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

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The *ume3-1* allele was identified as a mutation that allowed the aberrant expression of several meiotic genes (e.g. *SPO11*, *SPO13*) during mitotic cell division in *Saccharomyces cerevisiae*. Here we report that *UME3* is also required for the full repression of the *HSP70* family member *SSA1*. *UME3* encodes a non-essential C-type cyclin (Ume3p) whose levels do not vary through the mitotic cell cycle. However, Ume3p is destroyed during meiosis or when cultures are subjected to heat shock. Ume3p mutants resistant to degradation resulted in a 2-fold reduction in *SPO13* mRNA levels during meiosis, indicating that the down-regulation of this cyclin is important for normal meiotic gene expression. Mutational analysis identified two regions (PEST-rich and RXXL) that mediate Ume3p degradation. A third destruction signal lies within the highly conserved cyclin box, a region that mediates cyclin–cyclin-dependent kinase (Cdk) interactions. However, the Cdk activated by Ume3p (Ume5p) is not required for the rapid destruction of this cyclin. Finally, Ume3p destruction was not affected in mutants defective for ubiquitin-dependent proteolysis. These results support a model in which Ume3p, when exposed to heat shock or sporulation conditions, is targeted for destruction to allow the expression of genes necessary for the cell to respond correctly to these environmental cues.

Keywords: cyclin/heat shock/meiosis/protein degradation

Introduction

Cyclins are a conserved family of proteins required for the activation of a class of protein kinases termed Cdk (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 TFIIF complex that is required for transcription initiation (Roy *et al.*, 1994). A similar activity has been purified from yeast that contains the Ccl1p–Kin28p cyclin–Cdk

complex (Feaver *et al.*, 1994). In addition, a more distantly related cyclin (Pho80p) complexes with the Pho85p 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 (Srb11p–Ume5p) is required for the repression of genes involved in sucrose utilization (Kuchin *et al.*, 1995) and meiotic development (Strich *et al.*, 1989; Surosky *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.*, 1989; 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 meiotic genes (e.g. *SPO11*, *SPO13*) transcribed early in development (Strich *et al.*, 1989, 1994; Vidal *et al.*, 1991; Surosky *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

Table I. Repression of meiotic and heat shock genes by *UME3* and *UME5*

Strain	Genotype	<i>spo13-lacZ</i>		<i>ssal-lacZ</i>	
		β -Gal activity	Fold increase	β -Gal activity	Fold increase
RSY333	<i>UME3 UME5</i>	0.03 $\pm$ 0.01	1.0	208 $\pm$ 43	1.0
RSY383	<i>ume3Δ UME5</i>	0.19 $\pm$ 0.02	6.3	747 $\pm$ 107	3.6
RSY440	<i>UME3 ume5Δ</i>	0.17 $\pm$ 0.02	5.7	809 $\pm$ 108	3.9
RSY441	<i>ume3Δ ume5Δ</i>	0.18 $\pm$ 0.01	6.0	758 $\pm$ 11	3.6
RSY127	<i>ume3-1 UME5</i>	0.09 $\pm$ 0.01	3.0	1003 $\pm$ 230	4.8

The complete genotypes for the strains are listed in Table II. The *spo13-lacZ* and *ssal-lacZ* activities were determined from mid-logarithmic phase cultures (see Materials and methods). Activities presented were averaged from 2–3 independent transformants assayed at three extract concentrations.

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 *ume3 Δ* , *ume5 Δ* or *ume3 Δ ume5 Δ* 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 *ssal-lacZ* reporter gene (Slater and Craig, 1987) in the *ume3* and *ume5* single and double mutants described above. Similarly to *spo13-lacZ*, *ssal-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 9E10 (Evan *et al.*, 1985) was inserted at the amino-terminus of Ume3p (pJB323-myc, see Materials and methods). The *UME3-myc* construct is able to complement a *ume3 Δ* 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* 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

Fig. 1. Regulation of Ume3p during vegetative growth. (A) Ume3p levels in synchronized mitotic cells. Haploid strain MMY317 was synchronized using α -factor pheromone arrest and release. Samples were taken prior to (0') and following the release as indicated (in minutes). The culture was followed for two generations (80' and 160'), as determined by the budding index of the cells. The vector alone lane controls for non-specific cross-hybridization of the myc monoclonal antibody. Ume3p was detected using Western blots of immunoprecipitates (Materials and methods). (B) Analysis of Ume3p levels during heat shock. MMY329 harboring the integrated *UME3-myc* construct was grown at 30°C to mid-logarithmic phase (0'), harvested, and subjected to heat shock at 37°C as indicated (minutes). Tub1p and *ACT1* were used to control for protein and RNA quantitation of each sample, respectively.

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 as 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.

Fig. 2. Ume3p regulation during heat shock. (A) Transient heat shock time course. RSY10 containing pKC337 was grown at 30°C to mid-logarithmic phase, harvested, then shifted to pre-warmed (37°C) growth medium and maintained at this temperature for the duration of the experiment. Samples were taken prior to (0') and following the shift (minutes). *SSA3*, *UME3* and *ACT1* mRNA levels were monitored by Northern blot analysis of total RNA preparations. Ume3p was detected using Western blots of immunoprecipitates (Materials and methods). (B) Regulation of Ume3p-lexA. Cultures harboring the *UME3-lexA* fusion gene (pEG3, upper panels) or *lexA* alone (pEG202, lower panels) were subjected to 42°C heat shock for the times indicated (minutes). Ume3p-lexA and *lexA* were immunoprecipitated from soluble extracts then Western blotted (left panels) or analyzed directly from crude lysates by Western blot analysis (right panels). The Ume3p-lexA bands are indicated by the arrow. The asterisk denotes the immunoglobulin heavy chain.

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 Lieberman, 1993). If reduced translational initiation is combined with a normally rapid turnover rate, as observed for other cyclins (e.g. Cln3p, Tyers *et al.*, 1992; Cross and Blake, 1993), the Ume3p expression profile described here could be produced. Alternatively, Ume3p may be stable under normal

Fig. 3. Analysis of Ume3p stability. (A) Ume3p stability in glucose medium. RSY10 harboring the *gal1-UME3* gene (pKC333) was grown in non-repressing (but non-inducing) raffinose-containing medium. *gal1-UME3* expression was induced with galactose (2%) for 3 h then repressed by addition of glucose (2%) and the culture followed for 6 h. Samples were taken as indicated and the Ume3p–myc levels determined. Extracts prepared from RSY10 harboring pYES2 (vector lane) grown in raffinose and galactose medium controlled for the non-specific cross-hybridization of the myc antibody. Ume3p was detected using Western blots of immunoprecipitates (Materials and methods). (B) Ume3p stability at 37°C. RSY10 harboring pKC333 was grown at 30°C in galactose/raffinose medium to induce Ume3p, switched to 37°C and levels of Ume3p monitored at the timepoints indicated. For both experiments, Tub1p levels were monitored as a control for protein quantitation of each sample.

growing conditions but targeted for destruction when cells are exposed to heat shock. To investigate these possibilities, 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 (2 μ *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 I), 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 Cdks (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 *ume5 Δ* (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 RXXLX₍₄₎N (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 an ~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/PEST Δ). In cultures exposed to elevated temperatures, the stability of this derivative was enhanced dramatically (>20-fold)

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-

Ume3p Mutation	Host Genotype	Half Life (min.)	Fold Increase
■ Wild type	<i>UME5</i>	1.5	1.0
○ PESTΔ	<i>UME5</i>	5.2	3.5
● Wild type	<i>ume5Δ</i>	1.5	1.0

Fig. 5. Analysis of the PEST region and Ume3p 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 (*UME5*, ■) or in an isogenic *UME5* deletion mutant RSY440 (*ume5Δ*, ●). The stability of a Ume3p derivative lacking a PEST-rich sequence (PESTΔ) was assayed in a *UME5* host (○). 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/PESTA 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 a *ume3* null mutation, indicating that the A110V substitution does not significantly impair Ume3p activity. During the meiotic timecourse, 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 vacuole 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; Yaglom *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, Yaglom *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 Ume5p 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 mutagenesis (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 *galI* promoter (pYES2, Stratagene) to form plasmid pKC333 or the *ADHI* promoter in either a single copy (pBC102, B.Cormack and R.Brent, unpublished) or multi-copy plasmid (pRS424, Christianson *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 *SphI* and *BglII* fragment containing the cyclin box (amino acids 96–247) with either the *LEU2* or *TRP1* selectable markers derived from plasmids pJJ250 and pJJ246 respectively (Jones, 1991). *UBR1* was disrupted in RSY10 using pSOB29 (*ubr1::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-lacZ* 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 *ume3::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. Meiotic 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 bi- and tetranucleated 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 *myc* monoclonal antibody (mAb) 9E10 (Evan *et al.*, 1985) and analyzed by Western blotting with the same antibody. Bands were visualized using an anti-mouse secondary antibody conjugated to horseradish peroxidase (Sigma) and chemiluminescence (Dupont). Quantitation of the Ume3p signal was accomplished by substituting 0.4 μ Ci of 125 I-conjugated sheep anti-mouse IgG as the secondary antibody (ICN) followed by phosphorimaging (Fuji, Inc.). 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 32 P or 125 I 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 μ g of soluble protein. For overexpressing alleles of *UME3*, 200–500 μ g 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

Table II. Yeast strains

Strain	Genotype	Source
RSY10	<i>MATa ade2 ade6 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1</i>	Strich <i>et al.</i> (1989)
RSY127	<i>MATa his4-C leu2-3,112 trp1-1 ume3-1 ura3-1</i>	Strich <i>et al.</i> (1989)
RSY128	<i>MATa ade2 ade6 can1-100 his3-11,15 leu2-3,112 trp1-1 ume3-1 ura3-1</i>	Strich <i>et al.</i> (1989)
RSY335	<i>MATa/MATα cyh2'-z/cyh2'-z ho::LYS2/ho::LYS2 leu2::hisG/leu2::hisG lys2/lys2 trp1::hisG/trp1::hisG ura3/ura3</i>	this study
RSY391	<i>MATa ade2 ade6 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 ume3Δ::LEU2</i>	this study
RSY392	<i>MATa ade2 ade6 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 ume3Δ::TRP1</i>	this study
MMY317	<i>MATa ade6 his3-11,15 leu2-3,112 trp1-1 ura3-1 ume3Δ::LEU2::UME3-myc::TRP1 bar1::URA3</i>	this study
MMY329	<i>MATa ade6 his3-11,15 leu2-3,112 trp1-1 ura3-1 ume3Δ::LEU2::UME3-myc::TRP1</i>	this study
RSY333	<i>MATa cyh2'-z ho::LYS2 leu2::hisG lys2 trp1::hisG ura3</i>	this study
RSY383	<i>MATa cyh2'-z ho::LYS2 leu2::hisG lys2 trp1::hisG ura3 ume3Δ::TRP1</i>	this study
RSY440	<i>MATa cyh2'-z ho::LYS2 leu2::hisG lys2 trp1::hisG ura3 ume5Δ::LEU2</i>	this study
RSY441	<i>MATa cyh2'-z ho::LYS2 leu2::hisG lys2 trp1::hisG ura3 ume3Δ::TRP1 ume5Δ::LEU2</i>	this study
RSY405	<i>MATa ade2 ade6 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 ubr1::HIS3</i>	this study
EGY48	<i>MATα his3-11,15 leu2-3,112 trp1-1 ura3-1 6LexA-operators-LEU2-3</i>	Estojak <i>et al.</i> (1995)
MHY501	<i>MATa his3Δ200 leu2-3,112 lys2-801 trp1-1 ura3-52</i>	Chen <i>et al.</i> (1993)
MHY508	<i>MATa his3Δ200 leu2-3,112 lys2-801 trp1-1 ura3-52 ubc4Δ1::HIS3 ubc5::LEU2</i>	Chen <i>et al.</i> (1993)
MHY505	<i>MATa/MATα his3Δ200/his3Δ200 leu2-3,112/leu2-3,112 lys2-801/lys2-801 trp1-1/trp1-1 ura3-52/ura3-52 ubc4Δ1::HIS3/ubc4Δ1::HIS3 ubc6::HIS3/ubc6::HIS3</i>	M.Hochstrasser
MHY623	<i>MATa his3Δ200 leu2-3,112 lys2-801 trp1-1 ura3-52 doa4::LEU2</i>	Chen and Hochstrasser (1995)
MHY784	<i>MATa his3Δ200 leu2-3,112 lys2-801 trp1-1 ura3-52 doa3Δ1::HIS3 p(YCp50URADOA3)</i>	Chen and Hochstrasser (1995)
MHY792	<i>MATa his3Δ200 leu2-3,112 lys2-801 trp1-1 ura3-52 doa3Δ1::HIS3 (YCplac22doa3-1-TRP1)</i>	Chen and Hochstrasser (1995)
DDY112.15a	<i>MATa ade2-101 his3Δ200 leu2-Δ1 trp1-Δ1 ura3-Δ1::TRP1 sen3-1</i>	DeMarini <i>et al.</i> (1995)
MHY813	<i>MATa ade2-101 his3Δ200 leu2-Δ1 trp1-Δ1 ura3-52 cim3-1</i>	Ghislain <i>et al.</i> (1993)
MHY814	<i>MATa ade2-101 his3Δ200 leu2-Δ1 lys2-801 trp1-Δ1 ura3-52 cim5-1</i>	Ghislain <i>et al.</i> (1993)
BJ3501	<i>MATα can1 gal2 his3-Δ200 ura3-52 pep4::HIS3 prb1Δ1.6R</i>	Jones (1991)
SUB63	<i>MATα his3Δ200 leu2-2,112 lys2-801 gal trp1-1 ura3-52 ubi4-Δ2::LEU2</i>	K.Madura

presence of RIPA lysis buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris-HCl, pH 8) and PAGE 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 μ g of total soluble extract.

Northern and S1 analysis

S1 nuclease protection assays were performed essentially as previously described (Strich *et al.*, 1989). *SPO13* and *ACT1* probes have been described elsewhere (Surosky 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 *BbsI* and a single-stranded RNA probe was generated *in vitro* with SP6 polymerase. *SSA3* and *ACT1* Northern blot analyses were performed as described (Maniatis *et al.*, 1982) with 10 μ g of total RNA.

Ume3p stability assays

Cultures harboring either the integrated or plasmid-borne *UME3-myc* construct were harvested during logarithmic phase growth ($0.5\text{--}1 \times 10^7$ 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 then incubated with shaking at the same temperature. To measure the stability of Ume3p or its derivatives under normal growing 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 by diluting the culture with fresh medium containing 2% glucose. All samples were quick frozen at −80°C and total RNA or soluble protein extracts were prepared as described above.

Mutagenesis

To generate heat-stable *lexA-UME3* mutants, pEG3 was mutagenized *in vitro* (Sikorski and Boeke, 1991) then introduced into EGY48 which contains the *LEU2* gene under the control of *lexA* operators.

Transformants 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 *Escherichia coli* and the phenotype verified. Positive clones were subjected to sequence analysis to determine the position of the mutation. pKC354 (A110V), pKC206 (E170K) and pKC207 (A110V and E170K) were constructed by inserting a 1500 bp *EcoRI* containing the *UME3* ORF from heat-stable alleles into pBC102. pKC362 was constructed by inserting the 3000 bp *BamHI* fragment from pKC354 [containing the *ADH1* promoter, *UME3* (A110V) ORF and terminator] into the *BamHI* site of pRS424. The heat-stable alleles were recreated using site-directed mutagenesis on pKC342 single-stranded DNA as described earlier as follows: A110V, pKC202, (+313, AGGTTTTTAATAAAGGTTTCAGT-TAGAGAAATA, +345); E170K, pKC203, (+491, TTCTACTTACTA-GAAAAATTGGAAAGTTAC, +522). The internal deletion of the PEST-rich region (pKC359) was constructed by site-directed mutagenesis on pKC342 using the oligonucleotide (+766, GAAGCCATCAGAGATAT-CGTTCAAATAGCTTTT, +816) which replaces the PEST-rich region spanning amino acids 261–269 (PKNSSSPVQ) with an isoleucine. The RXXL single amino acid substitutions were made in a similar fashion using the oligonucleotides (+61, TTGGCTAAAGAGGCTCAGAAGTT-ATGG, +98) and (+52, AAGGCATCATTTGGCTGCAGCATTATGGC-TATTGGAG, +96) to form pKC220 (R25A) and pKC219 (L28A) respectively. The double mutants (L28A/A110V, pKC233; L28A/PESTΔ, pKC232) were constructed using oligonucleotide-directed mutagenesis as described above on pKC219 (L28A).

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 p(*spo13*)28 (*spo13-lacZ*, Buckingham *et al.*, 1990) or pZDO45 (*ssa1-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 cleared 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 insure that the assays were within the linear range. Units are presented as μ moles of *o*-nitrophenol produced/min/mg of soluble protein.

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