mik1$^+$ G1–S transcription regulates mitotic entry in fission yeast

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Abstract In the fission yeast *Schizosaccharomyces pombe* Mik1p, in combination with Wee1p, is an important inhibitor of mitosis through direct phosphorylation of Cdc2p. Here we present the observation that *mik1*$^+$ is transcribed during G1- and S-phase in normally dividing cells. *mik1*$^+$ transcription is regulated by the MCB–DSC1 system, which controls expression of other genes at the G1–S interval. *mik1*$^+$ is shown to be an important target of MCB–DSC1 as it is epistatic for the mitotic delay phenotype displayed in *cdc10-C4* cells, which are mutated in a component of DSC1. The mitotic delay in *cdc10-C4* cells is bypassed by *cdc2-1w*, suggesting that *mik1*$^+$ acts directly on *cdc2*$^+$, with no checkpoint function involved. Thus, *mik1*$^+$ represents a new type of MCB–DSC1 regulated gene in fission yeast, whose gene product is exclusively expressed during G1- and S-phase to prevent premature mitosis during this cell cycle stage. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Transcription; Cell cycle; Mitosis; *mik1*$^+$; *Schizosaccharomyces pombe*

1. Introduction

The cell cycle is the fundamental process by which cells duplicate and divide. In the budding yeast, *Saccharomyces cerevisiae*, microarray analysis has recently shown that ~800 genes, out of a total of ~7000, are differentially transcribed through the cell cycle [1,2]. In most cases genes are expressed at particular cell cycle times when their products are specifically required merely to maximise cell economy, with the implication that their expression pattern has no regulatory role. For example, a number of enzymes and polypeptides required for making DNA in budding yeast are expressed only during S-phase [3]. In a minority of cases, gene transcription controls cell cycle progress. For example the G1 cyclins *CLN1* and *CLN2*, which are expressed during G1, promote S-phase [4]. *CLB1* and *CLB2*, in contrast, expressed during G2 and M repress *CLN1* and *CLN2* transcription, so preventing S-phase at this cell cycle stage [5]. Thus budding yeast has both positive and negative transcription mechanisms to regulate cell cycle progress.

In the fission yeast, *Schizosaccharomyces pombe*, a group of at least four genes has been identified that are transcribed exclusively at the beginning of S-phase. This group includes *cdc22*$^+$, *cdc18*$^+$, *cig2*$^+$ and *cdt1*$^+$, all of which are involved, either directly or indirectly, in replicating DNA, and thus promote S-phase. The molecular components that regulate this G1–S transcription have been isolated and comprise a transcription factor complex named DSC1 (DNA synthesis control) binding to a common sequence present in all four genes' promoters. The promoter sequence is called an MCB, for MluI cell cycle box, and is conserved through evolution, being present in the promoters of some G1–S transcribed genes in budding yeast. DSC1 contains the products of the *cdc10*$^+$, *res1*$^+$, *res2*$^+$, *rep1*$^+$ and *rep2*$^+$ genes [6–11].

In this report we identify a new gene under MCB–DSC1 control in fission yeast, *mik1*$^+$, which has no known role in promoting S-phase, but instead inhibits entry into mitosis. We demonstrate that *mik1*$^+$ is an important target of MCB–DSC1 controls, as *mik1*$^+$ is epistatic to the mitotic delay phenotype of *cdc10-C4*, a mutant component of DSC1. Thus *mik1*$^+$ represents a new type of gene transcribed at G1–S in fission yeast during the normal cell cycle, whose function is to inhibit mitosis during S-phase.

2. Materials and methods

2.1. Media and general techniques

General molecular procedures and the media used for the propagation of *S. pombe* were as described by Moreno et al. [12]. The following strains were used in this study: *972* h−, wild-type (GG 1), h+ *cdc10-129* (GG 28), h− *res2*::*ura4+ ura4-D18* (GG 156), h− *cdc2-1w* (GG 54), h+ *cdc10-C4 cdc2-1w* (GG 184), h+ *cdc10-C4* (GG 249), h− *mik1*::*ura4+ ura4-D18 cdc10-C4* (GG 287); h− *mik1*::*ura4+ ura4-D18* (GG 290). For all physiological experiments cells were grown in minimal medium (EMM), with shaking, at 24°C, 32°C or 36°C. Populations of synchronously dividing fission yeast cells were prepared by use of a Beckman elutriator rotor. Cell number per ml of liquid culture was determined from a sample fixed in a 0.1% formaldehyde/0.1% sodium chloride solution. Following sonication, cells were counted electronically with a Coulter counter.

2.2. DNA and RNA manipulations

Fission yeast total RNA was prepared [13] and Northern blot analysis carried out using GeneScreen membrane (NEN, Life Science Products Inc., Boston, MA, USA), following the manufacturer's suggested protocol. DNA probes, corresponding to ~1 kb of the open reading frame for each gene, were amplified by PCR and labelled with [α^{32}P]dCTP using the random hexanucleotide labelling procedure.

2.3. Gel retardation analysis

Analysis was performed as previously described [6] using MCB promoter fragments from *cdc22*$^+$and *mik1*$^+$. The two MCB fragments were amplified using oligo's: *cdc22*$^+$ (1) GTAGTTCAAT CTCATAGA and (2) CTCTGTTTAC GCTGAATG. *mik1*$^+$ (1) 5′-TTTAATTAGT GGTTGGTTTA AGGCAAAAGA-3′ and (2) 5′-CAACCATTGT TGACATGCAC GTACACAAAC-3′.

Competition reactions were completed using MCB regions amplified with oligo's from the following genes' promoters: *cdc18*$^+$: (1) 5′-AGGTTCGCGC GTAGAACGCG ACACGACACG-3′ and (2) 5′-GCTTGGTGTT TAAGTTTTGT TTAAGAGCAG-3′. *cig2*$^+$: (1) 5′-ATTGCGTTAG TTTGTGTAGC ATGTAATGAC-3′ and

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(2) 5′-AGTGAAAAAG CAAAAGACAG GCATAGCAAG-3′. *cdt1*$^+$: (1) 5′-TAAGTAAGAC ATAAACTGAT TAGTCGGACA-3′ and (2) 5′-GTCGTGACTA TATAGGGCGC GACATTTCAT-3′.

3. Results

3.1. The *mik1*$^+$ promoter region contains four sequences resembling MCB elements

While searching the fission yeast genomic database for novel genes that contain MCB sequences (5′-$^{A}/_{T}$CGCG$^{A}/_{T}$-3′) in their promoters, we were surprised to identify four such sequences in the upstream region of *mik1*$^+$ (Fig. 1A). All four sequences lie within the intergenic region 5′ to *mik1*$^+$ and 3′ to the adjacent gene on the chromosome, *ssb1*$^+$ (predicted to encode replication factor-a protein 1).

mik1$^+$ has been extensively characterised and demonstrated to have an important role at the G2–M-phase of the fission yeast cell cycle, when it acts with *wee1*$^+$ to negatively regulate mitotic entry through phosphorylation of Cdc2p [14,15]. Wee1p protein is present at a constant level throughout the cell cycle [16], so *mik1*$^+$ seemed an unlikely gene to be transcribed specifically at the G1–S boundary. During the course of this work, however, further roles have been ascribed to *mik1*$^+$ during S-phase. These include an intrinsic function in normally dividing cells, and a checkpoint control function in cells where DNA has been damaged [17,18], see Section 4.

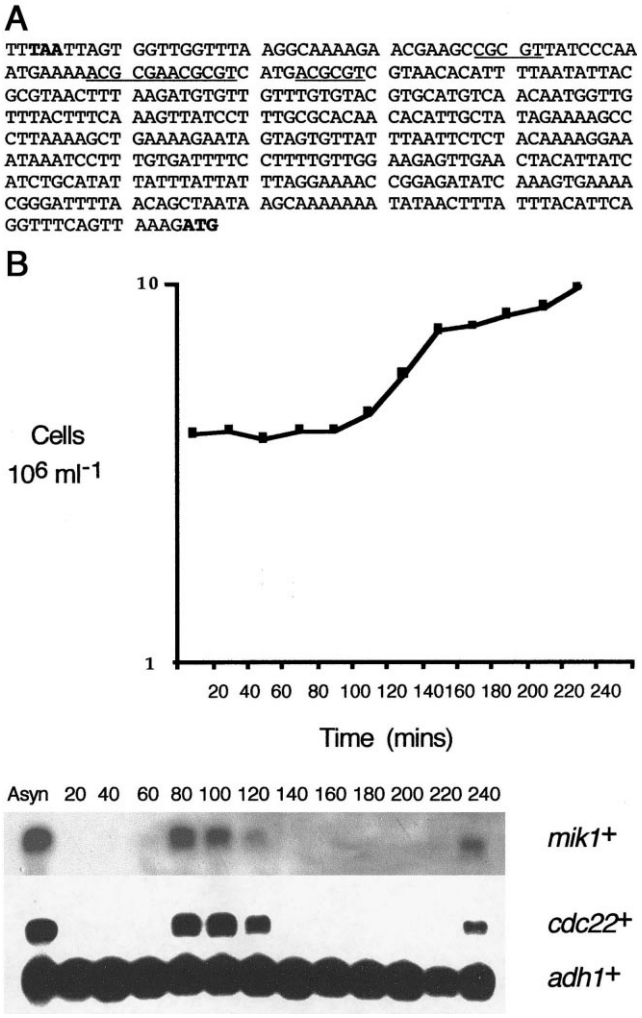


Fig. 1. Cell cycle regulation of *mik1*$^+$. A: The *mik1*$^+$ promoter region contains four sequences resembling MCB elements. DNA sequence taken from the fission yeast sequencing project. TAA in bold: stop codon of previous gene *ssb1*$^+$ 'replication factor-a protein 1'. ATG in bold: start codon of *mik1*$^+$. MCB elements underlined. B: *mik1*$^+$ transcript is cell cycle regulated with a peak at the G1–S boundary. A population of wild-type cells (*972* h−), synchronous for division, were size selected by elutriation, and RNA samples prepared for Northern blot analysis [13]. The blot was hybridised consecutively with *mik1*$^+$, *cdc22*$^+$ and *adh1*$^+$ probes. Asyn. indicates RNA prepared from the same cells prior to elutriation.

3.2. *mik1*$^+$ transcription is cell cycle regulated and peaks at the G1–S boundary

Previous reports have shown *mik1*$^+$ transcript and protein varied during the normal cell cycle [17,18]. We confirmed and extended this observation by directly comparing the pattern of *mik1*$^+$ transcript accumulation with another MCB regulated gene, *cdc22*$^+$, in elutriated wild-type cells. In a synchronous population of wild-type cells, size selected by elutriation, *mik1*$^+$ transcript was found to vary in abundance (Fig. 1B) coincident with *cdc22*$^+$ transcript, thus peaking at the G1–S boundary. MCB elements have been shown to confer cell cycle transcription at G1–S in fission yeast [6]; it is therefore likely that the MCB sequences in the *mik1*$^+$ promoter have a role in its cell cycle transcription in normally dividing cells.

3.3. *mik1*$^+$ MCBs bind to DSC1 in vitro and *mik1*$^+$ transcription is affected in vivo by *cdc10-C4*

A transcription factor complex has been identified in fission yeast that binds to MCB sequences, named DSC1 [6]. Furthermore, the product of the *cdc10*$^+$ gene has been shown to be a component of DSC1, as mutants of *cdc10*$^+$ affect DSC1, as well as transcription of genes containing MCBs in their promoters [6,13,19].

We next sought to determine whether *mik1*$^+$ MCBs bind DSC1 in vitro. Gel retardation studies were initially performed using *cdc22*$^+$ MCB DNA as labelled substrate, which revealed a single prominent retarded band that contains DSC1 (Fig. 2, lane 2, large arrow) [6]. This band disappears when the same DNA is added as unlabelled competitor (lanes 3 and 4), but remains when the core CGCG of the MCBs are mutated to CTAG (lanes 5 and 6), demonstrating that DSC1 specifically binds the MCB sequences in the *cdc22*$^+$ promoter. DSC1 also binds the MCB regions from the *cdc18*$^+$, *cdt1*$^+$ and *cig2*$^+$ promoters (lanes 7–12). Furthermore, on addition of unlabelled *mik1*$^+$ MCB DNA DSC1 disappeared (lanes 13 and 14). Finally, *mik1*$^+$ MCB DNA, when used as labelled substrate revealed a retarded band of similar mobility to that detected by *cdc22*$^+$ MCB DNA (compare lanes 16 and 21), which is lost in mutants of components of DSC1 (lanes 19 and 20). These combined results demonstrate that *mik1*$^+$ MCBs bind DSC1 in vitro.

An unusual mutant of *cdc10*$^+$ is *cdc10-C4* which at low temperatures (16–24°C) results in deregulated expression of all known MCB regulated genes through the cell cycle [13]. This phenotype is manifested in asynchronous cells as over-expression relative to wild-type, as all cells, irrespective of their cell cycle stage, express MCB regulated genes (Fig. 3A). We assayed *mik1*$^+$ transcription in *cdc10-C4* at 24°C in asynchronous cells, and found it to be over-expressed to a similar level to all other known MCB controlled genes (Fig. 3A). As the only known function of Cdc10p is in DSC1, this

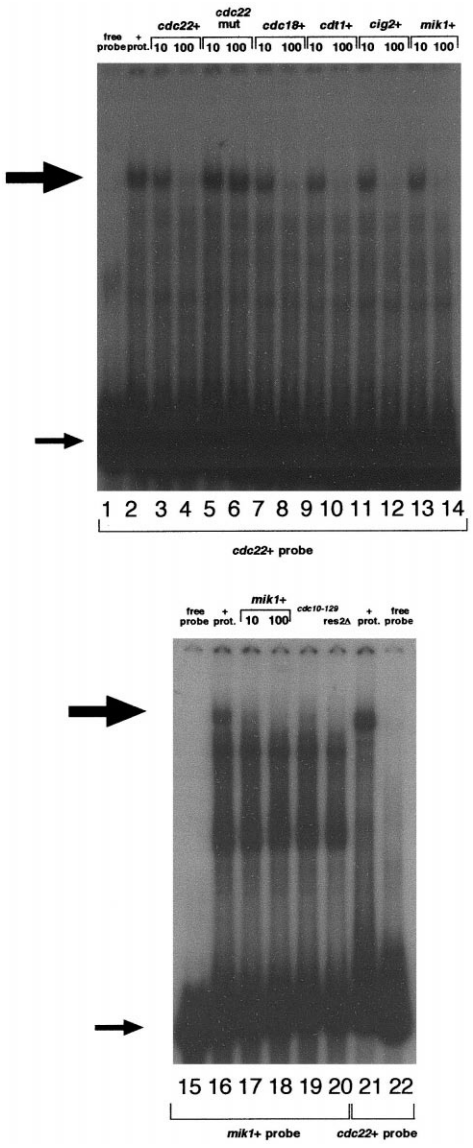


Fig. 2. *mik1*$^+$ MCB sequences bind to DSC1 in vitro. Gel retardation assay using *cdc22*$^+$ and *mik1*$^+$ MCB DNA promoter fragments as labelled probe with protein extracts from wild-type cells [6]. Large arrow indicates DSC1; small arrow free probe. In alternate lanes 10 and 100 M excess unlabelled competitor MCB DNAs from various fission yeast G1–S transcribed genes were added to the reaction mixture prior to electrophoresis. In lanes 5 and 6 unlabelled competitor DNA was the same as the labelled probe except the four MCB sequences were mutated from CGCG to CTAG. In lanes 19 and 20 protein extracts were prepared from *cdc10-129* cells grown at 36°C and *res2*Δ cells grown at 24°C, respectively.

observation suggests that *mik1*$^+$ is under MCB–DSC1 control.

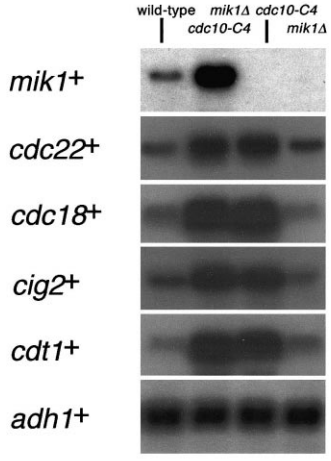
In summary, the combination of observations that *mik1*$^+$ transcription is affected by *cdc10-C4* in vivo and that *mik1*$^+$ MCB sequences bind DSC1 in vitro, confirms that *mik1*$^+$ is under direct DSC1 control.

3.4. *mik1*$^+$ *is epistatic to cdc10-C4 for cell length at division*

Another interesting phenotype of *cdc10-C4* is an increase in cell length during division at low temperature [13]. This increased length is due to a delay in mitosis, as FACs analysis of *cdc10-C4* demonstrates that the proportion of cells in G1- and S-phase is indistinguishable from wild-type (data not shown). Previously, we suggested that increased length at division might be due to the over-expression of one (or a group) of the genes regulated by DSC1 in this mutant. *cdc18*$^+$ seemed an obvious candidate, as its high over-expression is known to cause a mitotic delay, manifested as an increase in cell length at division [19]. However, in *cdc10-C4* at 24°C, *cdc18*$^+$ transcript is only ~8 times wild-type levels [13], Fig. 3A, which alone is unlikely to result in the observed mitotic delay, as this phenotype was only observed with much higher levels of over-expression *cdc18*$^+$ [20,21].

Ectopic over-expression of *mik1*$^+$in wild-type cells, like its redundant partner *wee1*$^+$, causes a mitotic delay [14], so we sought to test whether inappropriate expression of *mik1*$^+$ in *cdc10-C4* contributes to the cell length phenotype. *mik1*$^+$ is non-essential, so we combined a disruption of *mik1*$^+$ (*mik1Δ*) with *cdc10-C4* and examined cell length at division at 24°C. Interestingly, the *mik1Δ cdc10-C4* double mutant divided at wild-type size (Fig. 3B), even though *cdc18*$^+$ and the other known MCB regulated genes are still over-expressed (Fig. 3A). This observation suggests that over-expression of *mik1*$^+$ is required for the cell length phenotype in *cdc10-C4*. Over-expression of *cdc18*$^+$ and the other MCB regulated genes is not sufficient to cause this phenotype, as their transcript levels were similar in *cdc10-C4* and *mik1Δ cdc10-C4*

A



B

	generation time (mins)	cell-length (μm)
wild-type	620	12.3
cdc10-C4	600	23.4
mik1Δ cdc10-C4	660	11.3
mik1Δ	640	12.7

Fig. 3. Genetic interactions between *mik1*$^+$ and *cdc10*$^+$. A: *mik1*$^+$ transcript is over-expressed in *cdc10-C4*. RNA was prepared from cultures of wild-type, *cdc10-C4*, *mik1Δ* and *cdc10-C4 mik1Δ* cells grown at 24°C, and subjected to Northern blot analysis. The blot was hybridised consecutively with *mik1*$^+$, *cdc22*$^+$, *cdc18*$^+$, *cig2*$^+$, *cdt1*$^+$ and *adh1*$^+$ probes. B: Cell elongation at division in *cdc10-C4* is caused by over-expression of *mik1*$^+$. Samples from cultures in A were taken at suitable times to estimate cell numbers. The length of septated (dividing) cells was measured microscopically, and the mean calculated of 50 cells per culture.

cells. Thus we can conclude that *mik1*$^+$ is epistatic to *cdc10-C4* for cell length at division.

3.5. *cdc2-1w bypasses the cell length phenotype in cdc10-C4*

To establish whether the effect of over-producing *mik1*$^+$ in *cdc10-C4* cells directly acts on Cdc2p to cause the cell length phenotype, we combined the *cdc10-C4* mutation with *cdc2-1w*, which is insensitive to *wee1*+ and *mik1*+ levels [22], and examined cell length at division. This double mutant divided at small size (the 'wee' phenotype ~7.9 μm) at 24°C, comparable to the *cdc2-1w* single mutant (~7.7 μm), suggesting that mik1p acts directly on cdc2p in *cdc10-C4* cells.

4. Discussion

4.1. *Correct mik1*$^+$ *transcription is critical for normal cell cycle progression*

Initially, we were surprised to discover that *mik1*$^+$ is under MCB–DSC1 control, as previous work with this gene has suggested roles for *mik1*$^+$ exclusively at the G2–M boundary: Mik1p, in combination with Wee1p, inhibits Cdc2p activity (and consequent entry into mitosis) by phosphorylation [14]. However, recent work has shown that Mik1p levels vary during a normal cell cycle, with a peak during S-phase, suggesting that Mik1p mediates a functional link between S-phase and mitosis [17,18].

Other members of the group of MCB regulated genes in fission yeast include *cdc22*$^+$, *cdt1*$^+$ and *cdc18*$^+$, which encode the large subunit of ribonucleotide reductase and factors that bind and regulate the origin of replication complex [19,20,23–25]. All of these proteins are therefore directly or indirectly required for making DNA, so it is evident why they are expressed at the beginning of S-phase. Amongst these genes *cdc18*$^+$ appears to have a major role in controlling initiation of DNA replication, as its high over-expression results in multiple S-phases without intervening mitoses [20,21]. *cdc18*$^+$ and *cdt1*$^+$ have also been shown to have additional roles in preventing mitosis [19,24,25], through two different mechanisms, one checkpoint dependent, the other checkpoint independent [26].

mik1$^+$, in contrast, is not required for making DNA but instead its only known function is to prevent mitosis from occurring before S-phase is completed [14,15]. This function manifests in two different situations: in cells that are cycling normally, and in cells where the S-phase checkpoint is activated after damage to DNA [17,18,27].

In the experiments described here we address the regulation of *mik1*$^+$ transcription in normally dividing cells, where no checkpoint has been activated. We show that *mik1*$^+$ is a new type of gene under MCB–DSC1 control in fission yeast whose expression is not required for promoting DNA synthesis but instead is important for regulating correct cell cycle progress. The fact that *mik1*$^+$ is epistatic to *cdc10-C4* for cell length at low temperature, suggests that *mik1*$^+$ is an important target of DSC1. Over-expression of this gene alone amongst the MCB regulated genes is necessary for the delay in mitosis in *cdc10-C4* at low temperature. Significantly, this phenotype operates directly through cdc2p as the*cdc2-1w* mutation overrides the cell length phenotype in *cdc10-C4*, consistent with the suggestion that no checkpoint function is involved. Clearly, correct *mik1*$^+$ transcription is crucial for normal cell cycle progression, suggesting MCB–DSC1 regulation of gene expression plays an important part in cell cycle controls.

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