05	1.2	1.5	Ers1	C	SPCC1393.06c	0.9	0.9	Ipi1
chr1.10s.1	4.18	SPAC9.06c	0.6	1.5	adducin	C	SPAC9.05	N/A	N/A	mfh1
chr1.668.1	4.6	SPAC23H4.13c	0.7	1.1	dubious	C	SPAC23H4.12	1.3	1.7	Alp13
chr2.1112.1	4.73	SPBC4F6.12	2.2	0.2	Pxl1	C	SPBC4F6.13c	1.3	0.9	BOP1NT
chr2.mini.tile82:	4.59	SPCC1322.09	0.6	6.1	conserved					
chr2.mini.tile87:	5.32	SPCC1322.09	1.8	1.5	conserved fungal protein					
chr2.mini.tile37:	4.6	SPCC330.05c	0.5	1.3	Ura4		–			
chr2.mini.tile102:	2.99	SPCC1322.11	0.5	1	L23					

The analysis of the top 22 intergenic regions (tiles) on the microarray that had the highest ratios in the ChIP-chip experiment. The ChIP-chip ratios are shown, together with real-time PCR data for neighboring genes for Mtf1 deletion and overexpression strains. The genes adjacent to the tile have three possible orientations; divergent (D), convergent (C) or in tandem (T). Note that the upstream region of *srk1* gave a consistently high ratio by ChIP-chip and by RTPCR. N/A signifies genes for which no real-time PCR signal was detected. RecQ is located close to the end of chromosome 2.

deletion strain and in cells in which Mtf1 was overexpressed. These data suggest that Mtf1 may have multiple roles in regulating the transcription of certain nuclear genes.

Mtf1 is associated with transcription of the *srk1* gene *in vivo*

The ChIP-chip experiment identified *srk1* as a possible Mtf1 target gene. We confirmed this by real-time PCR experiments to analyze the transcript abundance of *srk1* in cells in which Mtf1 was overexpressed or deleted. We used the plasmid pREP3-Mtf1 in which the Mtf1 coding sequence was under the control of the thiamine repressible *nmt1* promoter. Initial experiments had confirmed that in the absence of thiamine, *mtf1* transcript abundance was induced; under these conditions Mtf1 protein levels were also elevated as detected by western blot analysis using the plasmid pREP3-Mtf1TAP which contained MTF TAP fusion. Overexpression of Mtf1 led to an increase in transcription of the mitochondrial genes, but transcription of the tubulin gene was unaffected in cells that over express Mtf1. Analysis of *srk1* mRNA by real-time PCR here showed that the transcript abundance of *srk1* mRNA was significantly increased in cells that overexpress Mtf1 compared with cells transformed with only the pREP3 vector (Figure 2A). We have previously shown that in the *mtf1* deletion mutant *mtf1Δ2* the transcription of *mtf1* was reduced to background levels and that transcription of the mitochondrial genes was almost completely abolished, in comparison, transcription of tubulin was relatively unchanged in the wild-type and the *mtf1* deletion cells. The analogous experiment in the *mtf1Δ2* mutant shows the transcript abundance of *srk1* mRNA to be reduced to background (Figure 2B). Thus overexpression of Mtf1 causes an increase in *srk1* transcription, and deletion of *mtf1* abolishes transcription of *srk1* almost completely. This supports the observations from the ChIP-chip analysis that Mtf1 may interact with the upstream sequence of *srk1 in vivo*.

Mtf1 overexpression causes cell elongation in a *srk1* dependent manner

Overexpression of Srk1 has been shown to inhibit cell entry into mitosis; cells that overexpress Srk1 are elongated and FACS analysis of these cells showed them to be arrested in late G2 phase (28). We observed that cells that overexpressed Mtf1 (transformed with the plasmid pREP3-Mtf1) became significantly elongated compared to wild-type cells or cells grown in the presence of 60 μM thiamine (Figure 3A), and show signs of cell division arrest when grown on solid plates (Figure 3B). We also observed that cells overexpressing Mtf1 GFP became elongated in the absence of thiamine, suggesting that the Mtf1 GFP fusion protein is probably functional (Figure 3A). To examine the protein levels of Mtf1 in cells over expressing *mtf1*, TAP was inserted into the 3′-end of

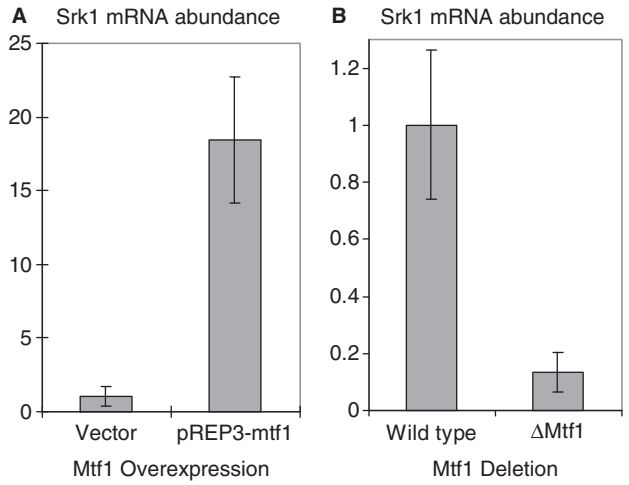

Figure 2. Mtf1 is associated with transcription of the *srk1* gene *in vivo*. (**A**) Real-time PCR of *srk1* transcripts abundance in cells transformed with vector only and cells transformed with pREP3-Mtf1 overexpressing Mtf1 in the absence thiamine. Note that *srk1* mRNA level is increased when Mtf1 is overexpressed. (**B**) Real-time PCR of *srk1* transcripts abundance in wild-type cells and Mtf1 deletion mutant cells, showing that transcription of *srk1* is reduced in Mtf1 deletion mutant cells. Act1 was used as a reference for the Real-time PCR experiment. Error bars were generated from the standard deviation (calculated by the software on Bio-Rad iQ5 Continuous Fluorescence Detector System) of four technical repeats.

Mtf1 (Mtf1TAP fusion) to generate the plasmid pREP3-Mtf1TAP and the TAP antibody was used to perform a western blot to detect protein levels of Mtf1. We confirmed that Mtf1 protein levels were induced in the absence of thiamine, but in the presence of 60 μM thiamine Mtf1 protein levels were very low (Figure 3C). We have shown that overexpression of Mtf1 caused an increase in *srk1* transcript abundance. If the increase in *srk1* transcription is matched by a proportional increase in Srk1 protein levels, then the elongation that we observe in cells in which Mtf1 is overexpressed is probably caused by Srk1 protein overexpression inhibiting cell entry into mitosis. To measure the effect of Mtf1 overexpression on Srk1 protein levels directly, we created a chromosomal *srk1* Tap (4) tagged strain, and then transformed the plasmid pREP3-Mtf1 in this stain. Tagged Srk1 was detected by western blotting in the presence or absence of thiamine using antibodies to protein-A. Figure 3D shows that in the absence of thiamine, in cells where Mtf1 is overexpressed, Srk1 protein levels are indeed induced compared to the Srk1 protein levels in cells transformed with pREP3-Mtf1 in the presence of thiamine. In comparison, controls for actin are relatively unchanged and independent of added thiamine. FACS analysis showed that overexpression of Srk1 causes cell cycle arrest in late G2 phase (28). Predictably, FACS analysis of cells that overexpressed Mtf1 showed that these elongated cells have a nucleus and 2n DNA content, indicating that the arrest may be in the late G2 phase (data not shown). To further investigate this, we transformed pREP3-Mtf1 into the *srk1* deletion strain RA112h$^-$ [a kind gift from Rosa Aligué (28)]. The *srk1* deletion strain was completely insensitive to Mtf1 overexpression; in the absence of thiamine, overexpression of Mtf1 no longer resulted in cell elongation in the *srk1* deletion strain (Figure 3F); these cells showed no changes with or without overexpression of Mtf1 when grown on solid plates (Figure 3E). This suggests that Mtf1 overexpression causes cell elongation in a *srk1* dependent manner.

Overexpression of Mtf1 causes cytoplasmic accumulation of Cdc25

To further analyze cellular function of Mtf1, we examined the effect of Mtf1 overexpression on the localization of Cdc25 *in vivo*. The plasmid pREP3-Mtf1 was transformed into a chromosomal Cdc25-GFP tagged strain (a kind gift from Rosa Aligué and Katherine Gould). In the presence of thiamine when Mtf1 overexpression is repressed, or in cells transformed with the vector only, Cdc25-GFP was restricted to the nucleus. In contrast, in the absence of thiamine, in cells where Mtf1 is overexpressed Cdc25-GFP is dispersed to the cytoplasm (Figure 4A). *mtf1* mRNA abundance is induced in Cdc25-GFP cells when *mtf1* is over expressed (Figure 4B), and western blotting of Cdc25-GFP cells transformed with pREP3-Mtf1TAP shows that Mtf1 protein levels are induced in the absence of thiamine (Figure 4C). We also detected an increase in *srk1* transcripts in Cdc25-GFP expressing cells (Figure 4D). However, whether the cytoplasmic accumulation of Cdc25 as a consequence of Mtf1 overexpression is completely or partially Srk1 dependent requires further investigation.

Biochemical evidence that Mtf1 may act as a transcription factor for *srk1*

We have identified a DNA fragment in the upstream region of the *srk1* gene that binds to Mtf1 *in vivo* by chromatin immunoprecipitation and microarray, and we have shown that Mtf1 is associated with transcription of the *srk1* gene *in vivo*. To gain biochemical evidence *in vitro* that Mtf1 can bind to the upstream sequence of the *srk1* gene, we used purified Mtf1 and the upstream sequence of the *srk1* gene to perform electrophoretic mobility shift assays (EMSA) with biotin and streptavidin-based detection (29). The upstream region of the *srk1* gene spans almost 2000 nucleotides and the transcription start site for *srk1* has not been mapped accurately. To identify the exact DNA sequence for EMSA from the upstream of the *srk1* gene, we first performed 5′ RACE *in vivo* to determine the transcription start site of the *srk1* gene, the *srk1* transcripts mapped to a position corresponding to a start site 963 nt upstream of the coding DNA sequence (CDS) of the *srk1* gene (Figure 5A and C). We then selected a region of the DNA sequence upstream of the transcription start site to perform EMSA (Figure 5B). Mtf1 was purified in *Escherichia coli* BL21 cells as described previously. A biotinylated DNA probe was generated by PCR from a 5′ biotinylated primer and purified by gel electrophoresis. By titrating increasing amounts of Mtf1, we could detect a weak dose-dependant retarded species in the gel (Figure 5D) suggesting the formation of an Mtf1-DNA complex.

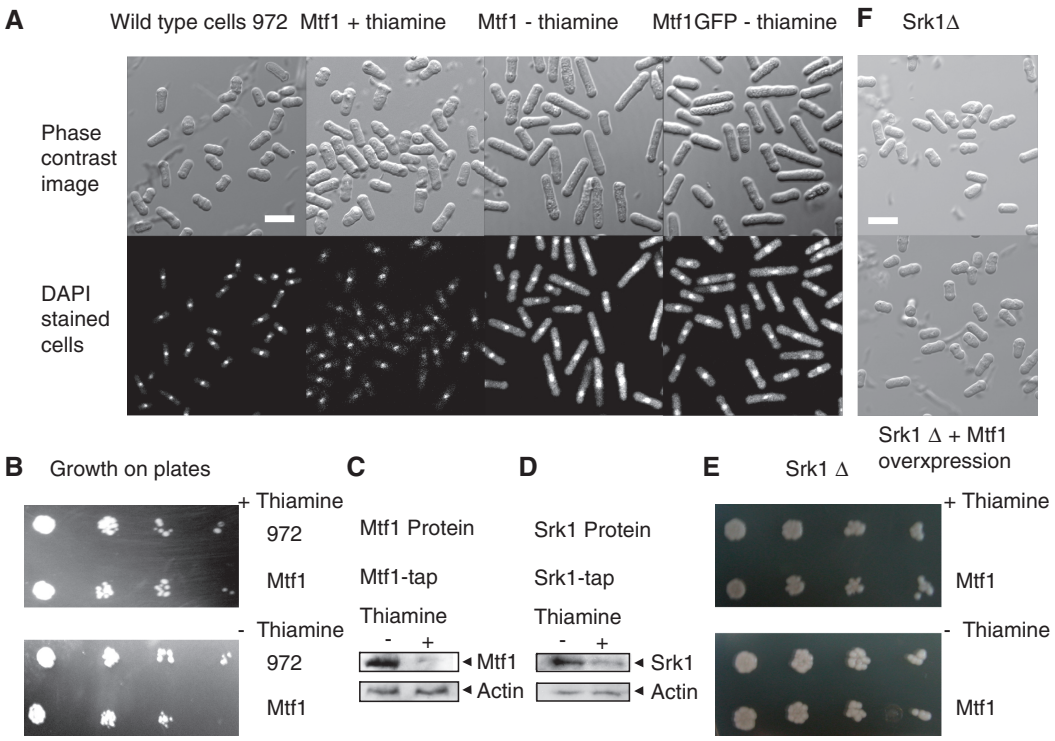

Figure 3. Mtf1 overexpression blocks progression into mitosis. (**A**) Wild-type cells and cells transformed with plasmid pREP3-Mtf1 or pREP3-Mtf1GFP encoding Mtf1 whole protein or Mtf1GFP were grown either in the presence or in the absence of thiamine in liquid medium. The top row shows phase contrast images of unstained cells and the bottom row DAPI stained cells both viewed by confocal microscope. Bar is 10 μm. (**B**) Wild-type cells and cells transformed with plasmid pREP3-Mtf1 were grown either in the presence or in the absence of thiamine on solid EMM plates. (**C**) Cells transformed with pREP3-Mtf1TAP was used to perform western blot to detect protein levels of Mtf1 using Rabbit anti-mouse IgG. Note that Mtf1-tap protein expression is induced in the absence of thiamine. Actin was used as a control for equal loading and detected with the β actin antibody (Abcam 8224). (**D**) The Srk1 TAP tagged strain was transformed with plasmid pREP3-Mtf1 in the presence or absence of thiamine. Srk1 protein levels were detected by western blot using Rabbit anti-mouse IgG, showing that Srk1 protein levels are induced when Mtf1 expression was induced in the absence of thiamine. Actin was used as a control for equal loading. (**E**) *srk1* deletion cells (RA112h^{-}) and *srk1* deletion cells transformed with pREP3-Mtf1 in the presence or in the absence of thiamine on solid EMM plates. (**F**) *srk1* deletion cells and *srk1* deletion cells transformed with pREP3-Mtf1 in the absence of thiamine viewed by confocal microscope. Note that overexpression of Mtf1 no longer leads to cell elongation in the *srk1* deletion strain.

The Mtf1-DNA complex was sensitive to competition by addition of a non-biotinylated probe with a corresponding reduction in the amount of retarded complex visible (Figure 5E, lanes 4 and 5). Although the initial EMSA experiments included poly-dIdC and NP40 in the binding reaction, we also observed an apparent increase in binding of Mtf1 to the probes in their absence of poly-dIdC or NP40 (Figure 5E, lanes 2 and 3). Binding by Mtf1 to the *srk1* fragment is highly reproducible (>10 times) and is consistent across batches of purified Mtf1 protein (>5 batches). Mtf1 is primarily regarded as and has been characterized as a mitochondrial transcription factor. To compare the interaction between Mtf1 and the *srk1* fragment with the mitochondrial promoter sequences, we performed EMSA experiments under the same experimental conditions. We also observed a weak band shift between the mitochondrial promoter Pmin and Mtf1 (Figure 5F). We have previously shown that Mtf1 and Rpo41 together can bind to Pmin much more efficiently. To investigate the specificity of the binding that we observed between Mtf1 and the *srk1* promoter, we selected a DNA fragment from the *srk1* CDS as a control for EMSA. Mtf1 with the *srk1* CDS sequence exhibited no band shift compared to the *srk1* promoter sequence (Figure 5G), suggesting that Mtf1 binding to the *srk1* promoter sequence is specific.

DISCUSSION

Mtf1 has been extensively characterized in *S. cerevisiae* and higher organisms as a mitochondrial transcription factor for the mitochondrial RNA polymerase (1,2,3,30). Thus far such studies have been limited to its mitochondrial activities. In *S. pombe* Mtf1 is also active as a mitochondrial transcription factor. Here we demonstrate for the first time that Mtf1 in fission yeast has a significant extra-mitochondrial role as a nuclear transcription factor. This is supported by several lines of evidence. (i) Using Mtf1-GFP we found that Mtf1 localizes to the nucleus and endogenously expressed Mtf1 was also detected in the nucleus (Figure 1 and Supplementary Figure S1). Moreover, over expression of either Mtf1-GFP or Mtf1 caused cell elongation indicating that Mtf1-GFP is probably functional (Figure 3). (ii) A genome-wide

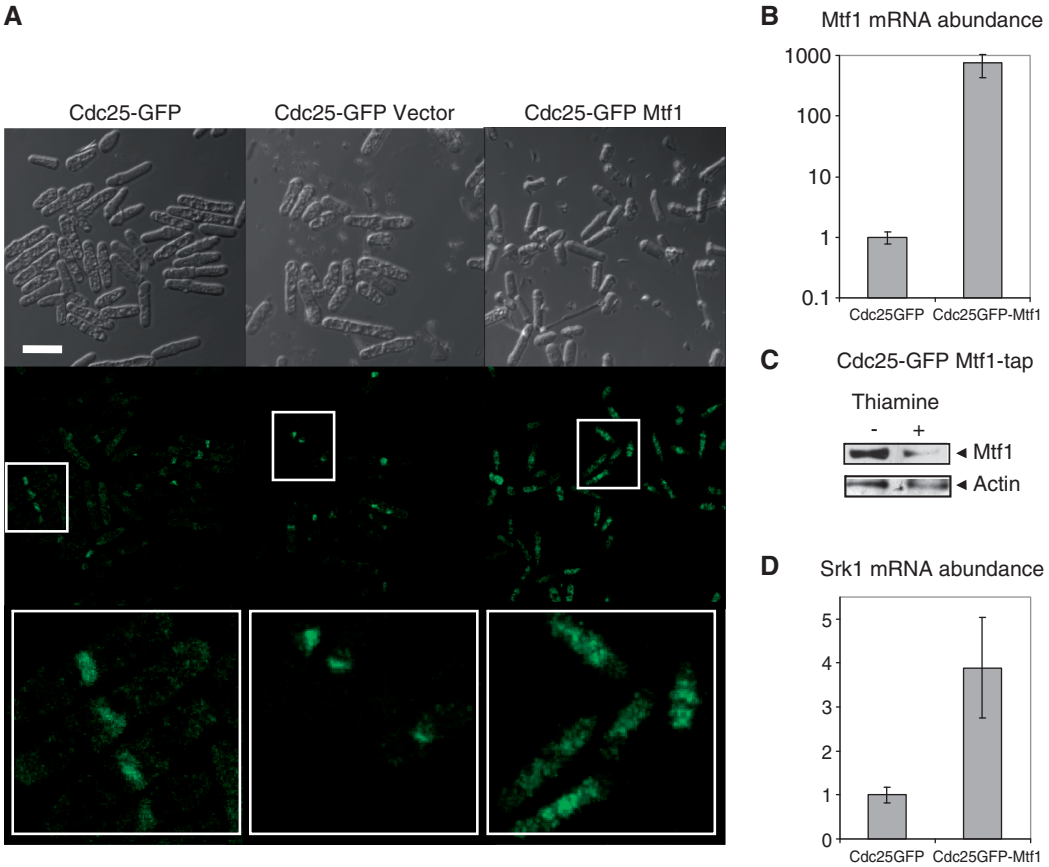

Figure 4. (**A**) Cdc25-GFP cells and Cdc25-GFP cells transformed with pREP3 or pREP3-Mtf1 were synchronized with HU and released cells were visualized by confocal microscopy 60 min after release. The boxed regions are shown at higher magnification in the lower panel. Note that Cdc25-GFP or Cdc25-GFP cells transformed with pREP3 vector only in cycling cells can be detected in the nucleus, but in cells overexpressing Mtf1, Cdc25-GFP is distributed throughout the cytoplasm. Bar is 10 μm. (**B**) Real-time PCR of *mtf1* transcripts abundance in Cdc25-GFP cells and Cdc25-GFP cells transformed with pREP3-Mtf1 in the absence thiamine. (**C**) Cdc25-GFP cells transformed with pREP3-Mtf1-TAP in the presence or absence of thiamine. Mtf1-tap protein levels were detected by western blot using Rabbit anti-mouse IgG. Actin was used as a control for equal loading. (**D**) Real-time PCR of *srk1* transcripts abundance in Cdc25-GFP cells and Cdc25-GFP cells transformed with pREP3-Mtf1 in the absence thiamine. For (B) and (D) Act1 was used as a reference for the Real-time PCR experiment. Error bars were generated from the standard deviation (calculated by the software on Bio-Rad iQ5 Continuous Fluorescence Detector System) of four technical repeats.

ChIP-chip analysis identified *srk1* as a target gene through the cross-linking of Mtf1 to the *srk1* sequence, suggesting that Mtf1 can interact with the *srk1* sequence *in vivo* (Table 2). (iii) Biochemical evidence showing that Mtf1 can bind to the *srk1* promoter sequence *in vitro* (Figure 5). (iv) Real-time PCR showed transcription of the nuclear gene *srk1* to be induced in cells where Mtf1 is overexpressed and abolished in cells where Mtf1 is deleted (Figure 2); a confirmation of the ChIP-chip analysis.

Further investigation of the cellular function of Mtf1 revealed that: (i) Mtf1 overexpression causes cell elongation. Srk1 protein levels are induced in cells that overexpress Mtf1, and Mtf1 overexpression leads to cell elongation in a *srk1* dependent manner (Figure 3). This is consistent with the observation of the Aligué group that Srk1 overexpression led to cell elongation and inhibition of entry into mitosis (28). (ii) We show that overexpression of Mtf1 causes cytoplasmic accumulation of Cdc25 (Figure 4) and transcription of *srk1* is induced (Figure 4). The exact mechanism by which the transcriptional regulation of *srk1* by Mtf1 contributes to the cytoplasmic accumulation of Cdc25 remains unknown. The Aligué group showed that overexpression of Srk1 led to the cytoplasmic accumulation of Cdc25 (28). We have shown that Mtf1 overexpression leads to an increase in *srk1* transcription and Srk1 protein expression. One possibility is that overexpression of Mtf1 results in the cytoplasmic accumulation of Cdc25 directly through increased Srk1 protein expression from the transcriptional activation of *srk1*. Transcription of *mtf1* is not cell cycle regulated (6). The localization of Mtf1-GFP in synchronized cells, did not appear to change during the cell cycle (data not shown) suggesting that Mtf1 expression may not be cell cycle regulated. The cellular function of Mtf1 during cell cycle control would benefit from further investigation. Srk1 is a stress response kinase. Transcription of *srk1* is induced upon exposure to all five environmental stresses (oxidative stress, heat stress, cadmium stress osmotic stress and MMS stress) (16) but

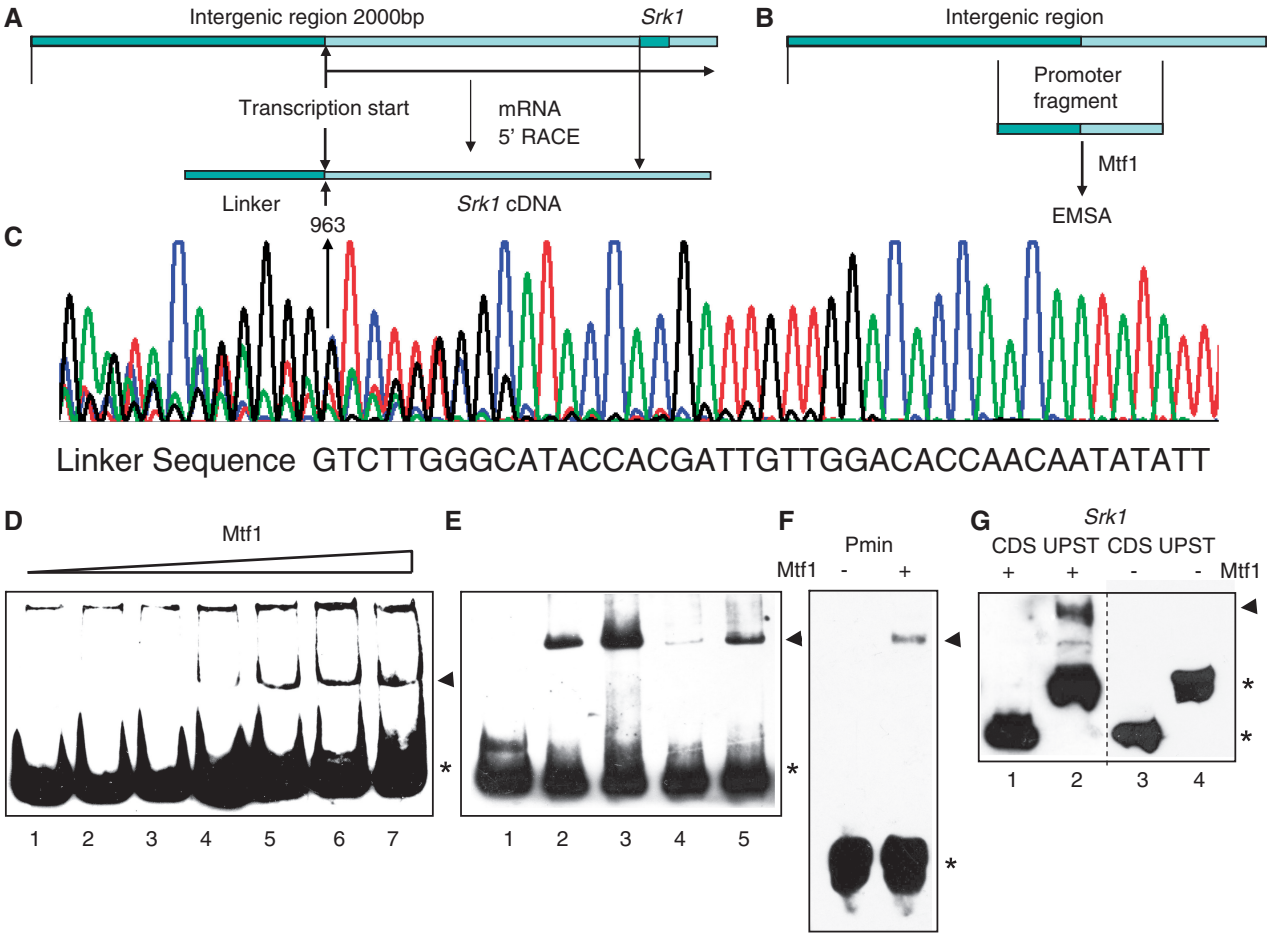

Figure 5. Biochemical evidence that Mtf1 may act as a transcription factor for *srk1*. (**A**) Schematic of the intergenic region of the upstream sequence of the *srk1* gene and the 5′ RACE strategy. (**B**) Location of the DNA fragments that were used for EMSA in the intergenic region of the upstream sequence of the *srk1* gene. (**C**) The sequence result of the 5′ RACE shows the transcription start site of the *srk1* gene. (**D–G**) EMSAs for Mtf1; the arrows show the position of the shifted material, the asterisks show the position of the unbound probes over a range of conditions. (D) 6% polyacrylamide native gels showing EMSA with different concentration of purified Mtf1. Lanes 1–7 contains 0.2, 2, 20, 100, 150, 200 and 400 ng of purified Mtf1. (E) EMSA with purified Mtf1 under different binding conditions. Lane 1 is free probe, lane 2 has no NP-40, lane 3 is the reaction in the absence of dIdC, lane 4 has 50 fMol of non-biotinylated cold probe added and lane 5 is the standard reaction conditions as described in materials and methods. (F) EMSA with a mitochondrial promoter Pmin with and without Mtf1. (G) EMSA with the upstream sequence (UPST) of the *srk1* DNA probe and a sequence taken from the coding sequence of the *srk1* gene (CDS) as a control with and without Mtf1. Lane 1 and 2 present CDS probe and *srk1* UPST DNA probe with purified Mtf1; Lane 3 and 4 present CDS probe and *srk1* UPST DNA probe only.

is not cell cycle regulated (6). The genome-wide transcriptional response profile of *mtf1* to the five environmental stresses showed that *mtf1* was also induced upon osmotic stress, cadmium stress and MMS stress (16). We speculate that as a nuclear transcription factor for *srk1*, Mtf1 may be involved in the response to these particular environmental stresses.

We have shown that Mtf1 is a nuclear transcription factor. Mtf1 has also been characterized as a mitochondrial transcription factor in *S. pombe*. Thus Mtf1 is able to function as a transcription factor in both the nucleus and the mitochondrion. This is consistent with our observation that Mtf1 can be detected in both organelles. In general, mitochondrial transcription is performed by the bacteriophage-like mitochondrial RNA polymerase and a mitochondrial transcription factor, and we have shown this to be the case in *S. pombe*; Mtf1 is the mitochondrial transcription factor for Rpo41, the mitochondrial RNA polymerase. Mtf1 and Rpo41 combine as a holoenzyme to recognize and initiate transcription at the core mitochondrial promoters. We have previously shown that Mtf1 and Rpo41 together bind to both of the mitochondrial promoters much more efficiently than Mtf1 or Rpo41 alone. Here we have shown weak but specific binding of Mtf1 to the *srk1* promoter sequence and under similar experimental conditions we could also detect Mtf1 binding to the mitochondrial promoters by EMSA. This is consistent with the recent observations that cross-links can be detected between mitochondrial promoter sequences and Mtf1 (31,32).

The observation that Mtf1 is also active as a nuclear transcription factor raises a number of intriguing questions; e.g. which of the nuclear RNA polymerases does Mtf1 associate with? What is the role of Mtf1 in nuclear

transcription? What other transcription factors cooperate to transcribe *srk1*? Nuclear mRNAs are normally produced by RNA polymerase II. Classical eukaryotic transcription by RNA polymerase II involves many general and gene specific transcription factors [reviewed in (33–35)]. We detected weak binding in EMSA by Mtf1 alone with the *srk1* promoter; suggesting that Mtf1 may retain its mitochondrial role in nuclear *srk1* promoter recognition. The molecular details of how Mtf1 acts as a nuclear transcription factor, what accessory factors it combines with and, perhaps most importantly, which RNA polymerase it associates with will benefit from further investigation. It is likely that the molecular details of Mtf1′s nuclear function will differ from the mitochondrial ones.

Mtf1 is nuclear encoded and made in the cytoplasm. For Mtf1 to be active both in the mitochondrion and the nucleus, it must be transported into both locations. Extra- mitochondrial proteins are imported by a number of mechanisms (36–39). Most mitochondrial proteins contain N-terminal mitochondrial-targeting presequences, that are removed after transport (40,41). Protein transport takes place through translocation channels in the mitochondrial outer and inner membranes and requires ATP hydrolysis and specific chaperones (40–43). Proteins can be imported co-translationally or post-translationally (41–44). The Mtf1 from *S. cerevisiae* has been shown to employ a unique mechanism for mitochondrial transport that does not require an N-terminal presequence, or an outer membrane receptor, is independent of the energy state of the mitochondria and can occur at low temperatures (45–47). The mechanisms of mitochondrial transport in *S. pombe* are not well understood. The possibility that Mtf1 in *S. pombe* might also employ a unique mechanism for mitochondrial entry may also explain its presence in the nucleus; the absence of a mitochondrial transport signal would allow Mtf1 to also be transported into the nucleus. The existence of a molecular mechanism whereby Mtf1 is not simply restricted to the mitochondrion but is also partitioned into the nucleus suggests that its nuclear role may also be important.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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