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Rme1, a negative regulator of meiosis, is also a positive activator of G_1 cyclin gene expression

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Control of G_1 cyclin expression in *Saccharomyces cerevisiae* is mediated primarily by the transcription factor SBF (Swi4/Swi6). In the absence of Swi4 and Swi6 cell viability is lost, but can be regained by ectopic expression of the G_1 cyclin encoding genes, *CLN1* or *CLN2*. Here we demonstrate that the *RME1* (regulator of meiosis) gene can also bypass the normally essential requirement for SBF. *RME1* encodes a zinc finger protein which is able to repress transcription of *IME1* (inducer of meiosis) and thereby inhibit cells from entering meiosis. We have found that expression of *RME1* from a high copy number plasmid can specifically induce *CLN2* expression. Deletion of *RME1* alone shows no discernible effect on vegetative growth, however, deletion of *RME1* in a *swi6Δ swi4*^{ts} strain results in a lowering of the non-permissive temperature for viability. This suggests that Rme1 plays a significant but ancillary role to SBF in inducing *CLN2* expression. We show that Rme1 interacts directly with the *CLN2* promoter and have mapped the region of the *CLN2* promoter required for Rme1-dependent activation. Consistent with Rme1 having a cell cycle role in G_1, we have found that *RME1* mRNA is synthesized periodically in the cell cycle, with maximum accumulation occurring at the M/G_1 boundary. Thus Rme1 may act both to promote mitosis, by activating *CLN2* expression, and prevent meiosis, by repressing *IME1* expression.

Keywords: cell cycle/G_1 cyclins/*Saccharomyces cerevisiae*/transcription

Introduction

In the budding yeast *Saccharomyces cerevisiae* the decision to enter a new cell cycle or commit to an alternative developmental fate occurs in late G_1 at a point called Start (Pringle and Hartwell, 1981). If growth conditions are appropriate cells will execute Start and become irreversibly commited to cell division. Prior to Start, in response to nutrient limitation or exposure to mating pheromone, cells will arrest the cell cycle in G_1. Thereafter, depending on its nutritional status and cell type, a yeast cell can pursue one of several developmental pathways, including sporulation, conjugation and pseudohyphal differentiation. The execution of Start requires activation of the Cdc28 cyclin-dependent kinase through its association with specific G_1 cyclins, encoded by *CLN1*, *CLN2* and *CLN3* (reviewed in Nasmyth, 1993). While any one of the *CLN* gene products is sufficient for Start, recent experiments indicate that their functions are not equivalent. *CLN1* and *CLN2* differ from *CLN3* in both their pattern of expression and the primary structure of their gene products. The *CLN1* and *CLN2* genes are expressed under cell cycle control, with both mRNA and protein levels peaking in late G_1. In contrast, *CLN3* expression does not oscillate in the cell cycle and the abundance of the Cln3 protein, which is unstable, is determined by cell size and the rate of protein synthesis. The dependence of Cln3 concentration on the growth status of the cell, coupled with the observation that ectopic expression of *CLN3* can induce expression of G_1 cyclin-encoding genes, including *CLN1* and *CLN2*, has led to a model wherein Cln3 acts as an indicator of the growth status of the cell, activating downstream cyclins which in turn catalyse Start (Tyers *et al.*, 1993).

Constitutive over-expression of *CLN2* from a heterologous promoter reduces the length of G_1 and in some strain backgrounds leads to premature entry into S phase and cell death (Amon *et al.*, 1993). Thus cell cycle-regulated transcription of *CLN2* and, probably, *CLN1*, is important for normal cell cycle progression. This regulation is dependent on Cdc28 kinase and is mediated primarily by the transcription factor SBF, composed of proteins encoded by *SWI4* and *SWI6* (Cross and Tinkelenberg, 1991; Dirick and Nasmyth, 1991; Ogas *et al.*, 1991). SBF may therefore be a target, directly or indirectly, for the Cln3 Cdc28 kinase activity. SBF was originally identified as a factor required for G_1 expression of the HO endonuclease gene and recognizes a sequence called the Swi4/6 cell cycle box (SCB; CACGAAA) (Breeden and Nasmyth, 1987; Andrews and Herskowitz, 1989). Another transcription factor that may have a role in expression of *CLN1* and *CLN2* is DSC1/MBF, which is composed of the Swi4 homologue Mbp1 and Swi6. This complex recognizes the *Mlu1* cell cycle box (MCB; ACGCGT) and induces expression of many genes in G_1 which are principally required for S phase (Lowndes *et al.*, 1991; Johnston and Lowndes, 1992). Considerable cross-talk is believed to occur between these transcription factors, since *in vitro* experiments demonstrate that SBF will recognize MCB elements and DSC1/MBF will recognize SCB elements (Dirick *et al.*, 1992; Breeden, 1995). Both MCB and SCB (or SCB-like) elements are found in the promoters of *CLN1* and *CLN2*. Furthermore, the lethality associated with inactivation of *SWI4* and *SWI6* or of *SWI4* and *MBP1* can be rescued by ectopic expression

of *CLN1* or *CLN2* (Nasmyth and Dirick, 1991). These observations suggest that the essential function of SBF and DSC1/MBF is activation of *CLN* expression. However, recent analyses of the *CLN2* promoter indicate that regulation of *CLN*s may involve other factors. Inactivation of *SWI4* or deletion of the SCB and MCB elements from the *CLN2* promoter reduces the overall levels of *CLN2* mRNA, but has no effect on periodic expression of the gene. Furthermore, periodic expression directed from a *CLN2* promoter devoid of SCB/MCB elements is dependent on *SWI4*, even though apparently all Swi4 binding sites have been removed. Thus it is probable that other factors, possibly regulated by Swi4, play a role in periodic expression of *CLN2* and, perhaps, *CLN1* (Cross *et al.*, 1994; Stuart and Wittenberg, 1994; Breeden, 1995). Indeed, in this report we demonstrate that *RME1*, a gene previously found to encode a negative regulator of meiotic gene expression, has a role in the activation of *CLN2* expression.

Upon starvation for nitrogen and carbon MAT**a**/α diploid cells can either arrest the cell cycle in G_1 or initiate a meiotic programme leading to sporulation. The ability of a yeast cell to enter the meiotic programme is controlled by the diploid-specific repressor a1-α2, composed of products from both haploid mating partners. The a1-α2 repressor controls entry into meiosis by repressing expression of *RME1* (regulator of meiosis), which encodes a negative regulator of *IME1* (inducer of meiosis) transcription (reviewed in Mitchell and Herskowitz, 1986; Kassir *et al.*, 1988; Covitz *et al.*, 1991; Covitz and Mitchell, 1993; Mitchell, 1994). The *IME1* gene product in turn is a transcriptional activator required for the induction of early meiotic gene expression. Thus haploid yeast cells which lack the a1-α2 repressor and consequently express the *RME1* gene are unable to sporulate. Disruption of *RME1* allows *IME1* expression, but does not alter the nutritional requirements for sporulation (Kassir *et al.*, 1988). Consequently, haploid cells deleted for *RME1* and starved for nitrogen and carbon can engage in premeiotic DNA synthesis, recombination and later steps in spore formation, however, they cannot complete meiosis because they lack homologous chromosomes. The current view of Rme1 function is that it acts as a protective measure to ensure that haploid cells do not enter a lethal meiosis.

The *IME1* promoter, to which Rme1 binds, is exceptionally large and is the known target of a number of pathways which transmit signals reflecting the nutritional status of the cell (reviewed by Mitchell, 1994). Rme1-induced repression of the *IME1* gene requires a sequence in the *IME1* promoter called the Rme1 response element (RRE), as well as an adjacent modulation region. Interestingly, in the absence of the modulation region the RRE can act as a UAS when fused to a reporter gene (Covitz and Mitchell, 1993). This ability of the RRE to act as a transcriptional UAS implies that Rme1 may be involved in both the activation and repression of gene expression.

In this report we demonstrate that over-expression of *RME1* can bypass the normally essential requirement for the SBF transcription factor. This abilty to bypass the requirement for SBF was found to be dependent on the presence of a functional allele of *CLN2*. Moreover, we show that Rme1 can specifically activate *CLN2* expression. Deletion of the *RME1* gene in combination with a partially inactive SBF complex results in a synergistically lethal phenotype. The *RME1* gene was found to be cell cycle regulated, with peak transcript levels occurring near the M/G_1 boundary. These results suggest that Rme1 may have a role as a stage-specific activator of *CLN2* gene expression.

Results

RME1 can suppress phenotypes associated with defects in G_1 cyclin expression

A *swi4*Δ yeast strain (BY604) was mutagenized with ethylmethanesulfonate and ~1500 temperature-sensitive mutants were collected. This screen was initiated with the aim of finding new mutations which were either synthetically lethal with *swi4*Δ (and which were rescued by re-introducing a single copy *SWI4* into the cell) or mutations which could be rescued by high copy *SWI4* (collectively called *rsf*, for requiring *SWI4*; manuscript in preparation). One of the mutations (*rsf11*) resulted in a temperature-sensitive lethal phenotype in a *swi4*Δ background but not in a $SWI4^+$ background, demonstrating that *rsf11* displays a synthetic interaction with *swi4*Δ. The temperature-sensitive growth defect in the *rsf11* mutant could be suppressed by both CEN-*SWI4* and by p*ADH-CLN2*, which provides ectopic expression of *CLN2* from the constituitvely active *Schizosaccharomyces pombe* promoter. The *rsf11* mutation was found to lie in the previously characterized essential gene *RAT1/TAP1/HKE1*, mutations in which show pleiotropic defects in RNA metabolism. In this publication we describe the isolation and characterization of a dosage-dependent suppressor of the *rsf11* (*swi4*Δ *rat1*) temperature-sensitive phenotype. The complete characterization of the *rsf11* mutant will be presented elsewhere.

Initial attempts to isolate the wild-type copy of the defective *rsf11* gene employed a 2μm-based genomic library and yielded a number of known genes, including *CLN1*, *CLN2*, the Pho85-associated cyclins *PCL1* and *PCL2* and known activators of G_1 cyclin expression such as *SWI4*. Thus the *rsf11* phenotype appears readily reversible by increased levels of G_1 cyclins. One library plasmid however, p1111-5, which did not appear by restriction analysis to encode a known cyclin or regulator of cyclin expression, was examined further. The gene encoding the suppressor activity of p1111-5 was localized by random insertions of Tn*1000* (Sedgwick and Morgan 1994). After introduction into an *rsf11* strain plasmids which had lost the ability to suppress the temperature-sensitive phenotype were identified. Restriction analysis showed that the transposons destroying suppressor activity had integrated within a 1 kb region of DNA. The sequences of the regions flanking the transposon insertions were obtained using oligonucleotide primers designed to hybridize to each end of the transposon. Database searches revealed that the gene inactivated by transposition and therefore responsible for the observed suppressor activity was the previously identified gene *RME1*.

The observation that the temperature-sensitive phenotype of the *rsf11* mutant strain could be suppressed by increased expression of genes encoding G_1 cyclins prompted us to test the ability of p1111-5 (hereafter called YEp-*RME1*) to suppress the growth defects in other strains

with known mutations affecting G_1 cyclin expression. G_1 cyclin expression in *S.cerevisae* is thought to be primarily under the control of the SBF transcription factor, whose subunits are encoded by *SWI4* and *SWI6* (Dirick and Nasmyth, 1991; Ogas *et al.*, 1991). Deletion of both *SWI4* and *SWI6* is lethal unless *CLN* expression is provided from a heterologous promoter. We used a *swi6Δ swi4*^{ts} strain (K2003) which is viable at 25°C but inviable at 37°C, presumably due to insufficient *CLN* expression (Nasmyth and Dirick, 1991). YEp-*RME1* was able to suppress the temperature-sensitive growth defect in K2003 as well as or better than a plasmid carrying *CLN2* under the control of the *S.pombe ADH1* promoter (Figure 1).

The ability of *RME1* to suppress the temperature-sensitive phenotypes of both the *rsf11* mutant strain and K2003 was found to be copy number dependent. Thus suppression of the associated temperature-sensitive phenotypes was observed when *RME1* was present on a 2μm-based plasmid, but not when *RME1* was subcloned onto a CEN plasmid (single copy number plasmid; data not shown). The ability of *RME1* to suppress the growth defects in *rsf11* and *swi4 swi6* strains, both of which require increased *CLN* expression for survival, suggests that *RME1* may have a previously unidentified role in the activation of mitotic gene expression.

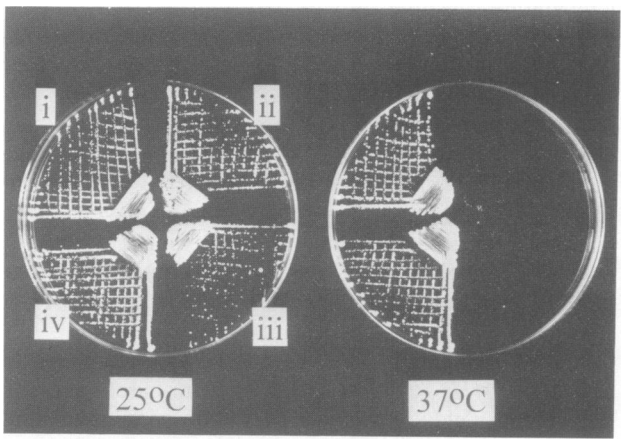

Fig. 1. YEp-*RME1* can bypass the essential requirement for Swi4/Swi6. Strain K2003 (*swi6Δ swi4*^{ts}) plated on YEPD at 25 and 37°C carrying either (i) YEp-*RME1*, (ii) YEp-*rme1*::Tn*HIS3*, (iii)YEp24 vector alone or (iv) p*ADH-CLN2*. Over-expression of *RME1* from a high copy number plasmid or ectopic expression of *CLN2* was found to suppress the temperature-sensitive phenotype in a *swi6Δ swi4*^{ts} strain. Inactivation of *RME1* by a transposon insertion blocks the ability of YEp-*RME1* to suppress the temperature-sensitive phenotype.

Induction of CLN mRNA by RME1

The observation that YEp-*RME1* can bypass the essential requirement for SBF implies that Rme1 can induce expression of at least a subset of the cyclin-encoding genes normally regulated by SBF. Swi4/Swi6 are required for maximal expression of *CLN1*, *CLN2*, *PCL1* and *PCL2*. To determine if Rme1 is acting through *CLN1*, *CLN2* or the constituitively expressed *CLN3* gene we introduced YEp-*RME1* into a triple *CLN* deletion strain maintained by *CLN3* on a galactose-inducible promoter. Normally this strain can only grow on galactose-containing media, so a gene which could bypass the requirement for *CLN*s would supplant the need for *CLN3* and allow growth on glucose-containing media. However, on a high copy number plasmid *RME1* does not allow growth of the triple *CLN* deletion strain on glucose, indicating that Rme1 sustains viability in a *swi4 swi6* strain through one or more of the *CLN* genes (data not shown).

To test directly whether or not *RME1* can induce expression of G_1 cyclins we introduced YEp-*RME1* into the *swi6Δ swi4*^{ts} strain K2003. Cells carrying YEp-*RME1* showed increased *CLN2* mRNA levels at both 25°C and after a temperature shift to 37°C (Figure 2). Levels of *CLN2* transcript accumulation were higher in the *RME1*-transformed strain than in the same strain carrying *SWI4* on a high copy number plasmid. In each case *CLN2* mRNA levels were higher than in strain K2003 carrying the vector alone. In contrast, a concomitant increase in *CLN1* mRNA levels was not observed in strains carrying YEp-*RME1*, although, as expected, high copy *SWI4* did lead to increased *CLN1* expression (Figure 2).

The ability of *RME1* to induce specifically *CLN2* expression was reflected in the suppression analysis of a number of mutant strains defective in G_1 transcription. We found that YEp-*RME1* could not only suppress a *swi6Δ swi4*^{ts} temperature-sensitive phenotype, but also the temperature sensitivity associated with both a *mbp1Δ swi4*^{ts} strain (BAM1; Figure 3) and a *mbp1Δ swi6Δ swi4*^{ts} strain (BAM2; data not shown). Furthermore, YEp-*RME1* could suppress the growth defect of a *mbp1Δ cln1Δ swi4*^{ts} strain (BAM3; data not shown), however, *RME1* was not able to suppress the temperature sensitivity of a *mbpΔ*

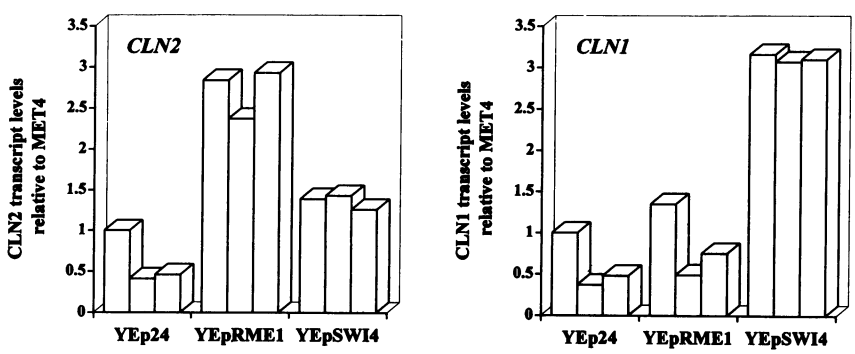

Fig. 2. *RME1* induces expression of *CLN2*, but not *CLN1*. Densitometric quantitation of a Northern blot analysis of RNA isolated from strain K2003 (*swi6Δ swi4*^{ts}) carrying either YEp24 vector alone, YEp-*RME1* or YEp-*SWI4* after 0, 2 and 4 h at 37°C. Levels of *CLN1* and *CLN2* transcript were normalized to a *MET4* loading control.

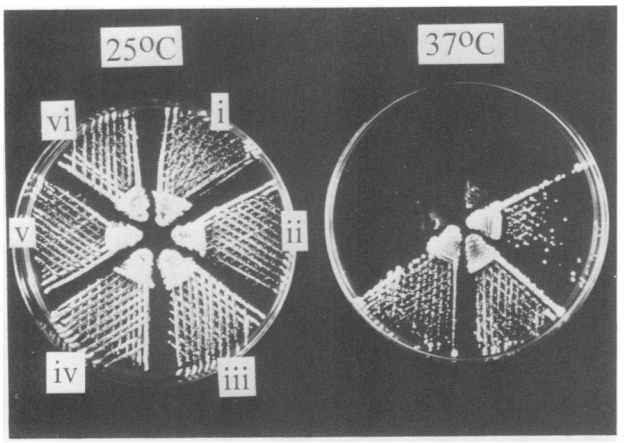

Fig. 3. YEp-*RME1* can rescue a *mbp1Δ swi4*^{ts} strain, but not a *mbp1Δ swi4*^{ts} *cln2Δ* strain at 37°C. The *mbp1Δ swi4*^{ts} strain (BAM1) plated on YEPD medium at 25 and 37°C carrying (i) YEp24 vector alone, (ii) YEp-*RME1* or (iii) p*ADH-CLN2*; the *mbp1Δ swi4*^{ts} *cln2Δ* strain (BAM4) carrying (iv) p*ADH-CLN2*, (v) YEp-*RME1* or (vi) YEp24 vector alone. *RME1* is dependent on a functional allele of *CLN2* for suppression of the *mbp1Δ swi4*^{ts} associated temperature-sensitive phenotype.

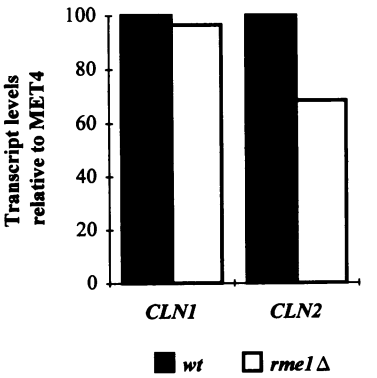

Fig. 4. *CLN2* and *CLN1* mRNA levels in a wild-type and *rme1Δ* strain. mRNA prepared from exponentially growing strains IH1783 and AMP143 (IH1783 *rme1Δ*) was blotted onto a nylon membrane and hybridized to probes representing either *CLN1* or *CLN2*. Hybridization levels were quantitated using a Molecular Dynamics PhosphorImager and normalized to the *MET4* mRNA as an invariant loading control (see Materials and methods). Expression of *CLN2*, but not *CLN1*, was found to decrease in an *rme1Δ* strain relative to an isogenic wild-type strain.

cln2Δ swi4^{ts} strain (BAM4; Figure 3). Thus, at least when present on a high copy number plasmid, *RME1* is able to bypass the requirement for the normally essential SBF and DSC1/MBF transcription factors in a *CLN2*-dependent manner.

RME1 null mutations display a synergistic phenotype with mutations which result in decreased SBF activity

The induction of *CLN2* expression following over-expression of *RME1* suggests that *RME1* may normally have a role in regulating *CLN2* activity. In order to examine the effect of deleting *RME1* on *CLN2* expression directly we prepared RNA from two exponentially growing strains. The two strains used, IH1783 and AMP143, are isogenic except that AMP143 carries a deletion of the *RME1* open reading frame (strains kindly provided by A.P.Mitchell). Interestingly, although there appears to be no difference in growth rates between the two strains, deletion of *RME1* results in a 30% decrease in steady-state *CLN2* mRNA levels, whereas no change in *CLN1* transcript levels was observed (Figure 4).

Since the observed decrease in *CLN2* levels in an *rme1Δ* strain does not result in a concomitant decrease in growth rate we decided to study the effect of deleting *RME1* in a strain which was already defective for *CLN2* expression. K2003, which is predicted to have no DSC1/MBF activity, due to the absence of *SWI6*, and a crippled SBF activity, grows at 25°C but is inviable at 37°C. We constructed a strain in which the *RME1* gene in K2003 was replaced by the *URA3* gene. Deletion of *RME1* in this strain resulted in a substantial reduction in the non-permissive temperature, from 37 to 30°C and even at 25°C the strain grew poorly (Figure 5). The reduction in the non-permissive temperature as a result of deleting *RME1* in K2003 could be reversed by ectopic expression of *CLN2* (data not shown). This result, together with the observation that *CLN2* mRNA levels are lowered in an *rme1Δ* strain, supports the conclusion that the effects of *RME1* on *CLN2* expression are not an artefact of over-expression, but a reflection of a wild-type physiological role in maintaing optimal levels of *CLN2* expression.

Identification of *cis*-acting sequences required for Rme1-induced expression of *CLN2*

Recently the *CLN2* promoter has been analysed for *cis*-acting sequences required for transcriptional activation and periodic expression (Cross *et al.*, 1994; Stuart and Wittenberg, 1994). The major upstream activating sequence (UAS) has been localized to a fragment of DNA of ~100 bp between –605 and –500 bases upstream of the translation start site. This region of DNA, called UAS1, contains three consensus SCB elements and two copies of a core MCB sequence. A second region immediately downstream of UAS1, called UAS2, was also found to be capable of conferring periodic expression on a reporter construct, albeit at lower overall levels than UAS1. Surprisingly, the ability of UAS2 to activate transcription was found to depend on *SWI4*, even though UAS2 has no recognizable binding sites for SBF/DSC1 (Stuart and Wittenberg, 1994).

Having shown that *RME1* in high copy can induce expression of *CLN2*, we examined the *CLN2* promoter for sequences required for this induction. Initially we determined whether or not *RME1* was acting through either the SCB or MCB UAS elements. YEp-*RME1* was introduced into yeast strains (wild-type W3031a or *swi6Δ* BY600) carrying a *lacZ* reporter construct driven by either synthetic MCB or SCB elements. Using an X-Gal blue colour assay we observed that *RME1* was unable to activate either MCB- or SCB-dependent expression (data not shown). To localize the region necessary for Rme1-dependent activation of the *CLN2* promoter we tested the ability of high copy *RME1* to induce expression of various promoter deletion mutants of *CLN2* using a *CYC1–lacZ* reporter construct (see Materials and methods). *RME1* was able to induce expression of *lacZ* driven by sequences from –728 to –256 upstream of the *CLN2* ATG (Figure 6A). Deletion of UAS2 had no apparent affect on the ability of Rme1 to induce *lacZ* activity (Δ –505 to –400).

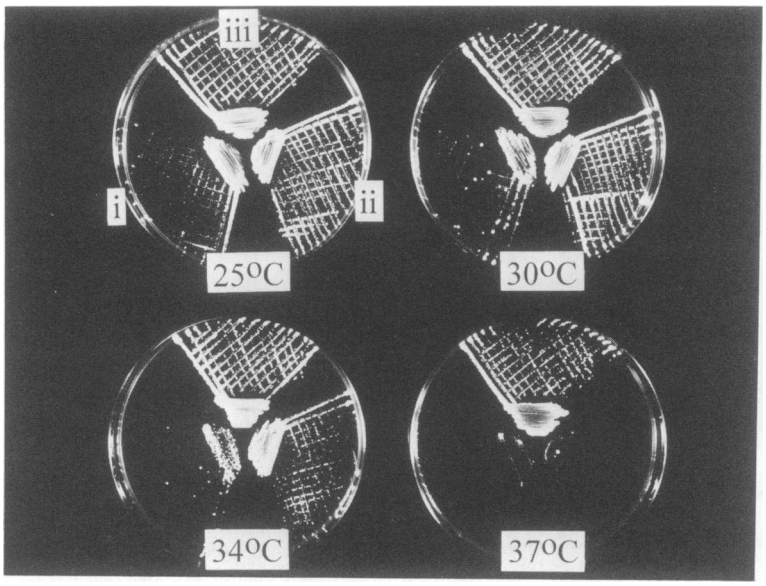

Fig. 5. Deletion of *RME1* in a *swi6Δ swi4*^{ts} background results in a lowering of the non-permissive temperature. The (i) *swi6Δ swi4*^{ts} *rme1Δ* strain, (ii) *swi6Δ swi4*^{ts} strain and (iii) *swi6Δ swi4*^{ts} strain + p*ADH-CLN2* were plated on YEPD medium at 25, 30, 34 and 37°C.

Similarily, Rme1 was unable to induce *lacZ* activity driven by UAS2 alone (–505 to –400), indicating that Rme1 does not activate expression through UAS2. Consistent with our initial experiments, Rme1 was able to activate reporter constructs in which all of the SCB and MCB elements have been specifically mutated. However, when UAS1 is entirely deleted from the reporter construct (Δ –605 to –500) *RME1*-dependent activation was reduced to very low levels. Therefore, it appears that *RME1* is acting within UAS1, but independently of the known MCB and SCB elements.

The RRE on the *IME1* promoter includes a 21 bp Rme1 binding site which consists of two imperfect direct repeat sequences (half-sites; Covitz and Mitchell, 1993). Sequence analysis of the *CLN2* promoter indicates that there is a region within UAS1 (–561 to –551) with good similarity to the RRE (Figure 6B). The sequence similarity, however, does not include a whole half-site, but rather a 12 bp region encompassing the core sequence between two potential half-sites. In addition, this element contains a G→A substitution at what would be position 5 of the second potential RRE half-site. In a previous study a synthetic RRE with the same base change was found to bind Rme1 with higher affinity and also acted as a more potent UAS (Covitz and Mitchell, 1993). A second potential RRE 5′ to UAS1 with good similarity to a complete half-site is found at position –672 to –662 (also carrying a G→A substitution at position 5). The presence of this second element may account for the remaining *RME1*-dependent activation observed when analysing the Δ –605 to –500 deletion construct.

Rme1 binds directly to the CLN2 promoter

To determine whether or not Rme1 binds directly to the *CLN2* promoter we carried out a gel retardation analysis. The *CLN2* fragment used in this assay contained the DNA sequence from –514 to –614, relative to the *CLN2* ATG, and encompassed all of UAS1. Incubation of this probe with an extract containing Rme1 transcribed and translated

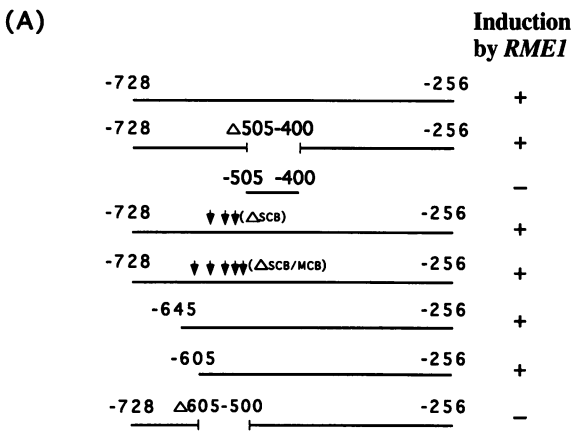

Fig. 6. Induction of *CLN2* promoter-dependent transcription by YEp-*RME1*. (**A**) *CLN2* promoter fragments, numbered relative to the *CLN2* ATG, were cloned in front of the *CYC1* minimal promoter driving *lacZ* (Stuart and Wittenberg, 1994). The *CLN2–lacZ* plasmids were co-transformed with YEp-*RME1* or YEp24 vector alone and assayed for Rme1-dependent activation of gene expression using an X-Gal blue colour assay. A + sign denotes clear induction of β-galactosidase activity relative to the vector alone control after incubation for 30 min. Arrows indicate inactivated MCB/SCB elements (see Stuart and Wittenberg, 1994). (**B**) Sequence of the *CLN2* promoter region encompassing UAS1 indicating potential Rme1 recognition sites (RREs), based on sequence similarity with the RRE of the *IME1* promoter; presented above the *CLN2* promoter sequence (Corvitz and Mitchell, 1993).

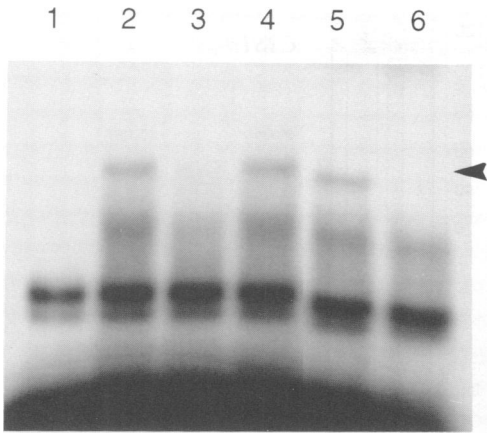

Fig. 7. Rme1 binds directly to the CLN2 promoter. Gel mobility retardation assays were carried out with a labelled *CLN2* fragment from –515 to –614 (relative to the ATG). The Rme1 protein was synthesized *in vitro*. Lane 1, no protein; lane 2, Rme1 protein; lane 3, Rme1 protein and unlabelled *CLN2* competitor DNA; lane 4, Rme1 protein and unlabelled phage λ competitor DNA; lane 5, Rme1 protein and a 249 bp unlabelled competitor DNA containing bp –496 to –634 of the *CLN1* promoter region; lane 6, protein from the *in vitro* transcription/translation of vector DNA alone.

in vitro from a vector containing the *RME1* gene resulted in formation of a single protein–DNA complex (Figure 7, lane 2). When extracts were prepared from the same vector without *RME1* no detectable complex was evident (Figure 7, lane 6). Thus the complex is specific to Rme1. To determine whether this complex was specific to *CLN2* DNA we added unlabelled competitor DNA to the binding reactions. Rme1–*CLN2* complex formation could be competed by the addition of competitor *CLN2* promoter DNA, but not by the addition of a competitor *CLN1* promoter fragment nor competitor phage λ DNA (Figure 7, lanes 3–5). These results indicate that Rme1 can bind directly and specifically to the *CLN2* promoter within the UAS1 region. Furthermore, the observation that Rme1 synthesized *in vitro* can bind UAS1 indicates that neither post-translational modification nor additional yeast proteins are required, at least *in vitro*, for binding.

RME1 is expressed periodically in the cell cycle

The ability of *RME1* to activate *CLN2* transcription implies that *RME1* has a role in cell cycle control, at least in haploid cells. Since *CLN2* is expressed specifically in late G_1, the *RME1* gene itself may be cell cycle regulated. To determine if *RME1* levels are regulated in the mitotic cell cycle a DNA fragment internal to the *RME1* open reading frame was used to probe an RNA blot prepared from samples extracted at specific time intervals following α factor synchronization of the cell cycle. *RME1* mRNA accumulation is strongly cell cycle regulated (Figure 8). Its expression peaks ~15 min after *DBF2*, a gene known to be expressed in late M phase, and ~15 min before *RNR1*, an MCB-regulated gene expressed in late G_1 phase. Therefore, *RME1* appears to be expressed near the M/G_1 boundary. This expression pattern is superimposable with that of another gene, *SIC1/SDB25*, which is expressed in late M phase/early G_1 with a functional role in ending mitosis and at G_1/S (Figure 8) (Donovan *et al.*, 1994; Schwob *et al.*, 1994).

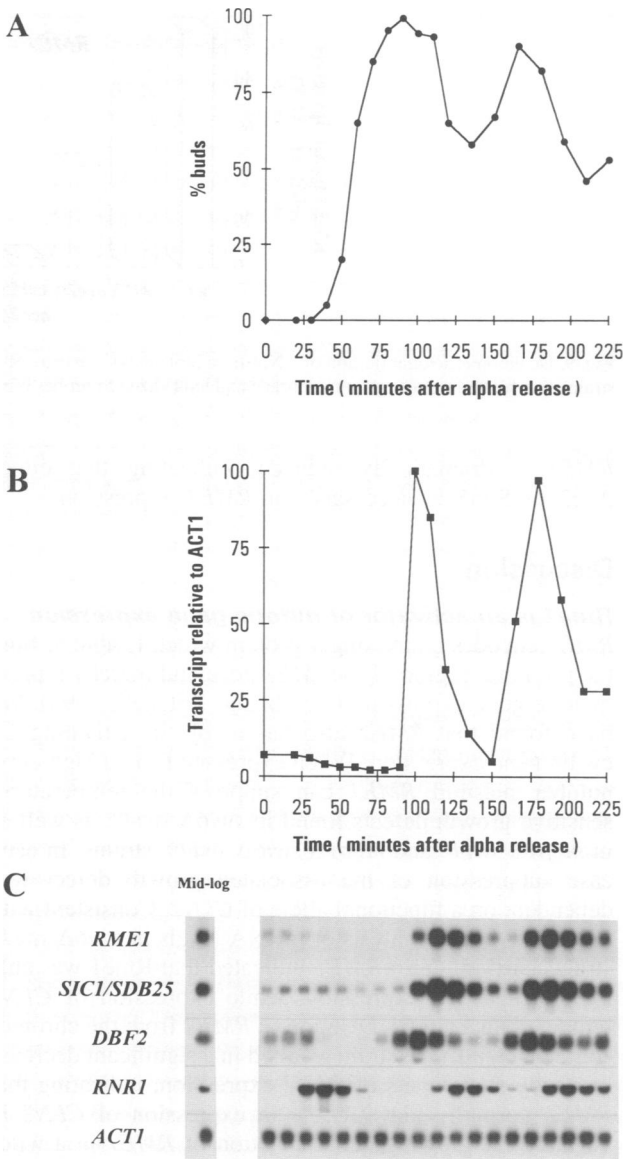

Fig. 8. *RME1* is expressed periodically in the cell cycle. Regulation of *RME1* mRNA levels in cells synchronized with α factor. (**A**) Budded cells, (**B**) *RME1* mRNA levels normalized to *ACT1* and (**C**) RNA blot analysis comparing the timing of *RME1* mRNA peaks with *SIC1/SDB25* (M/G_1 phase), *DBF2* (M phase) and *RNR1* (G_1/S phase).

The *SIC1* gene encodes an inhibitor of the Cdc28 cyclin-dependent kinase (Mendenhall, 1993). The stage-specific expression of *SIC1* is reported to be under the control of the Swi5/Ace2 transcription factors (Koch and Nasmyth, 1994; J.Toyn, unpublished observations). To test whether or not *RME1* is also under the control of Swi5, Ace2 or both we examined the levels of *RME1* mRNA in yeast strains carrying chromosomal deletions of *SWI5*, *ACE2* or both *SWI5* and *ACE2*. The *CTS1* gene, encoding chitinase, has been shown previously to be under the control of the Ace2 transcription factor and is used here as a control for Ace2-dependent transcription (Dohrman *et al.*, 1992). As shown in Figure 9, expression of *RME1* is reduced in an *ace2Δ* strain and to a lesser extent in a *swi5Δ* strain. However, in a *swi5Δ ace2Δ* strain expression of

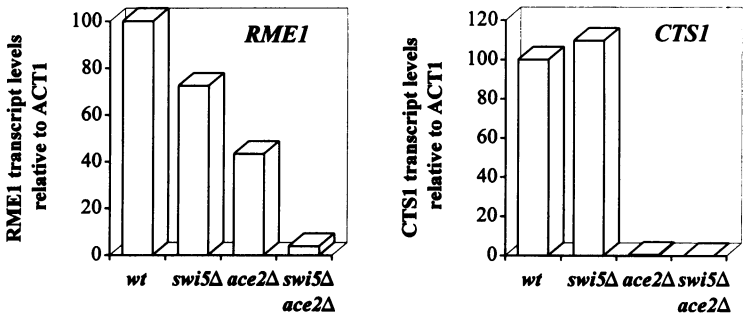

Fig. 9. Densitometric quantitation of a Northern blot analysis using mRNA prepared from exponentially growing wild-type, *ace2Δ*, *swi5Δ* or *ace2Δ swi5Δ* strains was blotted onto a nylon membrane and hybridized to probes representing *RME1* and *CTS1* and normalized to *ACT1* as a loading control.

RME1 is dramatically reduced, indicating that either Ace2 or Swi5 is necessary for *RME1* expression.

Discussion

Rme1 is an activator of mitotic gene expression

RME1 encodes a zinc finger protein which is able to bind the promoter region of the *IME1* gene and thereby repress meiotic gene expression (Covitz and Mitchell, 1993). We have found that *RME1* also has a role in activating G_1 cyclin gene expression. When expressed from a high copy number plasmid *RME1* can suppress the temperature-sensitive growth defects found in *swi6Δ swi4*^{ts}, as well as in *mbp1Δ swi4*^{ts} and *mbp1Δ swi6Δ swi4*^{ts}, strains. In each case suppression of the associated growth defect was dependent on a functional allele of *CLN2*. Consistent with this observation, analysis of mRNA levels in *swi6Δ swi4*^{ts} strains carrying YEp-*RME1* indicated that Rme1 was able to induce *CLN2* expression, while expression of *CLN1* remained unchanged. Deletion of *RME1* from the chromosome of a wild-type strain resulted in a significant decrease in steady-state levels of *CLN2* expression, indicating that *RME1* normally plays a role in expression of *CLN2* in vegetatively growing cells. Deletion of *RME1* in a wild-type strain did not, however, result in an observable decrease in growth rate, but strains such as K2003 (*swi6Δ swi4*^{ts}), in which the G_1 transcription apparatus is already attenuated, were found to be particularly sensitive to inactivation of *RME1*. This last observation is similar to observations made for other transcription factors that control G_1 cyclin expression in *S.cerevisae*. In most wild-type strain backgrounds inactivation of either *SWI4*, *SWI6* or *MBP1* results in a viable phenotype and only when mutations are present in combination is the growth of the cell severely affected. Thus there is considerable functional redundancy built into the mechanisms that control G_1 progression in *S.cerevisiae* (Breeden, 1995).

Previous studies have shown that the RRE when removed from the context of the *IME1* promoter and placed upstream of a reporter gene can act as a UAS element. Furthermore, the N-terminus of Rme1 shows similarity to an acidic activation domain. Thus, like the yeast Rap1 protein, Rme1 appears to be able to function as either an activator or a repressor of transcription, depending upon the context of its binding site (Covitz and Mitchell, 1993). Our analysis of the *CLN2* promoter indicated that sequences necessary for Rme1-dependent *CLN2* induction were located within the UAS1 region of the *CLN2* promoter. The UAS1 region contains both SCB and MCB elements required for maximal *CLN2* expression. Rme1-dependent *CLN2* expression was found to be independent of these elements, consistent with the observation that *RME1* is able to bypass the requirements for *SWI4*, *SWI6* and *MBP1*. The ability of Rme1 to bind directly and specifically to the UAS1 region was demonstrated by gel mobility retardation assays and, furthermore, sequence analysis of UAS1 revealed a potential RRE-like element located at position −561 to −551 relative to the *CLN2* ATG. In the light of these and previous findings we believe the most likely mechanism for *RME1*-induced *CLN2* expression involves direct binding of Rme1 to the *CLN2* promoter.

RME1 is expressed in late M/early G_1 phase of the cell cycle

The G_1-specific expression of *CLN2* is important for normal cell cycle progression, since mutations which stabilize the Cln2 protein accelerate the G_1 to S phase transition and constitutive over-expression of *CLN2* can result in premature entry into S phase and subsequent cell death (Hadwiger *et al.*, 1989; Amon *et al.*, 1993). We have shown that the *RME1* gene product is a potential regulator of *CLN2* expression and we therefore examined expression of *RME1* itself. The *RME1* transcript was clearly cell cycle regulated, with levels peaking in late M/early G_1 phase. Furthermore, we demonstrated that *RME1* expression was controlled by the transcription factors Swi5/Ace2, with maximal expression dependent primarily on Ace2. The *SIC1* gene, which encodes an inhibitor of the Clb Cdc28 kinase, was also found to be expressed at this time and in a Swi5/Ace2-dependent manner (J.Toyn, unpublished observation). Sic1, as a cdk inhibitor, appears to play a role both in exit from mitosis and in controlling the correct timing of S phase in the following cell cycle (Donovan *et al.*, 1994; Schwob *et al.*, 1994). Since *RME1* and *SIC1* expression are coincident, these genes may represent a group of genes expressed late in the previous cell cycle which have roles in the following G_1 period.

rme1Δ is not synthetically lethal with swi4Δ

All of our work concerning the role of Rme1 in activating *CLN2* expression has been performed in haploids. Indeed, since expression of *RME1* is repressed in MAT**a**/α diploids it is conceivable that its role as both an activator and repressor of transcription is confined to haploid cell types.

Evidence for a haploid-specific role for Rme1 or an Rme1-like activity is suggested by the observation that homozygous deletion of *SWI4* in diploid cells results in a far more severe phenotype than inactivation of *SWI4* in haploid cells (Nasmyth and Dirick, 1991; Ogas *et al.*, 1991). Why diploid cells are more sensitive to loss of Swi4 activity is not clear, although one suggestion is that a component of a Swi4-independent pathway for *CLN* expression is repressed in MATa/α cells (Ogas *et al.* 1991). Since *RME1* has a positive affect on *CLN* expression (independent of *SWI4*) and is also down-regulated in MATa/α diploid cells it is possible that Rme1 may be a component of this haploid-specific, *SWI4*-independent pathway. Following a cross between an *rme1*Δ strain and an isogenic *swi4*Δ strain we identified spores which had inherited both deletion alleles. Although we observed an abnormally high level of spore inviability amongst *swi4 rme1* double deletions, those germinating showed none of the growth defects observed previously for diploid homozygous *swi4*Δ cells (unpublished results).

What role does RME1 play in the mitotic cell cycle?

The lack of an obvious phenotype for *rme1*Δ strains may reflect the functional redundancy observed in the G_1 transcriptional machinery or, alternatively, it may suggest that Rme1 is necessary for inducing a Cln2 kinase activity independent of that required for normal cell cycle control. Covitz and Mitchell (1993) have observed that *RME1* mRNA levels increase 10-fold under starvation conditions in haploids. Thus in haploid cells under conditions of nutrient limitation Rme1 could both block the meiotic programme by repression of *IME1* expression and induce Cln2 kinase activity. Whilst expression of *CLN* genes has not been examined under these conditions, there is an interesting parallel between starvation-induced *RME1* expression and expression of the *S.pombe* G_1-like cyclin genes *puc1* and *cig2/cyc17*. Expression of the *cig2/cyc17* gene is induced when sexual development is initiated and *puc1* gene expression is induced in response to nutrient limitation. Analysis of cells in which *cig2/cyc17* or *puc1* are deleted suggests that these cyclins play a negative role in cell cycle exit (Forsburg and Nurse, 1994). Thus a paradox exists, wherein the cell is initiating events to leave the cell cycle at a point where it is also inducing mitotic cyclins. One possible explanation is that *cig2/cyc17* and *puc1* are induced as a 'double-check' to ensure that cell cycle exit is appropriate (Forsberg, 1994). A similar role could be postulated for *RME1*. The Rme1-dependent induction of Cln2 kinase activity may function to ensure that haploid cells do not enter an off-cycle stationary phase state prematurely or, alternatively, may be required for an alternative developmental programme instigated in response to nutrient limitation (see below and the model in Figure 10).

The Cln Cdc28 kinases are responsible for three known cell cycle events: (i) initiation of DNA replication; (ii) spindle pole body duplication; (iii) bud site selection and morphogenesis. How these processes are regulated by the Cln kinase are for the most part unknown. Control of the timing of DNA replication, for example, is thought to be indirect, involving both inactivation of the Sic1 cdk inhibitor and induction of *CLB5/6* transcription, both of

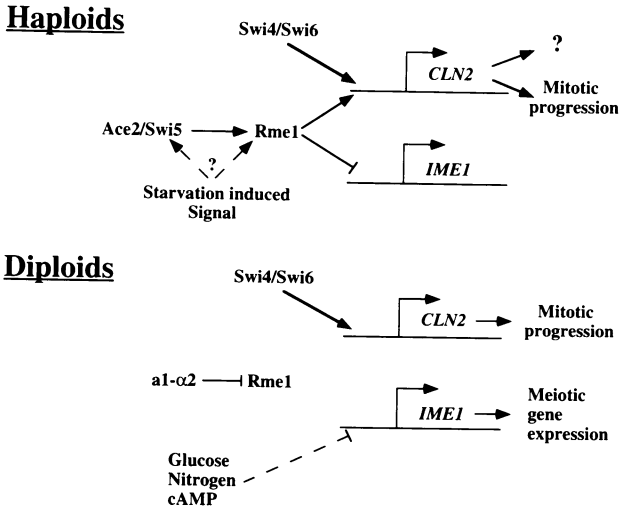

Fig. 10. Model for Rme1 function. Rme1 has previously been shown to inhibit meiotic gene expression by repressing transcription of the *IME1* gene in haploids. In haploids Rme1 is also a transcriptional activator of *CLN2* expression, suggesting that *RME1* plays a positive role in mitotic progression. Periodic expression of *RME1* is controlled by the transcription factors Ace2/Swi5. Furthermore, *RME1* gene expression is induced under conditions of nutrient limitation (Covitz and Mitchell, 1993), although it is unknown whether the signal for starvation-induced *RME1* expression (?) is acting through Swi5 and/or Ace2 or through a distinct activation pathway. Induction of *RME1* by starvation, coupled with the results presented here, suggests that *RME1* may play a role not only in the mitotic regulation of *CLN2*, but also at times in the yeast life cycle when cell cycle-independent *CLN2* expression may be required; for example, during starvation-induced filamentous growth (?). In MATa/α diploids *RME1* expression is repressed, therefore it is unlikely to regulate gene expression in diploids.

which appear to be Cln-dependent events. In contrast, the effect of the Cln1/2 Cdc28 kinase on polarized cell growth and bud site assembly may be more direct. For example, Lew and Reed (1993) have shown that Cln1 and Cln2 can trigger actin polarization to the pre-bud site even in the absence of *de novo* protein synthesis. This finding has possible implications for the regulation of a number of developmental pathways which include alterations in budding or cell morphology. Nutrient limitation can cause MATa/α diploid cells to undergo a dimorphic transition to pseudohyphal growth and cause haploid cells to initiate a programme called the invasive growth response (Gimeno *et al.*, 1992; Roberts and Fink, 1994). Both of these responses require alterations in budding pattern, cell morphology and cytokinesis which subsequently lead to filamentous growth. The role, if any, of *CLN2* in each of these developmental pathways is not known, however, these observations may indicate that there are times in the yeast life cycle when expression of *CLN2*, and perhaps other G_1 cyclin encoding genes, needs to be altered relative to their expression in the normal cell cycle. In these circumstances factors such as Rme1 might play a crucial role.

Materials and methods

Strains, media and yeast genetic manipulations

The haploid yeast strains used in this study were as follows: W3031a (MATa *ade2 his3 trp1 leu2 ura3*); BY600 (MATa *ade2 his3 trp1 leu2 ura3 swi6::TRP1*); BY604 (MATα *ade2 ura3 met⁻ trp1 leu2 can1 his3 ho-lacz swi4::LEU2*); K2003 (MATa *ade2 his3 met⁻ leu2 trp1 ura3 swi4^{ts} swi6::TRP1*); BAM1 (*ade2 leu2 his3 met⁻ ura3 trp1 swi4^{ts} mbp1::URA3*); BAM2 (*ade2 leu2 ura3 trp1 his3 met⁻ swi4^{ts} mbp1::URA3*

swi6::TRP1); BAM3 (*trp1 leu2 ura3 ade2 his3 met⁻ swi4^{ts} mbp1::URA3 cln1::HIS3*); BAM4 (*trp1 leu2 ura3 ade2 his3 swi4^{ts} mbp1::URA3 cln2::LEU2*); IH1783 (MATa *his4 trp1 ura3 leu2 can1*); AMP143 (IH1783 *rme1Δ5::LEU2*); CG378 (MATa *ade5 leu2 trp1 ura3 can1*).

Standard genetic techniques were used for manipulating yeast strains (Guthrie and Fink, 1991). Cells were grown in complete medium (YEPD; 1% Difco yeast extract, 2% Difco bacto-peptone and 2% glucose) or, for plasmid selection, synthetic medium supplemented with amino acids (0.67% YNB, 2% glucose and appropriate amino acids). Unless otherwise stated the growth temperature used was 25°C. Yeast transformations were performed using a modification of the lithium acetate method (Gietz and Sugino, 1988). Disruption of the chromosomal copy of *RME1* was performed using plasmid pHH1-2, kindly provided by G.Simchen (Hugerat and Simchen, 1993).

Transposon mutagenesis and DNA sequencing

The plasmid 1111-5 (YEp-*RME1*) was isolated from a genomic library cloned into the YEp24 vector (library kindly provided by D.Botstein). Simultaneous localization and DNA sequence analysis was performed by transposon mutagenesis of p1111-5. A transposon, Tn*HIS3*, was randomly inserted into p1111-5 as described previously (Sedgwick and Morgan, 1994). Transposed plasmids were pooled and introduced into a temperature-sensitive *rsf11* strain, selecting for the *HIS3* marker. Those plasmids that had now lost the ability to suppress the temperature-sensitive phenotype were re-isolated and subjected to DNA sequence analysis. Dideoxy DNA sequencing was performed using a T7 sequencing kit from Pharmacia using primers designed to hybridize to the 5′- and 3′-ends of the Tn*HIS3* transposon (Sedgwick and Morgan, 1994). Sequencing gels were prepared using the Sequagel™ Sequencing System supplied by National Diagnostics.

RNA blot analysis

Total RNA was extracted from cells as described previously (White *et al.*, 1986). A 5 μg sample of total RNA was denatured with glyoxal, separated on a 1.2% agarose gel and transferred to a GeneScreen hybridization membrane (Dupont NEN Rsearch Products, Boston, MA). Probes for RNA–DNA hybidization were restriction fragments internal to the genes concerned. Levels of hybridization were quantitated using either a Personal Densitometer PD-130 (Molecular Dynamics) or a PhosphorImager (Molecular Dynamics). Levels of hybridization were normalized to either the *ACT1* transcript or the *MET4* transcript (Thomas *et al.*, 1992), which we have found to be invariant following various perturbations to the cell cycle.

Cell cycle analysis of *RME1* transcript levels was performed as described above following synchronization of CG378 MATa cells with α factor as described previously (Johnston *et al.*, 1990).

CLN2 promoter analysis

lacZ reporter plasmids for *CLN2* promoter analysis are derivatives of pCZD and have been described previously (Stuart and Wittenberg, 1994). *RME1*-dependent induction of *lacZ* expression from these reporter plasmids was performed by co-transformation of YEp-*RME1* and the indicated reporter plasmid or co-transformation of YEp24 (vector alone) and the indicated reporter plasmid into either a wild-type yeast strain (W3031a) or a *swi6Δ* yeast strain (BY600). Induction of the *lacZ* gene was determined using a qualitative X-Gal blue colour assay performed on colonies grown on Hybond-N (Amersham) filters as described in Breeden and Nasmyth (1985). The ability or inability of YEp-*RME1* to induce expression from the various *CLN2* promoter fragments was determined by comparison with the vector alone control for each reporter construct. *CLN2*-dependent expression was decreased in a *swi6Δ* strain, but this had no effect on the relative ability of YEp-*RME1* to induce expression, although the background levels of *lacZ* expression were down.

Gel mobility shift assay

RME1 was cloned on a 1789 bp *Xba*I–*Xho*I fragment into pBluescriptKS. Rme1 protein was synthesized directly from this plasmid using a Promega TnT T7-coupled reticulcyte lysate system. A ^{35}S-labelled translation product of the predicted size of Rme1 was detected using PAGE. A control transcription/translation reaction was performed using empty pBluescriptKS vector. *In vitro* transcibed/translated [^{35}S]Rme1 or control extract was incubated in a binding reaction containing 50 mM Tris–HCl, pH 7.5, 50 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 0.2 mM sodium molybdate, 10% glycerol, 50 mg/ml phenylmethylsulfonyl fluoride, 5 mg/ml leuprotin, 5 mg/ml aprotin, 5 mg/ml pepstatin A, 10 mM benzamidine, 100 ng/ml poly(dI·dC) and 0.5 ng ^{32}P 5′-end-labelled DNA probe. The 109 bp probe contained positions −515 to −614 of the *CLN2* promoter. Competitor DNA was present in a 100-fold excess over the labelled probe. The reactions were incubated at 25°C for 5 min and then on ice for a further 20 min, when they were loaded directly onto a 4% (40:1) non-denaturing polyacryamide gel and electrophoresed in 0.6% TBE buffer at 7°C. Gels were dried onto Whatman 3MM paper and autoradiographed.

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