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Positive and negative roles for cdc10 in cell cycle gene expression

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ABSTRACT

In this paper we describe properties of the *cdc10-C4* mutant of the fission yeast *Schizosaccharomyces pombe*. The *cdc10$^+$* gene encodes a component of the DSC1^{Sp}/MBF transcription complex, which is required for cell-cycle regulated expression at G_1–S of several genes via *cis*-acting MCB (*Mlu*I cell cycle box) elements. At permissive temperatures *cdc10-C4* causes expression of MCB-regulated genes through the whole cell cycle, which in asynchronously dividing cells is manifested in overall higher expression levels. This overexpression phenotype is cold sensitive: in *cdc10-C4* cells, MCB genes are expressed at progressively higher levels at lower temperatures. In heterozygous *cdc10-C4/cdc10$^+$* diploid strains, MCB-regulated genes are not overexpressed, suggesting that loss, rather than alteration, of function of the *cdc10-C4* gene product is the reason for unregulated target gene expression. Consistent with this, the *cdc10-C4* mutant allele is known to encode a truncated protein. We have also overexpressed the region of the cdc10 protein absent in *cdc10-C4* under the control of an inducible promoter. This induces a G_1 delay, and additionally causes a reduction of the overexpression of MCB genes in *cdc10-C4* strains. These results suggest that DSC1^{Sp}/MBF represses, as well as activates, MCB gene expression during the cell cycle.

INTRODUCTION

Genetic analysis of the regulation of the eukaryotic cell cycle has revealed the existence of certain crucial events during the cycle, which are usually associated with the action of particular cyclin-dependent kinases (reviewed in 1). The yeasts *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* have been widely used in these studies. In *S.cerevisiae*, whose cell cycle has a long G_1 interval but indistinct S–G_2 and G_2–M transitions (reviewed in 2), the major point of cell cycle control appears to reside in G_1, at a point termed 'Start' (see below). In *S.pombe*, whose cell cycle contains a short G_1 phase and a long G_2 (reviewed in 3), entry into mitosis appears to be the main point at which control of the cell cycle resides, but Start is also an important point of cell cycle regulation.

Start is the point in the cell cycle at which the yeast cell makes a decision to enter the sexual or vegetative life cycle, or to remain in G_1/G_0. Before Start, cells can enter any of these developmental pathways, but once Start has been traversed cells are committed to complete the subsequent S-phase and mitosis. In *S.cerevisiae*, the Cdc28 cyclin-dependent kinase is required at Start (reviewed in 4), as well as a number of structurally homologous, partially redundant transcription factors, including Swi4, Swi6 and Mbp1. Swi6 associates with either Swi4 (5) or Mbp1 (6) to form alternative DNA binding complexes [named SBF (5,7) and DSC1/MBF (8,9)], which recognise different specific DNA sequence motifs [SCBs (10) and MCBs (see below)] to activate transcription of downstream genes. In particular, SBF binds SCBs to promote transcription of cyclins required for passage through Start, while DSC1/MBF binds MCBs to promote transcription of genes required during S phase, including cyclins required after the passage of Start (11,12). Progression from Start through S phase may be dependent on a series of interacting feedback loops (12).

In *S.pombe* the first genes to be identified as required for the passage of Start were *cdc2$^+$* and *cdc10$^+$* (13). *cdc2$^+$* encodes a cyclin dependent kinase (reviewed in 14); a number of potential cyclin partners for cdc2 have been identified, which may possess a role in G_1 (15). The cdc10 protein shows significant structural homology to Swi6 (16) and is also a transcription factor involved in the expression of genes during the G_1–S transition. A number of *S.pombe* genes are specifically expressed at this time during the cell cycle, including *cdc22$^+$* (17), *cdc18$^+$* (18) and *cdt1$^+$* (19) (all required in S phase) and *cig2$^+$* (which encodes a B-type cyclin) (20). *cdc22$^+$*, *cdc18$^+$* and *cdt1$^+$* contain MCB (*Mlu*I Cell-cycle Box) elements in their promoter regions similar to those found in *S.cerevisiae* genes under DSC1 control. MCBs are motifs either identical to the *Mlu*I recognition sequence (ACGCGT), or $^5/_6$ matches to this in which the central CGCG is always conserved (21); placed upstream of a heterologous reporter gene, they can confer periodicity of expression in either *S.pombe* (22) or *S.cerevisiae* (8). A protein complex, named DSC1^{Sp} [or MBF (23)], containing the cdc10 protein and which binds specifically to these elements has been identified by gel retardation assay (22). Although expression of these genes is periodic, this complex is detected throughout the cell cycle (22), and paradoxically, periodic binding is only seen in *wee1$^-$* mutant cells (24), in which the periodicity of transcription of MCB genes is reduced (our unpublished observations). It is possible that *in vivo* footprinting

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may be necessary to accurately observe DSC1^{Sp} binding during the cell cycle.

Two partners of cdc10 have been identified, res1/sct1 (25,26), which associates with cdc10 to form DSC1^{Sp} (26), and res2/pct1 (27,28). Both cdc10-containing complexes bind MCBs *in vitro* (26,28). res1 and res2 share substantial structural homology to each other and are partially redundant in function (26). However, the role of res1 is primarily concerned with the mitotic cell division cycle, whereas the role of res2 is mainly meiotic (25,27). The N-terminal region of res1 has been implicated in binding to cdc10 (23). The region of cdc10 involved in this interaction is not known, although cdc10 contains ankyrin motifs in the central region of the protein, which have been implicated in protein–protein interactions in other systems (16,29).

Strong overexpression of res1 induces G_1 arrest and expression of *cdc22*$^+$ at high levels; the arrest, although not the high-level expression of *cdc22*$^+$, is rescued by co-expression of *cdc10*$^+$ (23). This result is interesting, as it indicates that high level transcription of MCB genes may occur in G_1-arrested cells and is insufficient for progress into S phase.

The cdc10-Swi6 gene family shows some structural homology to higher eukaryotic transcription factors (30) involved in the periodic transcription of many genes during the G_1–S transition (31). However, there is no evidence yet to suggest an exact parallel between yeasts and higher organisms.

We have examined a mutant of *cdc10*$^+$, *cdc10-C4*, and show in this paper that it has unusual regulatory properties. MCB genes are overexpressed in the *cdc10-C4* mutant at low temperatures, permissive for cell cycle progress: this is because the genes are expressed throughout the cell cycle. The phenotype of overexpression of MCB genes conferred by *cdc10-C4* is recessive to *cdc10*$^+$. The cdc10-C4 protein is a C-terminal truncation (32). Overexpressing the C-terminus of cdc10 induces a G_1 delay, and in *cdc10-C4* strains additionally reduces the overexpression of MCB genes. These data suggest that DSC1^{Sp} has dual roles during the cell cycle, repressing as well as inducing MCB gene expression, and that these roles are differentially impaired by deleting or overexpressing the C-terminus of cdc10.

MATERIALS AND METHODS

Media and general techniques

General molecular procedures were performed as described by Sambrook *et al.* (33). Media for the propagation of *S.pombe* were as described by Moreno *et al.* (34). The standard genetical procedures of Gutz *et al.* (35) and Kohli *et al.* (36) were followed. The wild type (*cdc*$^+$) strain used in these experiments was *972 h*$^-$. For all physiological experiments cells were grown in minimal medium at temperatures between 16 and 36°C (specified in figure legends). Temperature sensitive mutants were grown at a permissive temperature then shifted to 35 or 36°C to display their mutant phenotype.

Populations of synchronously dividing fission yeast cells were prepared by use of a Beckmann elutriator rotor (37). Cell number per ml of liquid culture was determined from a sample diluted in Isoton (Coulter Electronics). Following sonication, cells were counted electronically with a Coulter Counter.

Flow cytometry analysis (FACS)

Flow cytometry was performed on ethanol-fixed cells as previously described (38), using the FACScan system and the Lysis II analysis program (Becton Dickinson); 10 000 cells were analysed for each time point.

Measurement of β-galactosidase activities

β-galactosidase activities were assayed by a procedure based on that of Miller (39). 200 µl culture was added to 800 µl Z-buffer. Cells were permeabilized with chloroform and SDS, and the suspension incubated at 28°C. 100 µl of a 4 mg/ml solution of ONPG was added, and after 10 min the reaction terminated by adding 250 µl 2 M Na_2CO_3. The suspension was then centrifuged, and the activity of the sample (pmol ONPG hydrolysed/min) calculated by measuring the A_{420} of a portion of the supernatant.

DNA and RNA manipulations

Schizosaccharomyces pombe total RNA was prepared essentially as described by Kaufer *et al.* (40), but without the proteinase K treatment. Northern blot analysis was carried out using GeneScreen (NEN) membrane following the manufacturer's suggested protocol. DNA probes were labelled with [α-^{32}P]dATP using the random hexanucleotide labelling procedure of Feinberg and Volgelstein (41).

Northern blots were hybridised with the following probes: *cdc22*$^+$ [equimolar amounts of the *Hin*dIII inserts of pCDC22-1; (17)]; *adh1*$^+$ [a 1.1 kb *Eco*RI fragment from the *S.pombe adh1*$^+$ gene; (42)]; *cdc18*$^+$ [nucleotides 19–1693 of the ORF amplified using the polymerase chain reaction with oligonucleotides 5′-GGTTGTCATACACCTCGAAG-3′ and 5′-CAACAGCTGTAATGACATCC-3′, (18)]; *cdt1*$^+$ [nucleotides 249–1247 of the ORF amplified using the polymerase chain reaction with oligonucleotides 5′-CCCCCAGTTAAAAATGAATC-3′ and 5′-GATCGCAAGTATGGTTTCCC-3′, (19)]; *cig2*$^+$ [nucleotides 7–1173 of the ORF amplified using the polymerase chain reaction with oligonucleotides 5′-CTCTATTCAATTTCAAAGCC-3′ and 5′-GAAGGTCATCATCGTCCGTACG-3′, (20)]; *cdc10*$^+$ [a PCR fragment containing the entire *cdc10*$^+$ ORF amplified using the oligonucleotides 5′-CGCGCGGGATCCTTATGCTTGATGTTCTTT-3′ and 5′-GCGCTCTAGACATATGGCTTCAGCCAATTTT-3′, (43)]; *cdc10*C (a 3′ probe made by amplification of the region of *cdc10*$^+$ from nucleotides 2117 to 2525 by PCR with the oligonucleotides 5′-CGAAATTCCAACATAAAA-3′ and 5′-TTAACTAATTCCAATAGGGATCCTTATTAACATATAATAAATTACG-3′) and *ura4*$^+$ [the *Hin*dIII fragment containing the *S.pombe ura4*$^+$ gene (44)].

Quantitative PhosphorImage analysis of Northern blots was performed using the Molecular Dynamics Image Quant software.

Construction of plasmids overexpressing fragments of *cdc10*$^+$

The plasmids listed in Table 2 were constructed, firstly by cloning a *Hin*dIII–*Sac*I fragment of DNA containing the entire *cdc10*$^+$ coding region (43) into pTZ19R (Pharmacia). Site directed mutagenesis reactions were then performed to introduce the following mutations. (**1**) Removal of the internal *Nde*I site within the *cdc10*$^+$ ORF, via a T–G change at nucleotide 436 with respect

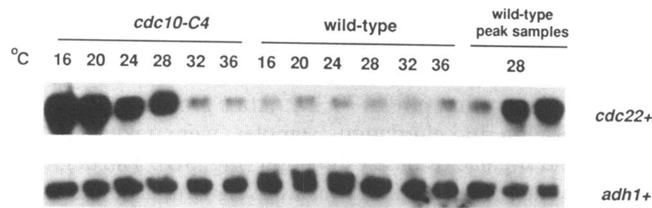

Figure 1. Effect of growth temperature on expression of *cdc22*+ in *cdc10-C4* cells. Cultures of *cdc10-C4* and *cdc10*+ were grown to mid-exponential phase of growth at 28°C, and portions were transferred to the temperatures indicated for 3.5 h. RNA was prepared from the cultures and subjected to Northern Blot analysis. The blot was hybridised with *cdc22*+ and *adh1*+ probes. Three consecutive RNA samples from *cdc10*+ cells grown in synchronous culture at 28°C, at the time of maximum *cdc22*+ expression, were processed in parallel (wild-type peak samples).

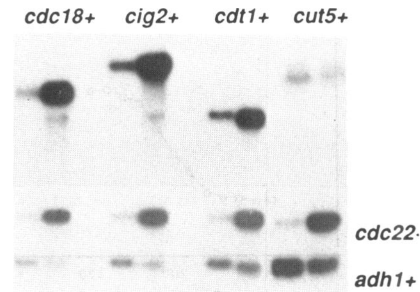

Figure 2. Other *S.pombe* MCB genes are also overexpressed in *cdc10-C4*. RNA was prepared from cultures of wild-type and *cdc10-C4* cells grown at 24°C, and subjected to Northern Blot analysis. Separate blots were hybridized with *cdc18*+, *cig2*+, *cdt1*+ and *cut5*+ probes; all the blots were then hybridised with *cdc22*+ and *adh1*+ probes. The left-hand lane of each pair contains RNA from *cdc10*+ cells; the right-hand lane RNA from *cdc10-C4* cells.

to translational start; (**2**) a *Bam*HI site was then introduced after nucleotide 2525; (**3**) one of the subsequent modifications was then carried out: (a) an *Nde*I site was introduced at the 5′ end of the ORF or (b) a stop codon was introduced via a C→T transition at nucleotide 2120 or (c) an *Nde*I site was introduced after nucleotide 1572 or (d) an *Nde*I site was introduced after nucleotide 2117; (**4**) in each case, an *Nde*I–*Bam*HI fragment was then cloned out of pTZ19R and into pREP1 (44). All mutagenised constructs were sequenced to confirm that all modifications had been introduced correctly.

The following oligonucleotides were used to perform the mutagenesis reactions described above: in step **1**, 5′-AATTGCT-CGATCCTATGGAACCCAT-3′, in step **2**, 5′-TTTAACTAATT-TCCAATAGGGATCCTTATTAACATATAATAAATTACG-3′, in step **3a**, 5′-TTGGCTGAAGCCATATGGTATATTAAAATA-AAAAATGCCGA-3′, in step **3b**, 5′-TGTTGGAATTTCATTGC-AAAGTTT-3′, in step **3c**, 5′-TGCGTTTCAACAAAATCATA-TGTCCAAAATTTGCTACGCT-3′, in step **3d**, 5′-TTTTAT-GTTGGAATTTCGCATATGTTGCCAAAGTTTGTTCGC-3′.

RESULTS

The *cdc10-C4* mutation leads to elevated expression of MCB-regulated genes at permissive temperatures

In the course of examining *cdc22*+ transcript levels in *cdc10*ts and wild-type strains, we were surprised to find a much higher level of transcript in one *cdc10*ts mutant, *cdc10-C4*. This strain is heat-sensitive for cell division, with a maximum permissive temperature <32°C, but strikingly, the level of *cdc22*+ transcript increased progressively at temperatures <28°C (Fig. 1), where *cdc10-C4* cells are able to proliferate. PhosphorImager analysis showed the transcript level in *cdc10-C4* at 16°C to be ~12 times that of the *cdc10*+ strain. In contrast, at 32 or 36°C, the level of *cdc22*+ transcript was very similar to that of the wild-type.

To investigate whether this phenomenon was restricted to *cdc22*+ or common to other MCB genes we tested the expression of three other genes known to be under cdc10 regulation: *cdc18*+, *cdt1*+ and *cig2*+ (18–20). The expression of these four genes was elevated in *cdc10-C4* (Fig. 2). *cut5* mutants have similar phenotypes to *cdt1* and *cdc18* mutants, and it has been suggested that the products of all three genes are functionally related (19,46). However, *cut5*+ is not under direct transcriptional control by cdc10 (47) and consistent with this, *cut5*+ expression in *cdc10-C4* was unaffected (Fig. 2).

To establish whether the elevated expression levels were due to the MCB elements, we tested the expression of a reporter construct, pSPΔ178.3M, in which the *E.coli lacZ* gene is under control of three *Mlu*I sequences arranged in tandem (described in 22). The plasmid was introduced into *cdc10*+ and *cdc10-C4* strains, and also into another *cdc10*ts mutant, *cdc10-129*. The specific β-galactosidase activity of these cells was determined in exponential culture. The specific activities at 24°C were 0.69 nmol/10^6 cells/min for *cdc10*+ and 6.2 nmol/10^6 cells/min for *cdc10-C4*, showing a 9-fold increase in the mutant. *cdc10-129* showed a modest but consistent increase in activity over the wild-type, at 0.90 nmol/10^6 cells/min.

Thus the mutant *cdc10-C4* allele has two distinct phenotypic effects: a heat-sensitive cell cycle block, such that *cdc10-C4* mutant cells are unable to undergo DNA synthesis or divide >32°C, and a cold-sensitive enhancement of MCB-driven transcription which increases progressively with reduced temperature.

Cell cycle expression of MCB genes in *cdc10-C4*

In *cdc10*+ cells, the *cdc22*+ transcript, and the *lacZ* transcript from the reporter plasmid pSPΔ178.3M, reach maximum levels around the G_1–S phase boundary (22). The minimum level of *cdc22*+ transcript, in mid-G_2, is at least 12-fold lower than the maximum (17). A possible explanation for the higher level of the *cdc22*+ and *lacZ* transcripts in *cdc10-C4* is that cell-cycle control over their transcription is lost, and expression occurs at a high level throughout the cell cycle. In this context it should be noted that the level of *cdc22*+ transcript in asynchronous *cdc10-C4* cells grown ≤ 24°C is at least as high as the peak level in synchronous *cdc10*+ cells (Fig. 1). To test this possibility directly, *cdc10-C4* cells were grown at 24°C and small cells in G_2 were selected by elutriation. These cells were allowed to undergo synchronous outgrowth, and samples were taken for RNA preparation, Northern blotting and hybridisation with a *cdc22*+ probe (Fig. 3A). The level of *cdc22*+ transcript varied very little during the experiment, consistent with a loss of periodicity of expression. *cdc18*+, *cdt1*+ and *cig2*+ were similarly found to be expressed throughout the cell cycle in *cdc10-C4* cells (data not shown).

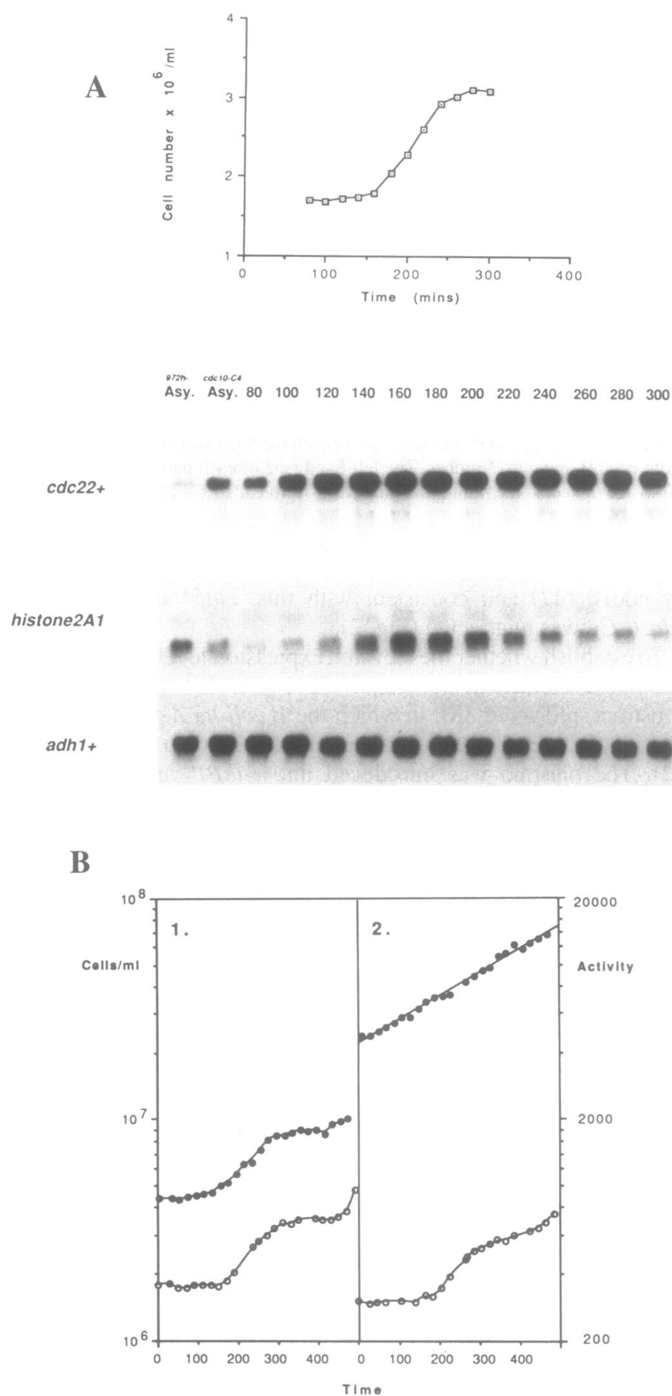

Figure 3. Continuous expression of *cdc22*$^+$ and reporter gene through the cell cycle of *cdc10-C4* (**A**) Continuous expression during the cell cycle of *cdc22*$^+$ in *cdc10-C4*. A synchronous culture of *cdc10-C4* cells was prepared by elutriation. Cells were allowed to undergo synchronous growth at 24°C, and samples were taken at intervals for cell number determination and preparation of RNA. The RNA was subjected to Northern Blot analysis, and the blot hybridised with *cdc22*$^+$, *adh1*$^+$ and histone H2A1 probes. Two control lanes were included containing RNA from asynchronous wild-type (*cdc*$^+$ Asy.) and *cdc10-C4* (Asy.) cells grown at 24°C. (**B**) Continuous expression of the *lacZ* reporter gene in *cdc10-C4*. Synchronous cultures of *cdc10*$^+$ (1) and *cdc10-C4* (2) strains carrying pSPΔ178.3M were prepared by elutriation, and allowed to undergo synchronous growth at 24°C. Samples were taken for determination of cell number ml^{-1} (o) and β-galactosidase activity (•) (pmol ONP produced/min/ml culture).

To determine whether the loss of cell cycle regulation in *cdc10-C4* was a specific property conferred by MCB elements, we investigated synchronous cultures of *cdc10-C4* and *cdc10*$^+$ strains, each carrying pSPΔ178.3M. After growth at 24°C and synchronisation by elutriation, samples were taken for cell number and β-galactosidase estimation (Fig. 3B). The degree of synchrony attained in both cultures was very similar, as judged by cell number increase. β-galactosidase activity in the *cdc10*$^+$ culture increased in a step-wise manner, consistent with a maximum in transcript level around the time of cell division, which is close to the G_1–S boundary, at which time *cdc22*$^+$ transcript is maximal. In contrast, β-galactosidase activity in the *cdc10-C4* culture showed no evidence of periodicity, but increased exponentially throughout the experiment, indicative of constitutive expression of the *lacZ* gene. A large difference in β-galactosidase activity per cell was again observed between the *cdc10*$^+$ and *cdc10-C4* cells.

In contrast to the behaviour of *cdc22*$^+$ and the MCB-driven *lacZ* reporter, the level of histone H2A1 message fluctuated periodically in this culture (Fig. 3A) just as in wild-type cells (48). This indicates that the loss of periodic expression caused by the *cdc10-C4* mutation does not extend to all periodically expressed genes, but is likely to be restricted to those containing MCB elements, which histone genes lack (49). It is known that in *S.cerevisiae* the histone genes are regulated independently of MCB genes (50,51).

cdc10-C4 is genetically recessive for elevated transcription of MCB genes

We investigated the dominance relationships among *cdc10* alleles with respect to their effects on *cdc22*$^+$ expression (note that all *cdc10*^{ts} alleles examined so far, including *cdc10-C4*, are recessive for temperature sensitive lethality; 32). A series of diploid strains comprising all pairwise combinations of the *cdc10*$^+$, *cdc10-129* and *cdc10-C4* alleles was constructed. Each strain was grown at 24°C, and RNA prepared and analysed by Northern hybridisation for *cdc22*$^+$ transcript. *cdc22*$^+$ transcript was present at the wild-type level in the *cdc10-C4*/*cdc10*$^+$ heterozygote (Fig. 4), indicating that *cdc10-C4* is recessive for increased MCB gene expression. Only in the *cdc10-C4* haploid and *cdc10-C4* homozygous diploid strains was the *cdc22*$^+$ transcript present at an elevated level. The significance of this observation is discussed below.

cdc10-C4 cells are delayed in G_2 progression

The unusual properties of the *cdc10-C4* mutant during growth at its permissive temperature led us to ask whether cell cycle progress was otherwise altered in this mutant. *cdc10-C4* strains showed substantially increased cell length at division during exponential growth at low temperatures, whereas doubling times for *cdc10-C4* were only slightly increased relative to wild-type (see Table 1). This strongly suggested a general effect of the *cdc10-C4* mutation on cell cycle progression at temperatures permissive for growth. Flow microfluorimetry (FACS) analysis of *cdc10-C4* cells growing at 24°C showed no G_1 delay during the cell cycle (see Fig. 5, profiles 14 and 15), so the observed increase in cell length at division is likely to be due to delay in G_2.

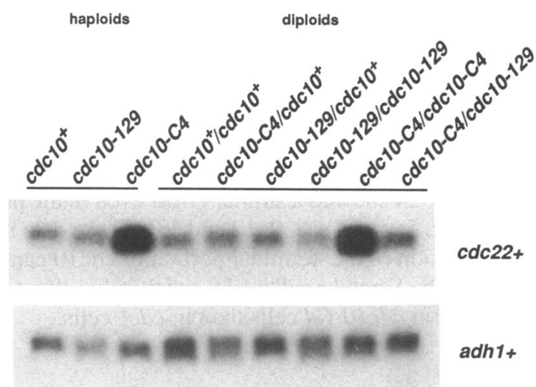

Figure 4. *cdc10-C4* is recessive to *cdc10*+ for overexpression of *cdc22*+ transcript. Diploid strains either heterozygous for combinations of *cdc10-C4*, *cdc10-129* and *cdc10*+ or homozygous for each allele, and the parental haploid strains, were grown at 24°C to mid-exponential phase. RNA was prepared from each culture and was subjected to Northern Blot analysis. The blot was hybridised with *cdc22*+ and *adh1*+ probes.

Table 1. Effect of growth temperature on cell length at division and growth rate in *cdc10-C4*

		28°C	24°C	20°C
cdc10+	cell length (μm)	15.3	15.4	15.3
	generation time (min)	200	270	600
cdc10-C4	cell length (μm)	19.6	24.7	30.7
	generation time (min)	210	330	660

Cultures of *cdc*+ and *cdc10-C4* were grown at the temperatures indicated, and samples were taken at suitable intervals for estimation of cell number. The lengths of septated (dividing) cells were measured microscopically, and the mean calculated of 30 cells per culture.

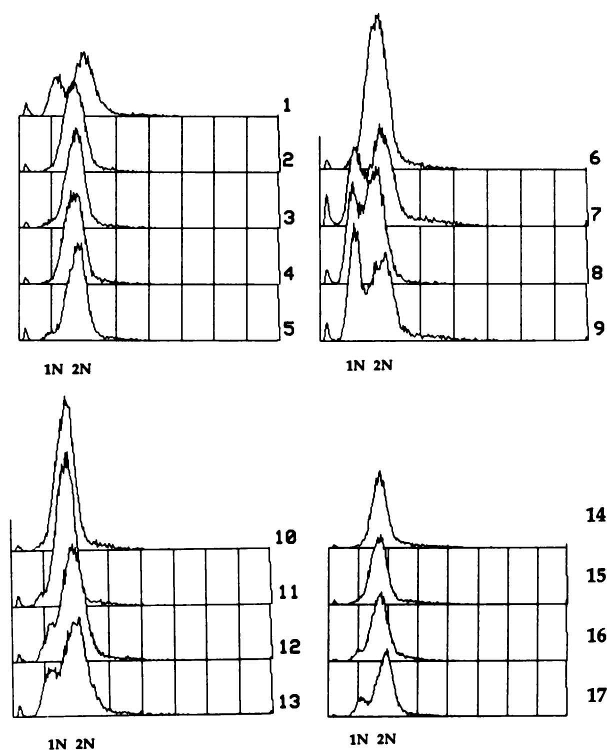

Figure 5. DNA content of cells overexpressing fragments of cdc10. Cultures of *S.pombe* strains were grown to early log phase in minimal medium at 28°C, in the presence and absence of 4 mM thiamine, and analysed for DNA content by flow cytometry; profiles indicate red fluorescence (x-axis) against cell number (y-axis). Strains analysed were: (1) *cdc10-29* cells grown at 28°C then shifted to their restrictive temperature of 36°C for 2 h, at which point roughly half the cells have arrested in G_1; (2), (3) *cdc*+[pREP1-*cdc10*+] +, – thiamine; (4), (5) *cdc*+[pREP1-*cdc10-C4*] +, – thiamine; (6), (7) *cdc*+[pREP1- *cdc10*Δ2-524] +, – thiamine; (8), (9) *cdc10-C4*[pREP1-*cdc10*Δ2-524] +, – thiamine; (10), (11) *cdc10*+[pREP1-*cdc10*Δ2-706] +, – thiamine; (12), (13) *cdc10-C4* [pREP1-*cdc10*Δ2-706] +, – thiamine; (14), (15) *cdc10-C4* +, – thiamine; (16), (17) *cdc10-C4* [pREP1] +, – thiamine.

Overexpression of the C terminus of cdc10 induces a G_1 delay

The *cdc10-C4* mutant encodes a C-terminally truncated protein, lacking 61 amino acids (32). Therefore, we decided to investigate the effects of over-expressing this C-terminal region under the control of a thiamine-repressible promoter, using the autonomously-replicating vector pREP1 (45). Four constructs were made (see Materials and Methods): pREP1-*cdc10*+ and pREP1-*cdc10-C4*, which express the wild type cdc10 protein and the cdc10-C4 mutant protein respectively, and pREP1-*cdc10*Δ2-524 and pREP1-*cdc10*Δ2-706, which express C-terminal regions of cdc10. The region of highest homology in cdc10 to *S.cerevisiae* Swi6 extends to amino acid 524, whereas amino acids 707–767 are absent in the cdc10-C4 protein (32). These plasmids, together with pREP1 (as a control), were transformed into *cdc10*+ and *cdc10-C4* strains, and in each case transformants were recovered at 28°C and grown on selective medium containing thiamine.

All the transformant strains remained viable at 28°C when shifted to medium lacking thiamine. Additionally, cells of each transformant strain were grown in liquid culture at 28°C in the presence and absence of thiamine and the cell length at division was measured. In no case was a significant difference in cell length observed between cultures grown in the presence or absence of thiamine. On solid medium, cells were also grown in the presence and absence of thiamine at 20 and 36°C. At 36°C, *cdc10-C4* cells containing pREP1-*cdc10*+ showed a cdc phenotype in the presence of thiamine but were viable if grown in the absence of thiamine, consistent with the recessive nature of the *cdc10-C4* mutation. None of the other strains showed a different cell length or cdc phenotype upon removal of thiamine at any of the temperatures examined (data not shown).

Asynchronous cultures of the transformant strains were then grown at 28°C and analysed for DNA content. Firstly, the *cdc*+ strains were examined. In the presence of thiamine, all strains had a wholly G_2 DNA content, typical of wild-type cells (Fig. 5, profiles 2, 4, 6, 10 and data not shown). In the absence of thiamine, this was also the case for cells carrying pREP1 (data not shown), pREP1-*cdc10*+, pREP1-*cdc10-C4* or pREP1-*cdc10*Δ2-706 (Fig. 5, profiles 3, 5 and 11). However, in cultures of cells carrying pREP1-*cdc10*Δ2-524, 36% of the population was located in G_1, indicating that these cells had reduced capacity to initiate DNA replication (Fig. 5, profile 7).

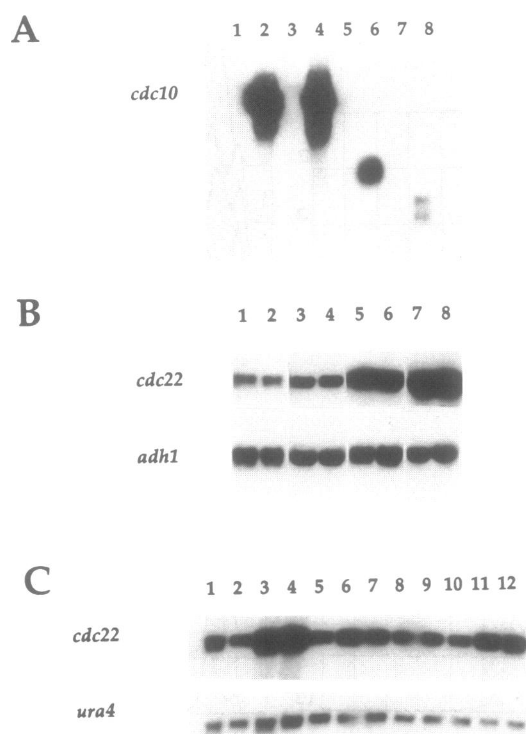

Figure 6. Effect of overexpression of fragments of cdc10 on expression of *cdc22*+. Cultures of *S.pombe* strains were grown to early log phase in minimal medium at 28°C, in the presence and absence of 4 mM thiamine, and analysed for RNA content by Northern blotting. **(A)** Cells carrying pREP1-*cdc10*+, *cdc10-C4*, *cdc10*Δ2-524 and *cdc10*Δ2-706 overexpress fragments of the *cdc10* gene in the absence of thiamine; this Northern blot was probed with a full length *cdc10* probe (see Materials and Methods). Strains analysed were: (1), (2) *cdc*+[pREP1-*cdc10*+] +, – thiamine; (3), (4) *cdc*+[pREP1-*cdc10-C4*] +, – thiamine; (5), (6) *cdc*+ [pREP1-*cdc10*Δ2-524] +, – thiamine; (7), (8) *cdc*+[pREP1-*cdc10*Δ2-706] +, – thiamine. Expression was also observed at lower levels in the presence of thiamine (data not shown; see text). **(B)** *cdc10-C4* cells carrying pREP1-*cdc10*+ do not overexpress *cdc22*+. Strains analysed were: (1), (2) *cdc*+ +, – thiamine; (3), (4) *cdc10-C4* [pREP1-*cdc10*+] +, – thiamine; (5), (6) *cdc10-C4* +, – thiamine; (7), (8) *cdc10-C4* [pREP1] +, – thiamine. **(C)** *cdc10-C4* cells carrying pREP1-*cdc10*Δ2-524 and pREP1-*cdc10*Δ2-706 show reduced overexpression of *cdc22*+. Strains analysed were: (1), (2) *cdc*+ +, – thiamine; (3), (4) *cdc10-C4* , +, – thiamine; (5), (6) *cdc*+[pREP1-*cdc10*Δ2-524] +, – thiamine; (7), (8) *cdc*+[pREP1-*cdc10*Δ2-706] +, – thiamine; (9), (10) *cdc10-C4* [pREP1-*cdc10*Δ2-524] +, – thiamine; (11), (12) *cdc10-C4* [pREP1-*cdc10*Δ2-706] +, – thiamine.

Next, the *cdc10-C4* mutant strains were examined. As noted above, *cdc10-C4* strains possess a predominantly G_2 FACS profile indistinguishable from that of wild-type strains (Fig. 5, profiles 14 and 15). However, in the presence of thiamine, the FACS profile of all five strains carrying a pREP1-based plasmid contained a small G_1 population. In the case of three strains, those carrying the plasmids pREP1 (Fig. 5, profiles 16 and 17), pREP1-*cdc10*+, and pREP1-*cdc10-C4* (data not shown), which have no phenotypic effect on wild-type cells, this G_1 peak did not increase in size in the absence of thiamine. This G_1 population in plasmid-carrying *cdc10-C4* cells may be a result of plasmid loss occurring against a background of altered cell cycle regulation.

However, in the absence of thiamine, the two *cdc10-C4* strains which overexpress a C-terminal fragment of cdc10 both contain significantly larger G_1 populations than the control strain carrying pREP1. Strains carrying either pREP1-*cdc10*Δ2-524 or pREP1-*cdc10*Δ2-706 showed a G_1 peak containing 35–40% of the population, a similar proportion to that seen in *cdc*+ cells carrying pREP1-*cdc10*Δ2-524 (pREP1-*cdc10*Δ2-706 has no effect on *cdc*+ cells). Interestingly, the strain carrying the pREP1-*cdc102*-524 showed a similar effect even in the presence of thiamine (Fig. 5, profiles 8, 9, 12 and 13). These data indicate that overexpression of a C-terminal portion of cdc10 can induce a G_1 delay into the *S.pombe* cell cycle, and that this effect is more readily induced in *cdc10-C4* cells than in *cdc*+ cells.

Overexpression of the cdc10 C-terminus reduces the overexpression of MCB genes in *cdc10-C4* cells

To investigate whether the G_1 delay observed in strains overexpressing C-terminal fragments of cdc10 was associated with altered levels of transcription of MCB genes, total RNA was prepared from the transformant strains and subjected to Northern blot analysis; the data from these experiments, together with the FACS analysis described above, is summarised in Table 2. A *cdc10* probe was first used to confirm strong overexpression of the C-terminal fragments of *cdc10* in the absence of thiamine (Fig. 6A); a second probe containing only the 3′ region of *cdc10*+ (see Materials and Methods) was also used to confirm that, in the presence of thiamine, the C-terminal constructs are expressed at a level comparable with wild-type *cdc10*+ transcript (data not shown). This is consistent with the existence of certain phenotypes in some strains even under repressing conditions. The levels of the MCB gene *cdc22*+ were then examined. No effect was observed on the level of *cdc22*+ in any of the wild-type strains. Overexpression of *cdc22*+ in *cdc10-C4* strains was unaffected by the presence of either pREP1 or pREP1-*cdc10-C4* . Interestingly, overexpression of *cdc22* was reduced in *cdc10-C4* strains carrying pREP1-*cdc10*+ under either repressing or inducing conditions (Fig. 6B), although the cdc defect at 36°C was only rescued in the absence of thiamine.

In *cdc10-C4* strains carrying pREP1-*cdc10*Δ2-524 the level of *cdc22*+ transcript is reduced compared with that normally found in *cdc10-C4* cells (Fig. 6C); quantitative PhosphorImage analysis comparing levels of the *cdc22*+ transcript to those of the *ura4*+ transcript revealed the level of *cdc22*+ to be reduced 3-fold with respect to *cdc10-C4* cells carrying no plasmid. pREP1-*cdc10*Δ2-706, which causes a G_1 delay only in *cdc10-C4* cells (but not in wild-type), had no effect on the level of the *cdc22*+ transcript relative to *ura4*+.

Interestingly, the reduction in *cdc22*+ expression in *cdc10-C4* cells is seen in the presence or absence of thiamine. This suggests that a very low level of expression of the cdc10 C-terminus is sufficient to reduce the MCB gene overexpression typical of *cdc10-C4* cells, and also, in the case of the longer C-terminus, to induce a G_1 delay in the cell cycle. One possible explanation for the fact that this reduction in *cdc22*+ transcript levels is seen only in *cdc10-C4* cells (and not in *cdc*+) is that the shortened cdc10-C4 protein may have weaker interactions with other components of the $DSC1^{Sp}$ complex, and thus formation of active $DSC1^{Sp}$ may be more sensitive to overexpression of C-terminal fragments (see Discussion). This is also consistent with the FACS data, which suggested that a G_1 delay was more readily inducible in *cdc10-C4* cells.

Table 2. Summary table of pREP1-based constructs over-expressing fragments of cdc10

Construct	pREP1-*cdc10*$^+$	pREP1-*cdc10-C4*	pREP1-*cdc10*Δ2-524	pREP1-*cdc10*Δ2-706
Region of cdc10 encoded	aa 1–767	aa 1–706	Met + aa 525–767	Met + aa 707–767
G_1 delay in *cdc*$^+$ cells?	+thi: No	+thi: No	+thi: No	+thi: No
	–thi: No	–thi: No	–thi: No	–thi: No
G_1 delay in *cdc10-C4* cells?[a]	+thi: No	+thi: No	+thi: Yes	+thi: No
	–thi: No	–thi: No	–thi: Yes	–thi: Yes
cdc phenotype in *cdc10-C4* cells at 36°C?[b]	+thi: cdc$^-$	+thi: cdc$^-$	+thi: cdc$^-$	+thi: cdc$^-$
	–thi: cdc$^+$	–thi: cdc$^-$	–thi: cdc$^-$	–thi: cdc$^-$
cdc22$^+$ over-expression in *cdc10-C4* cells at 28°C?[b]	+thi: No	+thi: Yes	+thi: Reduced	+thi: Yes
	–thi: No	–thi: Yes	–thi: Reduced	–thi: Yes

[a]Yes indicates G_1 FACS peak larger than that observed in *cdc10-C4* cells carrying control plasmid (see text).
[b]*cdc10-C4* cells show these phenotypes in the absence of plasmid, or in the presence of control plasmid pREP1.
For details of experimental results see text.

DISCUSSION

The transcription complex DSC1^{Sp} contains cdc10 and res1/sct1: previous observations have shown that functional alleles of both *cdc10*$^+$ and *sct1*$^+$ are required for the formation of DSC1^{Sp} *in vitro* (22,26). The transcriptional activating role of DSC1^{Sp} is demonstrated by the reduction in transcript levels of several MCB genes in *cdc10*^{ts} mutations (18,19,32). We report here that one mutant *cdc10* allele, *cdc10-C4*, shows unusual regulatory properties during growth at permissive temperatures, which suggests that DSC1^{Sp} also has a repressing role in the control of MCB gene expression.

While *cdc10-129* cells show reduced expression of MCB genes at restrictive temperatures, expression under permissive conditions is apparently normal. In contrast, in *cdc10-C4* cells, the expression of four endogenous genes under cdc10 control is greatly elevated: for *cdc22*$^+$ this increases progressively at lower temperatures with a maximum measured increase of 12-fold. A similar effect was observed for an MCB-driven *lacZ* reporter gene. Examination of *cdc22*$^+$ and *lacZ* transcript levels in synchronous cultures of *cdc10-C4* shows that expression of these genes persists throughout the cell cycle (including G_2). The level of expression is very similar to the maximum observed in *cdc10*$^+$ cells, around the G_1–S transition. Thus the elevated expression levels observed in exponential *cdc10-C4* cultures are likely to be largely due to continued expression during G_2, during which period *cdc10*$^+$ cells express MCB genes at a level at least 12-fold down with respect to expression during G_1–S.

We noticed that at reduced growth temperatures, *cdc10-C4* cells showed a substantial increase in cell length, an effect which increased progressively with lower temperatures. FACS analysis shows that this increase in cell length indicates a cell cycle delay specifically in G_2. It seems likely that this G_2 delay is an indirect consequence of overexpression or ectopic expression of one or more particular MCB genes. Certainly, increased expression of *cdc18*$^+$ leads to G_2 delay and cell elongation (18), and it is possible that *cdc10-C4* mediates this effect by stimulating *cdc18*$^+$ expression in G_2. In *cdc10*$^+$ cells, *cdc18*$^+$ expression is very low in G_2 (18), and increasing its level to that normally found at G_1–S might be sufficient to bring about a delay in G_2.

The unusual properties of *cdc10-C4* may shed some light on the molecular roles of cdc10 and sct1/res1 in regulating MCB gene expression. The mutation in the *cdc10-C4* allele introduces a stop codon 61 amino acid residues from the C-terminus, thus truncating the protein—in contrast, most *cdc10*^{ts} alleles are missense mutations (32). The temperature sensitivity of *cdc10-C4* for cell cycle progress may be due to the truncated protein retaining some ability to bind to res1 and form a functional DSC1^{Sp} complex at low temperatures, but with reduced stability. At higher temperatures, the complex is destabilised and becomes inactivated. Consistent with this explanation is our consistent failure to detect DSC1^{Sp} activity in extracts of *cdc10-C4 in vitro* (22; CJMcI and PAF, unpublished observations).

Explanations for the cold-sensitive phenotype of deregulated MCB gene expression in *cdc10-C4* are less obvious. One possibility is that cdc10 is primarily concerned with cell cycle regulation, while res1 binds to target DNA sites. This would be analogous to the roles of Swi6 and Swi4 in budding yeast (51–53), although the analogy may not be perfect since cdc10 contains a putative DNA-binding site towards its N-terminus (54). The C-terminal region of cdc10 that is absent in *cdc10-C4* might be involved in efficient binding to res1, analogous to the known role of the Swi4 C-terminus in interacting with Swi6 (55).

Alternatively, or additionally, the C-terminus of cdc10 might be directly involved in cell cycle regulation: according to this model, the partially functional complex formed in *cdc10-C4* at low temperatures would be insensitive to cell cycle signals. The observations that the *cdc10-C4* allele encodes a truncated protein, and that the allele is genetically recessive for overexpression of target genes at 24°C, indicate that some aspect of cdc10 function is lost in the mutant. Since this loss leads to increased target gene expression in G_2, the cdc10 protein seems likely to have a negative regulatory role in the cell cycle, in addition to being required for DSC1^{Sp} formation and gene expression. Conceivably, this negative regulatory function could lie within either the cdc10 C-terminus or another 'repressor' protein which binds to this part of cdc10.

If this were the case, it might have been expected that over-expressing the C-terminus of cdc10 in wild-type cells would titrate out such a repressor and thus mimic the *cdc10-C4* phenotype. This is not seen, and instead, a G_1 delay is induced. However, no effect is seen unless a large fragment of cdc10 is overexpressed, and it is possible that this fragment of the protein may bind, and titrate out, a required component of DSC1^{Sp} (such as res1) in addition to binding a repressor. It is also possible that the repressor is required to maintain the structural integrity of the complex as well as to mediate a negative signal.

Overexpression of the smaller cdc10 C-terminal construct does have an effect, however, in *cdc10-C4* cells, and overexpression of the larger construct reduces the normally exaggerated level of MCB gene transcripts seen in this strain. This is consistent with the idea, discussed above, that the cdc10-C4 protein, while hyper-active for transcription, may form part of a less stable complex, which is more susceptible to disruption.

Strong overexpression of res1 (or an N-terminal fragment of res1) has recently been shown to cause G_1 arrest, and to cause increased *cdc22*$^+$ expression to a similar (10–12-fold) extent (23) as we see in *cdc10-C4* cells. The growth defect is rescued if cdc10 is also over-expressed, although *cdc22*$^+$ over-expression is still observed (23). A closer parallel to our results is the observation that over-expressing the (C-terminal) cdc10-binding domain of res1 also induces G1 arrest, but accompanied by a fall in *cdc22*$^+$ expression, perhaps as a consequence of the titration of cdc10 away from full length res1 (23). We have shown that over-expression of the C-terminus of cdc10 causes similar, although less extreme, transcriptional and cell cycle phenotypes, while the behaviour of the *cdc10-C4* mutant suggests an additional role for the C-terminus in mediation of negative cell cycle regulation. Our work thus suggests cdc10 has distinct structural and regulatory roles within the DSC1^{Sp} complex, and that disruption to the wild-type system interferes differentially with the two outputs of DSC1^{Sp} function, namely MCB gene transcription and cell cycle progression.

NOTE ADDED IN PROOF

We state above that the cdc phenotype at 36°C of *cdc10-C4* strains containing the pREP1-based plasmids described is not altered by the absence of thiamine, except in the case of pREP1-*cdc10*$^+$. We have re-tested this observation and now report that the cdc defect is also rescued in the the absence of thiamine by cells containing pREP-*cdc10*Δ2-524, consistent with related observations made by Reymond, A. and Simanis, V., *Nucleic Acids Res.* (1993) **21**, 3615–3621.

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