Stress and developmental regulation of the yeast C-type cyclin Ume3p (Srb11p/Ssn8p)

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Introduction

Cyclins are a conserved family of proteins required for the activation of a class of protein kinases termed Cdns (cyclin-dependent kinases). Originally, cyclin–Cdk complexes were identified as activities required for the transition from one stage of the mitotic cell cycle to the next (for review, see Solomon, 1993). In addition, a subset of cyclin–Cdk complexes are also involved in transcriptional regulation. For example, the human cyclin H–Cdk7 comprises the CAK (Cdk-Activating Kinase) activity (Solomon et al., 1993; Fisher and Morgan, 1994; Makela et al., 1994; Tassan et al., 1994) and is a component of the TFIIH complex that is required for transcription initiation (Roy et al., 1994). A similar activity has been purified from yeast that contains the Cc11p–Kin28p cyclin–Cdk complex (Feaver et al., 1994). In addition, a more distantly related cyclin (Pho80p) complexes with the Pho5p Cdk to repress the yeast acid phosphatase gene by inhibiting the transcriptional activator Pho4p (Hirst et al., 1994; Kaffman et al., 1994). Finally, the yeast cyclin C–Cdk complex (Srb1p–Ume5p) is required for the repression of genes involved in sucrose utilization (Kuchin et al., 1995) and meiotic development (Strich et al., 1989; Suros et al., 1994). This complex co-purifies with the RNA polymerase II holoenzyme (Liao et al., 1995), suggesting that this cyclin-Cdk represses gene expression by modifying components of the transcription machinery.

As indicated by their name, the protein and transcript levels of most cyclins that drive cell cycle progression oscillate, peaking when their activity is required (for review, see Nasmyth, 1993). This regulation occurs at the level of both transcription and protein stability. Cyclin proteolysis is mediated through ubiquitin pathways that target at least two known motifs, PEST (proline, glutamic acid, serine and threonine) and the destruction box. Destruction boxes are required for the destruction of S phase (A-type) and G2 (B-type) cyclins (Murray et al., 1986; Glotzer et al., 1991), while PEST regions (Rogers et al., 1986) are carboxy-terminal domains important for the destruction of the yeast G1 cyclins (Cln1-3p) (Tyers et al., 1992; Cross and Blake, 1993; Yaglom et al., 1995).

Phosphorylation of the PEST regions by their associating Cdk appears to trigger the degradation progress (Yaglom et al., 1995; Lanker et al., 1996) and, in the case of Cln3p, requires the yeast DnaJ homolog Ydj1p (Yaglom et al., 1996). However, the mRNA levels of the human or Drosophila C-type cyclins do not fluctuate during mitotic cell division or embryonic development (Leopold and O’Farrell, 1991; Lew et al., 1991; Li et al., 1996). These findings may reflect their role in regulating transcription rather than cell cycle progression.

We have identified six genes (UME1–6) required for the mitotic repression of several mitogenic genes (e.g. SPO11, SPO13) transcribed early in development (Strich et al., 1989, 1994; Vidal et al., 1991; Suros et al., 1994). This study focuses on UME3. The UME3 gene was isolated and subsequently found to encode a protein that is identical to Srb11p/Ssn8p, the yeast C-type cyclin. We report that this cyclin, along with Ume5p, is required for the full repression of the inducible HSP70 family member SSA1. The examination of Ume3p regulation indicated that this cyclin is rapidly destroyed when cells enter the meiotic pathway or are exposed to heat shock. This destruction is significant since mutations that stabilize Ume3p result in a 2-fold reduction in SPO13 mRNA levels. Ume3p destruction is regulated through a PEST-rich region, a destruction box-like motif (RXXL) as well as the highly conserved cyclin box. However, unlike other cyclins, Ume3p degradation was not affected in strains mutant.
for ubiquitin-mediated protein destruction. These results represent the first observed regulation of a C-type cyclin and suggest the presence of a pathway, different from that controlling other cyclins, mediating the destruction of Ume3p.

Results

**UME3 encodes a protein with similarity to cyclin C**
The UME3 gene was cloned by complementation and found to encode a non-essential 37.7 kDa protein (Ume3p) that shares 38% identity with the human and *Drosophila* C-type cyclins (see Materials and methods). Subsequent analysis revealed that UME3 is identical to *SSN8/SRB11*, a negative regulator of the yeast invertase gene *SUC2* (Kuchin *et al.*, 1995) that co-purifies with the RNA polymerase II holoenzyme (Liao *et al.*, 1995). These reports also presented evidence that the Cdk UME5 (*SSN3/ SRB10*), a gene isolated in the same screen as UME3 (Strich *et al.*, 1989), is the Cdk activated by Ume3p. However, a previous study presented data that *UME5* regulates *SPO13* mRNA stability while *UME3* controls transcription initiation (Surosky *et al.*, 1994). To address this question, epistasis analysis was performed by measuring the expression of a *spo13–lacZ* reporter gene in isogenic *ume3Δ, ume5Δ* or *ume3Δ ume5Δ* mutant hosts. The *ume3* and *ume5* mutants exhibited a 3- and 6-fold increase in *spo13–lacZ* expression over the wild-type control (Table I). This derepression is consistent with that observed for the original *ume3* and *ume5* alleles (Strich *et al.*, 1989) and represents ~10% of the fully induced meiotic levels in this strain background (data not shown).

No significant difference in β-galactosidase activity was observed in the double mutant strain compared with the single mutants, formally suggesting that these two genes function in the same regulatory pathway. These results are consistent with the model that Ume3p and Ume5p form a cyclin–Cdk complex to repress the expression of *SPO13* during vegetative growth.

**Ume3p represses SSA1, a member of the HSP70 superfamily**

Several early meiotic genes, including *SPO13*, are controlled through a common upstream regulatory element termed URS1 (Buckingham *et al.*, 1990; Vershon *et al.*, 1992; Bowdish and Mitchell, 1993). In addition, repression of *SSA1*, a member of the HSP70 superfamily of heat shock proteins, requires URS1 (Park and Craig, 1989). The analysis of *SSA1* expression in a *ume3–1* mutant revealed a partial derepression of this gene under non-inducing conditions (E.Craig, personal communication). To investigate this observation further, we measured the expression of an *ssa1–lacZ* reporter gene (Slater and Craig, 1987) in the *ume3* and *ume5* single and double mutants described above. Similarly to *spo13–lacZ, ssa1–lacZ* expression was elevated in these mutant strains under non-inducing conditions (Table I). These results identify a new role for Ume3p–Ume5p in the repression of a member of the heat shock family of proteins.

**Ume3p levels do not fluctuate during mitosis**
The periodic transcription of many cyclins that drive mitotic cell division has been helpful in identifying when in the cell cycle these proteins are required. Analysis of *UME3* mRNA levels revealed that, similarly to other C-type cyclins (Leopold and O'Farrell, 1991; Lew *et al.*, 1991; Li *et al.*, 1996), no significant differences in synchronous mitotic and meiotic cultures occurred (data not shown). In addition to oscillating mRNA levels, most cyclins are also subjected to post-translational control through protein destruction. To determine if Ume3p is regulated in this manner, a 10 amino acid myc epitope tagged *UME3* mRNA construct is able to complement a *ume3Δ* null mutation, indicating that this gene is functional (data not shown). To monitor Ume3p levels during the mitotic cell cycle, a strain was constructed (MMY317) in which the *UME3–myc* reporter gene (Slater and Bowdish, 1994) was integrated into its normal chromosomal location under the control of its natural promoter. Using α-factor arrest/release protocols to obtain synchronous mitotic cultures (Oehlen and Cross, 1994), Ume3p levels were monitored through two cell divisions. Similar to the mRNA results, Ume3p levels did not fluctuate significantly through two cell generations (Figure 1A).

**Ume3p is degraded rapidly in response to heat shock**

Our genetic data indicate that Ume3p negatively regulates *SSA1* expression at non-inducing temperatures. Therefore, it is likely that Ume3p repressor activity must be inactivated to allow normal expression of *SSA1* at high temperatures. To investigate whether this control occurred through transcriptional or post-transcriptional mechanisms, the endogenous *UME3* mRNA and protein levels were monitored in MMY329 cultures subjected to a 37°C heat shock (see Materials and methods). These experiments...
revealed that Ume3p levels declined rapidly following heat shock (Figure 1B) while its transcript levels remained constant. These results indicate that UME3 is regulated through post-transcriptional mechanisms and demonstrate for the first time conditions that regulate C-type cyclin levels. Furthermore, the finding that Ume3p is down-regulated by an environmental stress stimulus represents a novel signal by which any cyclin is controlled.

To explore the relationship between the stress response and Ume3p degradation further, a transient heat shock experiment was performed. Rather than placing cell pellets into a water bath at before, a mid-log phase culture was harvested, resuspended in fresh pre-warmed medium, then incubated with shaking at 37°C. Under these conditions, cell division is temporally arrested and the heat shock genes are induced. As the cells adapt to the higher temperature, the heat shock genes are repressed and growth resumes (for review, see Craig, 1993). If the down-regulation of Ume3p is coupled to the stress response, then the pattern of Ume3p destruction and resynthesis should coincide with transient HSP expression. To test this possibility, RSY10 harboring pKC337 (CEN Adh-UME3) was grown to mid-log phase at 30°C and then transferred to fresh growth medium pre-warmed to 37°C. To monitor the transient HSP gene expression, SSA3 transcript levels were followed. SSA3 was chosen to follow the heat shock response due to its low basal mRNA levels and its strong induction. As observed with the endogenous gene, Ume3p levels expressed from the ADH1 promoter rapidly decreased following exposure to 37°C. These results suggest that, as observed for other cyclins (Yaglom et al., 1995), the system regulating Ume3p destruction is not acutely sensitive to protein levels. Strikingly, Ume3p levels declined then returned to pre-heat shock concentrations, inversely mirroring the transient accumulation of SSA3 mRNA. These findings suggest that the regulation of Ume3p is tightly coupled to the cellular response to heat stress.

Since these experiments were conducted using Western blots of immunoprecipitates, it was possible that the apparent loss of Ume3p was due to the inaccessibility of the myc epitope rather than to protein degradation. To address this possibility, UME3 was fused to the DNA-binding domain of the bacterial transcriptional repressor lexA (pEG3). High affinity polyclonal antibodies directed against lexA (a gift from E.Golemis) allow the detection of this fusion protein in straight Western blot analysis. Therefore, lexA–Ume3p levels were monitored following heat shock by immunoprecipitation/Western as before or by straight Western blot analysis (see Materials and methods). Both methodologies produced reductions in lexA–Ume3p levels compared with the lexA controls (Figure 2B). The higher levels of lexA–Ume3p observed at the later timepoints in the straight Western analysis may reflect the more rigorous method employed to prepare Ume3p (boiling cell pellets in SDS) versus the soluble extracts used in the immunoprecipitation/Western. These findings indicate that Ume3p is indeed destroyed in cells exposed to heat shock.

**Ume3p stability is reduced 60-fold in response to heat shock**

Two models (not mutually exclusive) can explain the observed reduction in Ume3p levels during heat shock. First, translation initiation is known to be altered in cells subjected to stress (Hinnebusch and Liebman, 1993). If reduced translational initiation is combined with a normally rapid turnover rate, as observed for other cyclins (e.g. Cin3p, Tyers et al., 1992; Cross and Blake, 1993), the Ume3p expression profile described here could be produced. Alternatively, Ume3p may be stable under normal...
No differences in sporulation and a ~3- to 4-fold increase in Ume3p stability in cells not significantly impact normal meiotic development (data relationship between the RXXL motif and the PEST-rich region, a double mutant was constructed (L28A/PESTΔ) to investigate further the involvement of this residue in Ume3p degradation. To investigate this possibility, the half-life of Ume3p was determined under normal growing conditions by fusing the UME3 coding sequence to the galactose-inducible (glucose-repressible) promoter gal1 (pKC333). UME3 expression was induced by growing a strain harboring pKC333 in galactose/raffinose medium then repressed by the addition of glucose. Quantitation of Ume3p levels revealed a half-life of 1.5 h (three separate experiments, Figure 3A). Examination of the gal1–UME3 transcript revealed that the mRNA level was reduced by >95% within 1 h following addition of glucose (calculated half-life of 15 min, data not shown), suggesting that the half-life of Ume3p was not significantly influenced by new translation. In contrast, the half-life of Ume3p in galactose/raffinose cultures exposed to 37°C was only 1.5 min (Figure 3B, two separate experiments). These results are consistent with a model in which Ume3p is relatively stable under normal growing conditions but is specifically targeted for degradation during heat shock.

Ume3p is destroyed during meiosis

If relieving Ume3p repression through protein destruction is a general mechanism, then one prediction is that this cyclin should also be destroyed in meiosis prior to SPO13 induction. To provide an adequate signal to detect Ume3p during meiosis and sporulation, diploid strain RSY335 harboring pKC342 (2µ Adh–UME3) was employed (see Materials and methods). No differences in sporulation efficiency or the timing of the various landmark events were observed between cells harboring pKC342 or the vector alone, suggesting that UME3 overexpression did not significantly impact normal meiotic development (data not shown). Western blot analysis of immunoprecipitates revealed that Ume3p levels were reduced dramatically just prior to the peak accumulation of SPO13 mRNA (Figure 4A). As observed with the heat shock experiments, the decline in Ume3p levels occurred through post-transcriptional mechanisms since UME3 mRNA levels remained constant throughout the timecourse. This observation, coupled with the aberrant expression of SPO13 in ume3 mutants (Strich et al., 1989, Table 1), is consistent with our model in which the destruction of Ume3p is part of the transcriptional program designed to relieve repression of genes which it negatively regulates.

**PEST-rich region is required for the rapid destruction of Ume3p during heat shock**

Sequence analysis of Ume3p revealed that, similarly to other C-type cyclins, the carboxy-terminus of this protein contains a region that possesses a relatively high PEST score (Rogers et al., 1986). PEST regions have been identified in several regulatory molecules (G1 cyclins, transcription factors) and appear to be a substrate for Cdk (see Introduction). To determine if this motif is involved in Ume3p destruction, the heat shock decay kinetics were measured in a mutant lacking the highest percentage of PEST residues including the only potential Cdk target site (SP, see Materials and methods). This deletion resulted in a 3.5-fold increase in stability compared with wild type (Figure 5), indicating that, similarly to G1 cyclins, a PEST-rich region does contribute to Ume3p degradation. Analysis of Cln2p and Cln3p degradation has demonstrated that their PEST regions are activated when phosphorylated by their kinase partner Cdc28p (Yaglom et al., 1995; Lanker et al., 1996). To address this issue, Ume3p degradation was monitored during heat shock in wild-type (RSY10) and isogenic ume5Δ (RSY440) strains. Surprisingly, Ume3p was still degraded rapidly in this strain (Figure 5), suggesting that Ume5p activity is not required for the destruction of its cyclin. Taken together, these results indicate that this PEST-rich region is involved in Ume3p destruction, although its activation is independent of Ume5p function.

**Destruction box-like region is required for rapid degradation of Ume3p during heat shock**

The destruction of S phase and mitotic cyclins requires an N-terminal amino acid motif RXXLX₀₋₄N (destruction box) which serves as a target for ubiquitin-mediated protein degradation (Murray et al., 1989; Glotzer et al., 1991). Altering the arginine or leucine residues in Xenopus (Kobayashi et al., 1992) or the arginine in sea urchin (Glotzer et al., 1991) or clam (Lorca et al., 1992) cyclins reduced their destruction in vitro. Ume3p contains an amino-terminal motif starting at residue 25 (RQKL, asterisks, Figure 6A) that contains similarity to the destruction box consensus. Oligonucleotide-directed mutagenesis was employed to replace the two residues that share similarity with the destruction box (Arg25 or Leu28) with an alanine (see Materials and methods). Both mutations resulted in a ~3- to 4-fold increase in Ume3p stability in cells exposed to heat shock (Figure 6B). These results indicate that this destruction box-like motif (RXXL) is also involved in Ume3p degradation. To investigate further the relationship between the RXXL motif and the PEST-rich region, a double mutant was constructed (L28A/PESTAΔ). In cultures exposed to elevated temperatures, the stability of this derivative was enhanced dramatically (>20-fold)
Regulation of the yeast C-type cyclin

Fig. 4. Ume3p levels during meiotic development. (A) Meiotic timecourse with Ume3p. Samples were taken from a RSY335 culture harboring pKC342 during vegetative growth (0 h) and at subsequent times (in hours) following transfer to sporulation medium. Tub1p and ACT1 mRNA levels were used to standardize protein and mRNA quantitation, respectively. Ume3p was detected using Western blots of immunoprecipitates (Materials and methods). The levels of Ume3p and SPO13 mRNA were quantitated and graphed below. Total SPO13, UME3 and ACT1 mRNA values were obtained by summing the signals from all seven timepoints. (B) Meiotic timecourse with Ume3p–A110V. A meiotic timecourse experiment was performed with RSY335 harboring pKC362 as described in (A).

compared with wild type or either single mutant. Moreover, the steady-state levels of the double mutant protein were not altered compared with wild type under normal growing conditions, suggesting that overall stability was not affected (data not shown). These results indicate that the PEST-rich and RXXL motifs provide redundant activities and constitute the major pathway through which Ume3p is destroyed in heat-shocked cells.

Cyclin box integrity is required for Ume3p destruction

To search for additional cis-acting sequences that affect Ume3p turnover, we took advantage of the observation that the lexA–Ume3p fusion protein can activate transcription of a LEU2 reporter gene under the control of lexA operators. However, this activation is temperature sensitive due to the destruction of this fusion protein at 37°C (see Figure 2B). Therefore, variants of lexA–Ume3p that were resistant to heat shock-induced proteolysis were identified by their ability to activate the LEU2 reporter at 37°C (see Materials and methods). Using this protocol, eight isolates were recovered and the nature of the mutations determined by sequence analysis. Surprisingly, all isolates contained amino acid substitutions in the cyclin box region. Six contained an alanine to valine substitution at position 110, one possessed a glutamic acid to lysine change at position 170 and one isolate contained both (Figure 6A). To insure that this phenotype was not due to an additional alteration in the promoter or in lexA, two experiments were per-

Fig. 5. Analysis of the PEST region and Ume5p in the regulation of Ume3p destruction during heat shock. Mid-log cultures harboring UME3 expression plasmids were harvested and subjected to heat shock for the times indicated (see Materials and methods). The decay kinetics of Ume3p were measured in a wild-type strain RSY333 (UME3), or in an isogenic UME3 deletion mutant RSY440 (ume5Δ). The stability of a Ume3p derivative lacking a PEST-rich sequence (PESTA) was assayed in a UME5 host (C). The half-life determinations are given below and are averaged from at least two independent trials. Ume3p was detected using Western blots of immunoprecipitates. All Ume3p values were compared with Tub1p levels to control for protein quantitation (not shown).
Fig. 6. Analysis of the RXXL motif and the cyclin box in the regulation of Ume3p during heat shock. (A) Sequence analysis of the cyclin C cyclin box region. The amino acid sequences of the Ume3p and human cyclin C cyclin box regions are presented. Bold type indicates amino acid identity between the two proteins. The asterisks indicate the degradation signal motif (RQKL). The helical regions (1A–5A) are inferred from the structure of cyclin A (Jeffrey et al., 1995) and agree with secondary folding prediction algorithms (Garneir et al., 1978). The positions of the mutations R25A, L28A, A110V, E170K and ume3-1 are indicated below the sequence. (B) Analysis of degradation signal and cyclin box mutations on Ume3p stability during heat shock. The stability of the UME3 derivatives was determined during heat shock as described in the legend to Figure 5 and in the text. Representative results from three mutations and wild type are presented on the left. Quantitations of Ume3p half-life of the various derivatives are given on the right. aEstimated values, as the actual half-life of the L28A/PEST Δ mutant could not be determined in this experiment.

formed. First, recloning the UME3 coding region from one of the A110V mutant isolates into a different vector produced a protein that was also resistant to destruction in cells exposed to heat shock (Figure 6B). Second, the identical amino acid substitution was recreated in the wild-type UME3 gene using oligonucleotide-directed mutagenesis (see Materials and methods). This mutant was also stabilized during heat shock (data not shown), confirming that this alteration did affect Ume3p stability. These results indicate a new role for the cyclin box in regulating protein destruction. Similar destruction kinetics were observed for the E170K substitution (Figure 6B) and the derivative containing both mutations (data not shown), suggesting that these mutations affect the same regulatory pathway. Moreover, combining the RXXL L28A mutation with A110V did not increase the stability of the protein over either single mutation. These results suggest that the cyclin box and the RXXL motif function through the same regulatory pathway to control Ume3p levels.

Failure to destroy Ume3p reduces SPO13 expression during meiotic development

Our working model suggests that the destruction of Ume3p is required for the full induction of several early meiotic genes including SPO13. One prediction of this model is that the failure to destroy this cyclin would reduce SPO13 expression during meiosis. To test this hypothesis, RNA and protein samples were prepared at various times from a sporulating diploid strain harboring the A110V UME3 allele under the control of the ADH1 promoter. This allele complements the ume3 null mutation, indicating that the A110V substitution does not significantly impair Ume3p activity. During the meiotic time course, Ume3p-A110V remained at or near vegetative levels for 4.5 h following transfer to sporulation medium and was still at 25% by 9 h (Figure 4B). This result is in contrast to the wild-type protein whose levels were <25% by 4.5 h and below the levels of detection by 9 h (Figure 4A). SPO13 mRNA levels peaked at approximately the same time in both strains (6 h), but the total expression levels were reduced 2-fold in the A110V strain compared with wild type (two experiments). ACT1 and UME3 mRNA levels varied by ≤10% between strains, indicating that similar amounts of mRNA were recovered. Moreover, the 2-fold effect on SPO13 mRNA levels is consistent with the phenotype associated with ume3 mutations (see Table I). Taken together, these results indicate that the timely destruction of Ume3p is required for the normal accumulation of SPO13 transcript.

Mutations in ubiquitin- and vacuole-mediated proteolysis do not affect Ume3p destruction

Previous studies have found that both G1 and G2 cyclins are destroyed through the ubiquitin pathway and the 26S proteasome (Glotzer et al., 1991; Deshaies et al., 1995; Seufert et al., 1995). To investigate whether the ubiquitin...
pathway is involved in Ume3p degradation, wild-type UME3 under the control of the ADH1 promoter (pKC337) was transformed into strains containing mutations in various stages in this process. Due to the large number of ubiquitin-conjugating enzymes (12), we limited our analysis to ubc mutants defective in either the stress response and/or meiosis (for review, see Finley, 1993). No differences in Ume3p destruction were observed in ubc4 ubc5 (MHY508) or ubc4 ubc6 (MHY505) mutants compared with wild type. Similarly, Ume3p degradation was not altered in mutants lacking the stress-induced polyubiquitin gene UBI4 (SUB63) or the N-end rule ubiquitin-mediated degradation (ubr1, RSY405, data not shown). These results suggested either that ubiquitin is not required in Ume3p destruction or that additional UBC genes are involved. To address this issue, the effects of mutations in components of the 26S proteasome itself were tested. Similarly to the ubc mutant strains, no effect on Ume3p degradation was observed in doa3 (MHY792), doa4 (MHY623), sen3 (DDY112.15A), cim3 (MHY813) or cim5 (MHY814) mutants subjected to heat shock (data not shown). To verify that these strains were expressing a proteolysis-defective phenotype, the turnover of a ubiquitin–LacZ fusion protein was determined in these strains (see Materials and methods). As expected, this fusion protein was stabilized (as determined by increased β-galactosidase activity) in the mutant strains compared with their wild-type controls (data not shown). In addition to the ubiquitin system, we examined Ume3p regulation in a strain (BJ3501) lacking two major vacuolar proteases (Pep4p, Prb1p). Functionally equivalent to the mammalian lysosome, the vacuole provides the bulk of the non-specific protein degradation observed in the cell. Again, no significant effect on Ume3p stability was observed during heat shock compared with wild-type controls (data not shown). Taken together, these results suggest that the destruction of Ume3p occurs through mechanisms outside the normal pathways previously identified for cyclin degradation.

Discussion

We report the isolation of UME3, a C-type cyclin that is required for the full repression of several early meiotic genes (e.g. SPO13) and SSA1, a member of the HSP70 superfamily. Similarly to other cyclin C family members, UME3 mRNA and protein levels remained unchanged throughout the mitotic cell cycle. However, under conditions that induce SSA1 or SPO13 transcription, we demonstrate that Ume3p is subjected to degradation. This destruction is required for normal meiotic gene induction, as a mutation that stabilizes Ume3p resulted in a 2-fold reduction in SPO13 mRNA accumulation. These findings reveal the first observed regulation of a C-type cyclin. Moreover, the destruction of Ume3p in response to heat shock or developmental cues represents a new set of regulatory signals by which any cyclin is controlled. We identified three cis-acting domains (PEST-rich, RXXL and the cyclin box) that contribute to the destruction of Ume3p during heat shock. In cultures exposed to heat shock, Ume3p half-life is decreased ~60-fold. Altering any one of the three regions produces a 3- to 4-fold increase in stability. However, combining the PEST-rich and RXXL mutations produced a protein that displayed a turnover rate under heat shock conditions similar to that obtained at normal temperatures. These results suggest that these domains play largely redundant roles in mediating Ume3p destruction in response to heat shock. In addition, unlike cyclins that drive mitotic cell cycle progression, we find no evidence for the involvement of the ubiquitin pathway in Ume3p degradation. Although these are negative results, they raise the possibility that Ume3p is destroyed through a mechanism different from those described for other cyclins.

This study demonstrates the requirement for a PEST-rich region for efficient destruction of Ume3p during heat shock. Similarly, PEST regions direct the destruction of G1 cyclins in yeast (Salama et al., 1994; Yoglom et al., 1995; Lanker et al., 1996). However, the mechanism by which these sequences are activated may be different. Recent studies have shown that the activation of the Cln2p and Cln3p PEST regions requires phosphorylation by their Cdk partner, Cdc28p (Yaglom et al., 1995; Lanker et al., 1996). Several pieces of data suggest that the PEST region of Ume3p does not depend on the activity of its Cdk (Ume5p). During vegetative growth when the G1 cyclins and Ume3p are active, there is a significant difference in turnover rate among these cyclins. Ume3p is relatively stable (half-life 1.5 h) compared with Cln2p (8 min, Yoglom et al., 1995) or Cln3p (4 min, Lanker et al., 1996). Moreover, deletion of the PEST region does not increase the stability of Ume3p under these same conditions. Second, Ume3p destruction is not affected in mutants lacking Ume5p activity. These findings suggest that Ume3p-dependent activation of Ume5p is not required for its own destruction. Rather, if phosphorylation is required to activate the Ume3p PEST domain, then a protein kinase other than Ume5p is required to perform this function.

In addition to the PEST region, a sequence (RXXL) with similarity to the previously described destruction box also regulates Ume3p stability. Destruction boxes are found in all G2 cyclins and serve as a target for ubiquitin-mediated protein degradation (Murray et al., 1989; Glotzer et al., 1991). We examined several strains harboring mutations in the ubiquitin-conjugating pathway including ubc4 ubc5 double mutants that are required for stress resistance (Arnason and Ellison, 1994) and cyclin degradation (Yaglom et al., 1995), but found no evidence for the involvement of these genes in Ume3p destruction. However, due to the redundancy observed with this gene family (reviewed in Hockstrasser, 1995), a definitive conclusion concerning the role of ubiquitin is difficult to draw. Nearly all ubiquitin-mediated destruction occurs through a specialized structure termed the proteasome (Finley, 1993). We found no effect on Ume3p destruction in mutants for components of the core 20S particle (doa3) or the 26S complex (doa4, sen3, cim3, cim5). Therefore, our data indicate that although a sequence with similarity to a destruction box is involved in Ume3p regulation, the nature of the machinery that destroys this cyclin may be different. We have isolated several mutants that do not degrade Ume3p at 37°C (K.F.Cooper and R.Strich, unpublished results). The characterization of these strains may provide insight into the mechanism(s) by which Ume3p is destroyed.

A surprising result was obtained with the identification
of single amino acid substitutions within the cyclin box that stabilized Ume3p during heat shock and meiosis. The cyclin box directs the association with the protein kinase and is essential for cyclin function (Morgan, 1995). The simplest explanation of these results is that the association of the Cdk with Ume3p is required for cyclin destruction. Mutations interfering with this binding (e.g. A110V, E170K) would in turn stabilize Ume3p. However, several pieces of data suggest that this explanation is not satisfactory. Structural analysis of the cyclin A cyclin box found five helical domains, with helices 3A and 5A providing most of the contact with Cdk2 (Jeffrey et al., 1995). Consistent with the importance of these helices for cyclin–Cdk interaction, mutations E170K and ume3-1 fall within helix 5A and 3A (Figure 6A) respectively and are not functional in vivo. This phenotype is most likely the result of the inability of the cyclin to associate properly with the kinase, although this possibility has not been tested directly. Conversely, the A110V mutation lies within a loop region between helices 2A and 3A and this protein is still functional. While these mutations have the opposite impact on cyclin activity, both alleles stabilize Ume3p to equivalent degrees. These results suggest that cyclin function and perhaps cyclin–Cdk interaction is not a direct effector of stability. A caveat to this interpretation is the possibility that the association of Ume3p with another Cdk may be influencing the stability of this cyclin. Although phenotypic analysis of other Cdk mutants does not point to a candidate gene, this possibility cannot be formally excluded at present. An alternative possibility is that these mutations may affect the secondary or tertiary structure of the molecule, making Ume3p less susceptible to proteolysis. Partially misfolded Ume3p may not be recognized efficiently by the destruction machinery or may be stabilized by the Hsp70p molecular chaperone as observed for p53 (Finlay et al., 1988). A role for Hsp70p in regulating Ume3p destruction would provide a mechanism by which stress signals could influence the stability of this cyclin. This possibility is currently under investigation.

To conclude, UME3 is a member of an emerging subclass of cyclins (C- and H-type) that are involved in regulating gene expression. Unlike the cyclins required for the transition between one phase of the mitotic cell cycle and the next, the C- and H-type cyclins activate kinases that may serve as a switch between on and off transcription states. The finding that C-type cyclins associate with the RNA polymerase II holoenzyme, coupled with the study presented here, suggests that this complex is a direct target of environmental signaling pathways. We propose that under normal growth conditions, Ume3p activates Ume3p and, in conjunction with trans repressors, inhibits the transcription of heat shock or meiotic genes. However, when subjected to stress signals or differentiation cues, Ume3p is destroyed, allowing full induction of these genes by trans activators. This system would provide a rapid, flexible mechanism to alter gene expression programs in response to changing environmental signals.

Materials and methods

Plasmids/strains

The strains used in this study are described in Table II. The epitope-tagged derivative of UME3 was constructed by inserting the myc epitope (Evan et al., 1985) at the amino-terminus of the UME3 coding region using oligonucleotide-directed mutageneis (Kunkel, 1985), generating pJB323-myc. Functional overexpression alleles of UME3 were constructed by engineering an EcoRI site 32 bp upstream of the initiator ATG to generate a 1500 bp EcoRI fragment containing the myc-tagged UME3 open reading frame (ORF) and transcriptional terminator. This fragment was placed under the control of the gal1 promoter (pYES2-Stragatene) to form plasmid pKC333 or the ADH1 promoter in either a single copy (pBC102, B.Cormack and R.Brent, unpublished) or multicopy plasmid (pRS424, Christianon et al., 1992) to form pKC337 and pKC342 respectively. The lexA–UME3 gene was constructed by fusing this 1500 bp EcoRI fragment to the carboxyl end of the bacterial repressor lexA in pEG202 (Gyuris et al., 1993), forming plasmid pEG3. The UME3 disruption allele was constructed by replacing the Sp6l and BglII fragment containing the cyclin box (an amino acids 96–247) with either the LEU2 or TRP1 selectable markers derived from plasmids pJ1250 and pJ246 respectively (Jones, 1991). LER1 was disrupted in RSY10 using pS0B29 (abr::HIS3, a gift from K.Midura, Bartel et al., 1990). The disruption of UME5 was accomplished using pPL144-13 as described (Surosky et al., 1994).

Isolation of UME3

The UME3 gene was isolated by complementing the aberrant mitotic expression phenotype of a spo13 lac–Z reporter gene in a ume3-1 mutant strain (Strich et al., 1989). A genomic library (a gift from M.Rose) was introduced into strain RSY127, and transformants were identified that exhibited reduced expression of the reporter in qualitative colony filter lift assays. The genomic fragment from one positive clone was able to direct homologous recombination of a selectable marker to the ume3-1 locus, indicating that the UME3 gene was isolated (data not shown). The nucleotide sequence was determined using dideoxy methodology. The DDBJ/EMBL/GenBank accession No. for UME3 is U16428. The ume3-1 point mutation was identified by sequencing a PCR fragment generated from the chromosomal copy of the mutant allele in strain RSY128.

Mitotic and meiotic cell synchrony

Haploid strain MMY317 contains the UME3–myc construct under the control of its own promoter integrated into the umes–LEU2 disruption locus (confirmed by Southern blot analysis). This strain was arrested in G1 with α-factor then released and samples taken as described (Oehlen and Cross, 1994). Progression through the cell cycle was monitored by following the budding index of the cells for two generations. Mitotic timecourse experiments were performed with diploid strain RSY335 as described (Klapholz and Esposito, 1980). In addition, a portion of each sample was fixed and stained with the nucleic acid-specific stain 4′,6′-diamidino-2-phenylindole (DAPI). The execution of meiosis I and II was determined by the appearance of meiotic cells respectively, with at least 200 cells counted per time point.

Western blot analysis

To visualize Ume3p in strain MMY317, extracts were prepared as described (Pfeifer et al., 1989) from mid-logarithmic cultures. Ume3p was immunoprecipitated from 2 mg of total soluble extract using the lexA polyclonal antibodies (Sigma) and chemiluminescense (Dupont). Quantitation of the Ume3p signal was accomplished using 0.4 μCi of 125I-conjugated AffiniPure sheep anti-mouse IgG as the secondary antibody (ICN) followed by autoradiography. Background signals, as determined from an identical sized area within each lane, were subtracted from the individual Ume3p signals prior to half-life calculations. Half-life calculations were performed by linear regression analysis of curves with a linear correlation coefficient value >0.9. All half-life values are averages of at least two independent trials. The standard deviation varied by 30% or less for all experiments. Results from the phosphorimager are presented in PSL units, which are derived from the accumulated disintegrations of 125I detected by the apparatus. Figures were generated using the digital images corresponding to the signals obtained from these experiments that were downloaded into a graphics program (MacDraw Pro, Claris Inc.) for annotation and printed without further modification. LexA–Ume3p was immunoprecipitated using lexA polyclonal antibodies (a gift from E.Golemis) from 100 μg of soluble protein. For overexpressing alleles of UME3, 200–500 μg of total soluble extract was used for immunoprecipitation/Western analysis as described above. Extracts were prepared for straight Western analysis by boiling cell pellets in the

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presence of RIPA lysis buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris–HCl, pH 8) and PAGEL loading buffer. Ume3p–lexA or lexA was detected with alkaline phosphatase-conjugated secondary antibodies (Sigma) directed against the rabbit polyclonal lexA antibodies. Tub1p was detected by straight Western blot analysis using mouse anti-β tubulin (Boehringer Mannheim) and 20 μg of total soluble extract.

**Northern and S1 analysis**

S1 nuclease protection assays were performed essentially as previously described (Strich et al., 1989). SP031 and ACT7 probes have been described elsewhere (Suroyko and Esposito, 1992). The UME3 probe was constructed by inserting the 580 bp HindIII fragment (–331 to +249) into SP65 (Promega Inc.) to form pRS302. This construct was digested with BstH1 and a single-stranded RNA probe was generated in vitro with SP6 polymerase. SS41 and ACT7 Northern blot analyses were performed as described (Maniatis et al., 1982) with 10 μg of total RNA.

**Ume3p stability assays**

Cultures harboring either the integrated or plasmid-borne UME3–myc construct were harvested during logarithmic phase growth (0.5–1×10^7 cells/ml) at 30°C; cell pellets were subjected to heat shock at 37°C. For the transient heat shock experiment, cells were resuspended in fresh growth medium pre-warmed to 37°C and then incubated with shaking at the same temperature. To measure the stability of Ume3p or its derivatives under normal growth conditions, RSY10 harboring these constructs under the control of the galactose-inducible promoter was grown to mid-log phase in raffinose-based minimal medium at 30°C. Galactose was added (2% final concentration) and the culture incubated for 3 h to induce gal–UME3 expression. Glucose was added to a final concentration of 2% to repress gal–UME3 transcription, and samples taken as indicated. For the later timepoints, the cells were maintained in log phase growth.

Transfectants growing at 30°C were replica-plated onto medium selecting for the activation of the LEU2 reporter then incubated at 37°C. Plasmids bearing the heat-stable mutations were rescued into this study.

**Regulation of the yeast C-type cyclin**

### Table II. Yeast strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
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<td>RSY10</td>
<td>MAtα ade2 ade6 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1</td>
<td>Strich et al. (1989)</td>
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<td>RSY127</td>
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<td>Strich et al. (1989)</td>
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<td>MMY317</td>
<td>MAtα ade6 his3-11,15 leu2-3,112 trp1-1 ura3-1 ura3-1 ΔTRP1 LEU2::UME3-myc::TRP1 bar1::URA3</td>
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<tr>
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<td>EGY48</td>
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<td>Chen et al. (1993)</td>
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<td>DDDY112.15a</td>
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<td>MHY813</td>
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<tr>
<td>MHY814</td>
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</tr>
<tr>
<td>BJ5301</td>
<td>MAtα can1 gal2 his3Δ200 ura3-52 pep4::HIS3 prb1Δ6R</td>
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</tr>
<tr>
<td>SUB63</td>
<td>MAtα his3Δ200 leu2-3,112 lys2-801 gal trp1-1 ura3-52 ubc4Δ2–Δ2–Δ2 –LEU2</td>
<td>K Madura</td>
</tr>
</tbody>
</table>

**β-Galactosidase assays**

β-Galactosidase (β-gal) activities were measured essentially as described (Miller, 1972). At least three independent cultures of each strain tested were grown to mid-logarithmic phase in synthetic dextrose medium selecting for either plasmid pspo13 (spo13–lacz, Buckingham et al., 1990) or pZDO45 (sasl–lacz, Slater and Craig, 1987) at 30 or 23°C respectively. Cells were harvested and disrupted by vigorous vortexing in the presence of glass beads. The extracts were clarified by centrifugation and soluble protein concentrations were determined using the micro BioRad assay (BioRad Inc.). β-Gal activities were determined from three extract dilutions to ensure that the assays were within the linear range. Units are presented as μmole of o-nitrophenol produced/min/mg of soluble protein.
References


Rogers, S., Wells, R. and Rechsteiner, M. (1986) Amino acid sequences of 26S protease and a helpful discussions. We also thank E.Craig for the swai–luc reporter construct and Jones for the vacuole-defective strain. We thank J.Jaehning for helpful discussions and J.Sherley, J.Cernhoff and T.Yen for critical reading of the manuscript. This work was supported in part by grants from the American Cancer Society, National Cancer Institute and the Pew Charitable Trust to R.S. and both a BBSRC Overseas Fellowship and The Fox Chase Cancer Center Board of Associates Post-Doctoral Fellowship to K.F.C.
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