The *S. pombe sep1* gene encodes a nuclear protein that is required for periodic expression of the *cdc15* gene

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Abstract The *Schizosaccharomyces pombe sep1* gene encodes a putative transcription factor that is required for cell separation. Among the genes required for septum formation and cytokinesis in fission yeast examined to date, the only one whose mRNA fluctuates significantly during the cell cycle is *cdc15*. In this study we have examined *cdc15* mRNA levels in *sep1* mutant and null backgrounds and have found that sep1p function is required for periodic accumulation of *cdc15* mRNA. We have also localised sep1p and find that it is a nuclear protein, consistent with its proposed role as a transcription factor.

Key words: Cytokinesis; Septum formation; Transcription; *cdc15*; *Schizosaccharomyces pombe*

1. Introduction

The yeast *Schizosaccharomyces pombe* provides a simple model for the study of cytokinesis. Fission yeasts grow mainly by elongation at their tips, and then divide by binary fission, after formation of a new cell wall, or division septum. Though it may appear that model organisms such as fission yeast, budding yeast and higher eukaryotic cells differ in their mode of division, common themes are emerging from all these systems, which suggest that the underlying mechanisms may be very similar, as is the case in other major cell cycle events, such as DNA synthesis and mitosis (reviewed in [1]).

The position of the future site of cell division is defined very early in mitosis, probably by a signal emanating from the nucleus, and an actomyosin-based medial ring is assembled at that position. This is the functional equivalent of the mammalian contractile ring, and persists until the end of anaphase, when a signal is delivered via the septation signalling network, to promote ring contraction and division of the cell. Septum synthesis is thought to be guided by contraction of the medial ring (reviewed in [1–3]).

A large number of *S. pombe* mutants have been identified that are defective in positioning, or assembly of the medial ring, signalling the onset of septation, and in cell separation (for reviews, see [2,3]).

A mutant in the *sep1* gene leads to problems in cell separation and a hyphal morphology. The gene has been isolated by positional cloning and shown to encode a member of the HNF-3 family of transcription factors [4]. Of all the genes implicated in septum formation and cytokinesis, at present only the *cdc15* mRNA has been shown to vary in abundance [5]. Mutants in *cdc15* do not form a division septum [6], and fail to construct a functional medial ring [5]. cdc15p is a component of the medial ring and the contractile ring during mitosis [5], and the abundance of *cdc15* mRNA changes through the cell cycle, peaking after the onset of mitosis [5], though it is not known which factors are responsible for regulating *cdc15* mRNA accumulation. Ectopic expression of *cdc15* from a strong promoter can promote F-actin ring formation in G2 arrested cells, indicating that inappropriate expression of *cdc15* can be deleterious to proper co-ordination of cell cycle events [5].

In this study we have investigated the subcellular localisation of sep1p, and the effects of loss of *sep1* function upon the level of *cdc15* mRNA through the cell cycle. We conclude that *sep1* function is required for periodic accumulation of *cdc15* mRNA.

2. Materials and methods

2.1. Yeast techniques

A list of *S. pombe* strains used in this study is given in Table 1. The media YE yeast extract and EMM2 minimal medium have been described previously. EMM2 was supplemented as required. Standard genetic methods were used for strain construction by crosses and tetrad analysis [7].

2.2. Synchronisation methods

sep^+ strains were synchronised through selecting G2 cells from exponential phase cultures (grown in EMM2) using a Beckman JS5.0 elutriation system [11]. This method cannot be used to synchronise cells that do not separate after division. Therefore the sep^- cultures were not synchronised by elutriation but by arrest–release based on the temperature sensitivity of *cdc25-22* which was crossed into *sep1-1*. The double mutant *sep1-1 cdc25-22* h^- was grown to mid-exponential phase at 25°C, shifted to 35°C for 4 h and then shifted back to 25°C. For the analysis of transcription, 10^8 cells were harvested from the synchronised cultures at 15 min intervals and total RNA was isolated. The progression through the cell cycle after return to the permissive temperature was monitored microscopically by determining the percentage of septated cells (septation index).

2.3. DNA techniques

Standard methods were used throughout for DNA manipulation. Plasmid pDW232 has been described previously [8]. DNA fragments were isolated by gel purification using a Geneclean kit according to the manufacturer's recommendation. *S. pombe* transformation was carried out as described [7]. The coding sequence of $sep1^+$ was amplified by standard PCR method (25 cycles) using the primers described in Section 3 and a pUR19-based *S. pombe* genomic library [9] as template.

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To tag the chromosomal *sep1*$^+$ gene, the PCR-based gene targeting method [10] was used. Two 100 nucleotide long primers were synthesised which contained sequences complementary to the *sep1*$^+$ coding sequence and sequences complementary to the tag modules of the plasmids pFA6a-3HA-kanMX6 and pFA6a-GFP(S65T)-kanMX6: VS 348 5′-CAGCCGCGCAAGATGATACTTACTTGCCTTCTCCTACCAAGAGGAAAATGCCACTTCTTCGTCAAACTTCAACACTATTCCGGATCCCCGGGTTAATTAA-3′ (the 3′ end of *sep1*$^+$ is underlined) and VS 349 5′-GCAAATGAACAAGTAACATCGGGGGTTTGAAAGTTTATTAAGCAACCAAGGTAAAAGATATGTGTAAAATTAGGAATTCCGAATTCGAGCTCGTTTAAAC-3′ (the *sep1*$^+$ downstream sequence is underlined). Using these primers and the pFA6a-3HA-kanMX6 and pFA6a-GFP(S65T)-kanMX6 plasmids as templates, PCR reactions were performed. The PCR products were used to transform *S. pombe leu1-32 h*$^-$ cells; G418-resistant transformants were identified, and tested for stable insertion of the fragment.

2.4. RNA techniques

Total RNA was isolated with the Qiagen RNA Kit. Northern blots were made using 10 μg RNA samples and by capillary transfer onto Gene Screen Plus membrane (NEN). Hybridisation and washing of the filters were done according to the manufacturer's protocol. Probes corresponding to the coding regions of each of the genes have been described previously (cdc15 [5]; sce3 [11]; histone H2A [12]). For *sep1*, a *Bam*HI–*Eco*RI fragment excised from the pDW232 clone was used.

2.5. Western blot analysis

Total soluble protein extract was prepared from exponential phase cultures using a previously described method [13] and the concentration of protein was determined using the Bio-Rad kit. 5–50 μg extract per lane was loaded on 10% SDS–PAGE and after running the gel, it was blotted to 0.2 μm nitrocellulose. The blots were stained with Ponceau S, and processed as recommended using ECL Western blotting kit with rabbit antiserum against green fluorescent protein (GFP) as primary and goat anti-rabbit horseradish peroxidase as secondary.

2.6. Microscopy

To visualise nuclei and septa, the cells were stained with DAPI (4′,6′-diamino-2-phenylindole, Sigma) and calcofluor (fluorescent brightener, Sigma) as described previously [7]. For sep1p localisation, *sep1-GFP leu1-32 h*$^-$ cells were fixed in 4% formaldehyde. After 20 min fixation, the cells were washed three times with PEM (100 mM PIPES pH 6.9, 1 mM EGTA, and 1 mM $MgSO_4$) and then digested for 30 min at 37°C in 1 ml of PEM plus 1.2 M sorbitol (PEMS) containing 1 mg/ml zymolyase 20T (ICN). The digested cells were washed once in PEMS plus 1% Triton X-100, three times in PEM. The pellet was resuspended in PEMBAL (PEM plus 1% bovine serum albumin [Sigma A-7030], 100 mM lysine hydrochloride, and 0.1% sodium azide) and gently shaken for 30 min. After centrifugation the cells (5×10^6) were resuspended in 90 μl of PEMBAL containing affinity-purified anti-GFP antibody diluted 1/500 and left on a rotating wheel overnight. After washing three times in PEMBAL, the cells were incubated for 5–7 h in PEMBAL containing a FITC conjugated goat anti-rabbit secondary antibody (1/100). This was followed by serial washes: once in PEM, once in phosphate-buffered saline (PBS) pH 8.0, and once in PBS (pH 8.0) containing 1 μg/ml DAPI. The stained cells were resuspended in 50 μl PBS (pH 8.0), containing 0.1% sodium azide, mounted in 50% glycerol containing 1 mg/ml *p*-phenylenediamine and observed with a Zeiss Axiophot microscope. Localisation of sep1p was also attempted in *sep1-HA leu1-32 h*$^-$ cells, but no detectable signal was found in any cell part.

3. Results

3.1. Cloning of full-length sep1$^+$

A previous paper [4] reported the sequence of a genomic DNA fragment which complemented the *sep1-1* phenotype. The fragment was isolated by positional cloning from a cosmid clone of the left arm of chromosome II. The *S. pombe* genome project has recently completed the sequence of this part of the genome and revealed that this fragment does not contain the entire coding sequence of *sep1*$^+$. The whole *sep1*$^+$ open reading frame (ORF) (SPBC4C3.05) was found to be 1992 bp long, encoding a 664 amino acid protein with a predicted molecular mass of 80 kDa. To clone the entire *sep1*$^+$ ORF, we amplified a 2.0 kb fragment by PCR from a wild-type genomic library using the primers VS 361 5′-CGGAATTCATGAATTTTAATTCTACTAACC-3′ and VS 356 5′-CGGAATTCTTAGAATAGTGTTGAAGTTTGA-3′. The fragment contained two *Eco*RI sites: one before the start codon and one after the stop codon. Using these sites, the PCR product was cloned into the *Eco*RI site of the pDW232 vector [8]. Transformation of the *sep1-1* mutant with pDW232*sep1* restored the wild-type phenotype (data not shown).

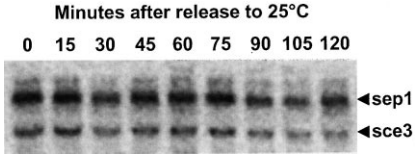

Fig. 1. The abundance of *sep1* mRNA level in synchronised culture. The cells were synchronised by elutriation, and samples were taken from the culture at time intervals for RNA extraction. The Northern blot was probed with *sep1* and *sce3*.

3.2. sep1 mRNA level is constant during the cell cycle

To determine whether the steady-state level of *sep1* mRNA changes as cells progress through the cell division cycle, a synchronous population of exponentially growing wild-type *S. pombe* cells was generated by arrest–release of *cdc25-22* [14], and RNA was extracted from samples of the culture at regular intervals. A Northern blot was probed for *sep1*, and *sce3* [13] as loading control. As shown in Fig. 1, the steady-state level of *sep1* mRNA did not vary through the cell cycle. The small variations observed reflect differences in loading, since the *sce3* mRNA shows similar fluctuations. We also examined whether genes implicated in controlling actin ring formation and regulating septum formation influenced the expression of *sep1*. Northern blots of RNA extracted from elutriation-selected populations of *cdc7-24, cdc11-136*, *cdc14-118*, *cdc15-140*, and *cdc16-116*, which were generated at 25°C and then shifted to 36°C, showed that none of these proteins is required for normal expression of *sep1* mRNA (data not shown).

3.3. Sep1p is a nuclear protein

Next, we explored the possibility that sep1p activity might be regulated by changes in its subcellular localisation, as has been demonstrated for transcription factors implicated in the stress response in fission yeast (for example [15]). To localise

Table 1
S. pombe strains used in this study

Strain	Source
ura4-D18 h$^-$	Lab collection
leu1-32 h$^-$	Lab collection
cdc25-22 h$^-$	Lab collection
cdc25-22 sep1-1 ura4-D18 h$^-$	this study
sep1-1 ura4-D18 h$^+$	[16]
sep1-GFP leu1-32 h$^-$	this study
sep1-HA leu1-32 h$^-$	this study
sep1-1 cdc25-22 h$^-$	this study

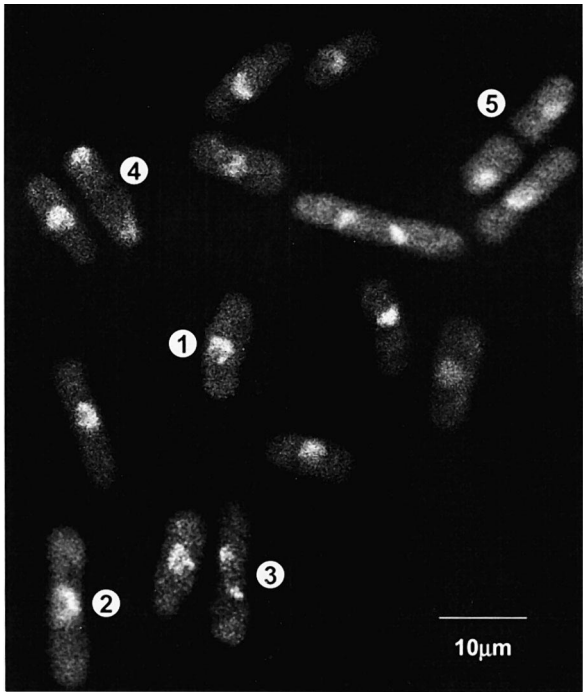

Fig. 2. Tagging and localisation of Sep1p in the cell. Cells in which the chromosomal copy of *sep1* had been tagged with the HA3 epitope were fixed and stained with the 12CA5 antibody. Cell 1 is in interphase, cell 2 is in early mitosis, cell 3 is in early anaphase, cell 4 is in late anaphase, cell 5 is septated.

sep1p the protein was tagged by oligonucleotide-mediated addition of either GFP from the jellyfish *Aequorea victoria*, or three copies of the 12CA5 epitope from the influenza virus haemagglutinin protein (HA), to the C-terminus of the protein by oligonucleotide-mediated tagging [10]. Proteins of the expected size were detected in Western blots of protein extracts probed either with a polyclonal anti-GFP antibody or with 12CA5 (not shown). Indirect immunofluorescence showed that the tagged sep1p is located in the chromatin region of the nucleus, at all stages of the cell cycle. No significant signal was observed in the nucleolar region of the nucleus (Fig. 2). Since the tagged allele of the *sep1* gene is the only one in the cell, and the cells have a normal morphology (Fig. 2), we conclude that the protein is biologically functional. We conclude that sep1p is located predominantly in the chromatin region of the nucleus at all stages of the cell cycle, consistent with its proposed role as a transcription factor.

3.4. *sep1 is not essential for cell viability*

Since *sep1-1* is recessive [16], the possibility exists that it is a hypomorphic allele. To test this, we constructed a complete deletion of the *sep1* gene by oligonucleotide-mediated targeting of the *ura4*$^+$ gene [10]. Viable *ura4*$^+$ colonies were obtained after transfection into both haploid and diploid strains, suggesting that the gene was not essential. Crossing of a haploid transformant to wild-type cells showed co-segregation of the *ura4*$^+$ marker and the branched *sep1* phenotype in 40 tetrads, suggesting that the cells were not viable due to the presence of a suppressor mutation. The morphological phenotype of the cells was similar to that produced by the *sep1-1* mutation (not shown), confirming the results of a previous study [17].

3.5. *Effects of sep1-1 and sep1::ura4*$^+$ *upon accumulation of cdc15 mRNA*

Sep1p shows significant homology to HNF-3 family transcription factors [4]. Since the *cdc15* gene, which is implicated in controlling medial ring formation, shows periodic accumulation of mRNA at the onset of mitosis (for example, *cdc15* [5]), we tested whether loss of sep1p function would affect its expression.

Since loss of *sep1* function leads to a hyphal morphology, these cells cannot be synchronised by elutriation. Therefore, we generated a synchronous population by induction synchrony. The double mutants *cdc25-22 sep1::ura4*$^+$ and *cdc25-22 sep1-1* were constructed and synchronised by arrest–release [14]. In the *cdc25-22 sep1-1* mutant, the abundance of *cdc15* mRNA showed a pattern similar to that observed in arrest–release-synchronised *sep1*$^+$ cells, peaking after release of the *cdc25-22* mutant [5]. However, the peak occurred earlier compared with a wild-type background, peaking 15–30 min after release, compared to 45–60 min for *sep1*$^+$ cells (Fig. 3A,B). When the level of *cdc15* mRNA was examined in a *cdc25-22 sep1::ura4*$^+$ background, it was found that the cell cycle variation was lost and that the levels, which were already elevated in G2-arrested cells, did not increase after release (Fig. 3C). The level of H2A increased at the time of septum formation (which coincides approximately with the S phase), as expected, thereby indicating that arrest–release had generated a synchronous cell population.

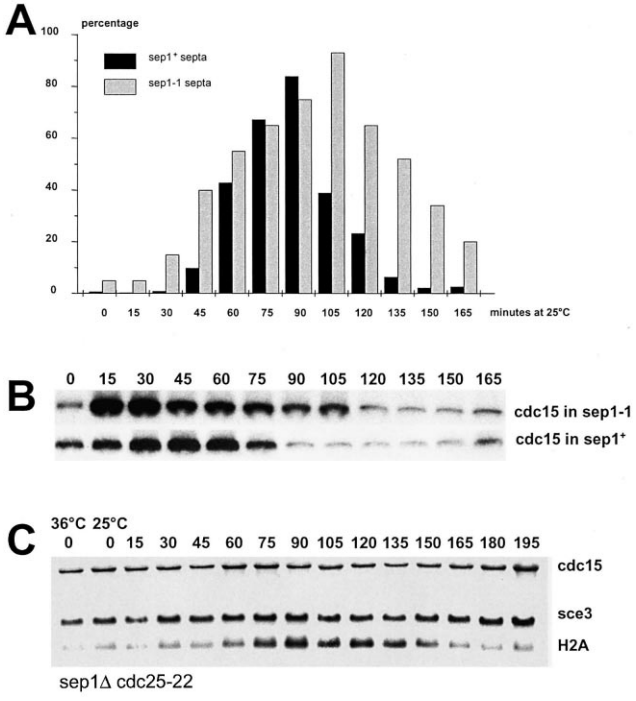

Fig. 3. Cycling of the steady-state level of *cdc15* mRNA in a synchronous population of *sep1-1 cdc25-22 h*$^-$ cells compared with *cdc25-22 sep1*$^+$. Synchrony was induced by arrest–release and samples were taken at time intervals for RNA extraction. A: Septation index for the two strains. B: Northern blot probed for *cdc15*. C: Northern blot of RNA extracted from *cdc25-22 sep1::ura4*$^+$ cells. The blots had been probed with *cdc15 sce3* and H2A probes. Note that the *cdc15* mRNA level does not cycle. In this experiment, the peak of septum formation was at 75–90 min. In these cultures, septation is coincident with DNA synthesis.

4. Discussion

Mutation of the *sep1* transcription factor produces defects in cell cleavage [16]. In the course of this study it became apparent that the *sep1* clone described originally was truncated at its 3′ end. Despite this, the truncated protein retains biological activity as it is able to rescue the *sep1-1* mutant [4]. A previous study showed that the steady-state level of *sep1* mRNA fluctuates upon release from a *cdc25-22* arrest [17]. In this study, we have found no evidence for significant fluctuation of the steady-state level of the mRNA. The reason for the discrepancy is unclear, but may reflect the different techniques employed for the assays.

Of all the genes implicated in medial ring formation and signalling the onset of septation, the only one whose steady-state mRNA level is known to fluctuate is *cdc15* [5]. Expression of *cdc15* increases sharply after the onset of mitosis, and declines at the time of septum formation. Inappropriate expression of *cdc15* mRNA in G2 cells can promote some of the rearrangements of the F-actin cytoskeleton that are associated with medial ring formation [5]. We therefore examined the expression of *cdc15* in both *sep1-1* and *sep1::ura4*$^+$ mutants.

In *sep1-1* we observed that *cdc15* mRNA still accumulated in a periodic fashion, but that the peak appeared earlier than in wild-type cells. In contrast, in a *sep1::ura4*$^+$ mutant, expression of *cdc15* was aperiodic. Reprobing of the Northern blot with histone H2A showed that the H2A mRNA accumulated at the time of septation which corresponds to the S phase, demonstrating that the culture had been synchronised by the arrest–release protocol. These data suggest that one of the roles of sep1p is to influence the accumulation of *cdc15* mRNA. Since *sep1-1* is recessive [16] this altered timing is likely to result from partial loss of function, and suggests that the *sep1-1* allele is hypomorphic.

Since a *sep1* null allele results in a constant level of *cdc15* mRNA throughout the cell cycle, one possibility is that it functions as a repressor of *cdc15* expression. Previous studies [17] have demonstrated that increased expression of *sep1* does not mimic the phenotype produced by loss of *cdc15* function [5]. It is possible that sep1p acts together with other proteins to influence *cdc15* mRNA levels, or that it regulates another gene, whose product in turn influences *cdc15* expression. Future analysis will address these possibilities through the use of DNA microarrays once the *S. pombe* genome sequence is completed, and attempt to identify other target genes whose expression is influenced by *sep1*.

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