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Regulated degradation of the transcription factor Gcn4

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We report that Gcn4, a yeast transcriptional activator of the bZIP family involved in the regulation of the biosynthesis of amino acids and purines, is rapidly turned over. This degradation is inhibited under conditions of starvation for amino acids. Degradation is also inhibited by single amino acid alterations in a region adjacent to the Gcn4 activation domain. Furthermore, we show that degradation of Gcn4 proceeds through the ubiquitin pathway, a major proteolytic system for cytoplasmic proteins, and is dependent on two specific ubiquitin conjugating enzymes, Cdc34 (Ubc3) and Rad6 (Ubc2). As a first step towards reconstituting the Gcn4 degradation pathway *in vitro*, we show that purified Cdc34 and Rad6 proteins are able to direct the specific ubiquitination of Gcn4.

Key words: Gcn4/protein degradation/starvation/ubiquitin

Introduction

The important role of protein stability in regulation is becoming increasingly apparent. Although most cellular proteins are metabolically stable, a subset of proteins is rapidly degraded (reviewed in Gottesman and Maurizi, 1992). This subset includes many regulatory proteins, presumably because rapid turnover allows effective modulation of the levels of a protein in response to changes in its rate of synthesis (Schimke, 1973; Rechsteiner, 1988). Control of degradation could also provide an additional level of regulation. A number of proteins are known to be conditionally degraded: in eukaryotes, mitotic cyclins are degraded at specific times during the cell cycle (Glotzer *et al.*, 1991); a plant phytochrome is degraded after activation by red light (Shanklin *et al.*, 1987); and a number of important biosynthetic enzymes are degraded more rapidly in the presence of their end product (Arad *et al.*, 1976; Jingami *et al.*, 1987; Li *et al.*, 1992; Murakami *et al.*, 1992). In prokaryotes, the heat-shock sigma factor, HtpR, is transiently stabilized after temperature up-shift (Strauss *et al.*, 1987).

Little is known about the structural features that distinguish short-lived proteins from their long-lived counterparts. The N-end rule, which relates the half-life of a protein to its N-terminal residue, constitutes the best-characterized degradation signal to date (Bachmair *et al.*, 1986). Unfortunately, virtually no natural substrates of the N-end rule degradation pathway are known and its physiological role remains unclear (reviewed in Varshavsky, 1992). Another general degradation signal has been suggested to consist of regions rich in the amino acid residues proline, aspartic acid, glutamic acid, serine and threonine, i.e. 'PEST' residues; this suggestion is based mostly on the observation that such regions are over-represented in rapidly degraded proteins (Rogers *et al.*, 1986; Rechsteiner, 1988).

In eukaryotes, the ubiquitin system is a major cytoplasmic pathway for selective protein degradation (see Finley, 1992; Hershko and Ciechanover, 1992; Hochstrasser, 1992; Jentsch, 1992; Rechsteiner, 1993, for recent reviews). This system mediates the covalent linkage of the 8 kDa ubiquitin protein to substrate proteins through an isopeptide bond. Thioester-linked ubiquitin is transferred from a ubiquitin activating enzyme (or E1) to a ubiquitin conjugating enzyme (E2) and subsequently to the ε-amino group of an internal lysine on the target protein. This latter reaction requires, at least in some cases, a ubiquitin ligase (E3), which mediates recognition of the substrate by the ubiquitin conjugating enzyme.

Substrate proteins can be conjugated to a single ubiquitin molecule or to chains of ubiquitin molecules linked to each other via lysine residues. Multi-ubiquitin chains, rather than the mono-ubiquitin adducts, probably constitute the signal required for degradation (Chau *et al.*, 1989; Deveraux *et al.*, 1994). Degradation of multi-ubiquitinated proteins occurs through a large multi-catalytic proteinase, the 26S proteasome (Heinemeyer *et al.*, 1991; Goldberg, 1992; Seufert and Jentsch, 1992).

The specificity of the system for particular substrates is thought to reside in the E2 and E3 enzymes. In yeast, 10 genes encoding E2 enzymes have been isolated to date (Jentsch, 1992). They include: *UBC4* and *UBC5*, which, along with *UBC1*, mediate turnover of the bulk of short-lived and abnormal proteins (Seufert and Jentsch, 1990) and are required, along with *UBC6* and *UBC7*, for the degradation of Matα2 (Chen *et al.*, 1993); *CDC34* (*UBC3*), a gene required for the G_1 to S transition of the cell cycle (Goebl *et al.*, 1988); and *RAD6* (*UBC2*), a gene involved in a number of processes, including DNA repair, sporulation, cell proliferation and N-end rule degradation (Haynes and Kunz, 1981; Kupiec and Simchen, 1984; Dohmen *et al.*, 1991; Sung *et al.*, 1991). Only one E3 encoding gene, *UBR1*, has been identified in yeast (Bartel *et al.*, 1990); two other E3s have been detected biochemically (Sharon *et al.*, 1991; Parag *et al.*, 1993).

Phenotypes of these yeast *ubc* mutants indicate that the ubiquitin pathway is involved in many cellular processes. Nevertheless, only a few of the *in vivo* substrates of the ubiquitin pathway have been identified to date

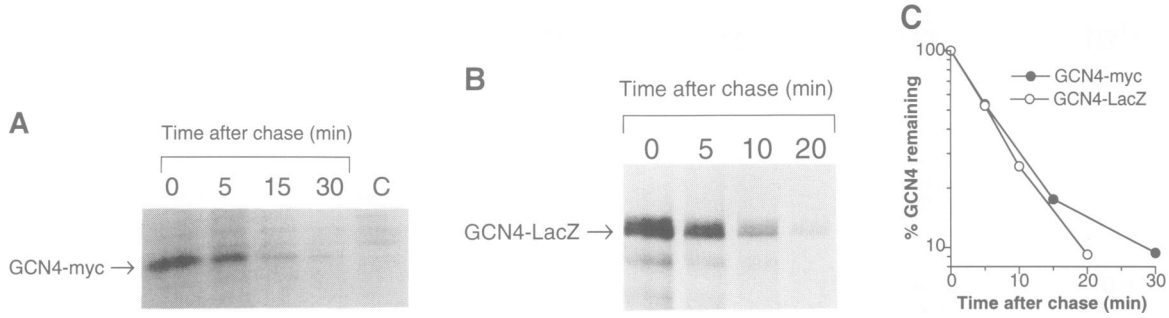

Fig. 1. Gcn4 is rapidly degraded. (**A**) Pulse–chase analysis of Gcn4 tagged with two myc epitopes (Kolodziej and Young, 1991) (GCN4–myc), inserted after residues 4 and 192. Lane C is a non-epitope-tagged control. (**B**) Pulse–chase analysis of a Gcn4–LacZ fusion protein. (**C**) Comparison of the decay rate of the epitope-tagged protein (GCN4–myc) versus that of the Gcn4–LacZ fusion protein.

(Rechsteiner, 1991). They include the yeast transcription factor Matα2 (Hochstrasser *et al.*, 1991; Chen *et al.*, 1993), cyclin B (Glotzer *et al.*, 1991), a plant phytochrome (Shanklin *et al.*, 1987) and the oncogene products p53 (Scheffner *et al.*, 1993) and Mos (Nishizawa *et al.*, 1993).

Gcn4, a well-characterized yeast transcriptional activator (Hinnebusch, 1986; Hope and Struhl, 1986), is required for high level expression of genes involved in the biosynthesis of amino acids and purines and its synthesis is induced at the level of translation by starvation for these compounds (Hinnebush, 1992). Gcn4 activity was also recently found to be induced by UV irradiation in a Ras pathway-dependent fashion (Engelberg *et al.*, 1994).

In this study, we show that Gcn4 is extremely unstable and identify both the *cis*-acting sequences as well as the *trans*-acting functions required for its degradation. We show that degradation of Gcn4 *in vivo* depends on the presence of two specific ubiquitin conjugating enzymes, Cdc34 and Rad6, and that these enzymes are able to direct ubiquitination of Gcn4 *in vitro*. Consistent with its role in regulation, starvation for amino acids stabilizes Gcn4.

Results

Sequences in Gcn4 required for degradation.

To analyse the stability of Gcn4 *in vivo*, we used two types of derivatives of the protein: constructs with myc epitopes introduced at various sites in the protein (Gcn4–myc) and constructs with the LacZ protein fused to the C-terminus of Gcn4 (Gcn4–LacZ). Pulse–chase experiments show that both Gcn4–myc and Gcn4–LacZ are highly unstable, each with a half-life of about 5 min (Figure 1). This short half-life may be the reason that Gcn4 activity has been difficult to isolate from yeast cells.

We used deletion analysis in an attempt to identify a specific region of Gcn4 required for normal degradation of the protein. To this end various segments of Gcn4 were fused to LacZ, transformed into yeast and assayed for their stability. Analysis of this deletion series (Figure 2) shows that deletions throughout Gcn4 stabilize the protein to varying degrees; no single region responsible for the instability was identified. It is possible that the majority of the Gcn4 sequence is necessary for normal rates of degradation. Alternatively, it is possible that the deletion analysis was simply too crude; the novel junctions created by each deletion could distort the protein so that it is a defective substrate for the degradation system.

We then attempted to identify by genetic analysis

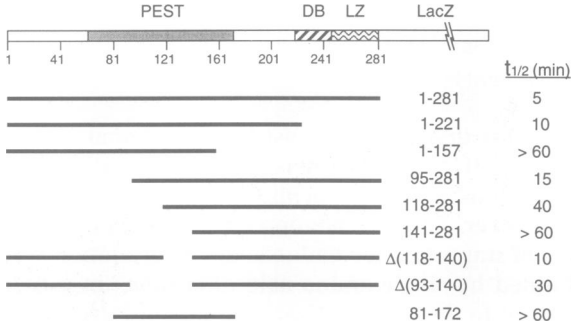

Fig. 2. Effect of deletions on the stability of Gcn4–LacZ. Schematic depiction of the Gcn4–LacZ fusion protein: PEST, region with high proportion of PEST residues; DB, DNA binding domain; LZ, leucine zipper. The numbers indicate the position in the Gcn4 amino acid sequence.

specific residues in Gcn4 important for its degradation. We mutagenized a plasmid containing *GCN4* and screened for *cis*-acting mutations that increased the transcriptional activity of Gcn4, assuming that such an increase could result from stabilization of the protein (see Materials and methods). Of the seven Gcn4 mutants that were isolated, five had a mutation in the threonine residue at position 105. This mutation greatly increased the stability of Gcn4; when Thr105→Ala (T105A) was present in either Gcn4–myc or Gcn4–LacZ the half-life was increased 2- to 3-fold (Figure 3A and C).

Gcn4 is rich in the amino acid residues P, E, D, S and T ('PEST') in the region flanking Thr105 and extending from position 59 to 172. 'PEST'-rich sequences have been suggested to function as degradation signals (Rogers *et al.*, 1986; Rechsteiner, 1988). In the region of Gcn4 from 59 to 172, Thr105 is located within the longest contiguous sequence of PEST residues, between positions 99 and 106 (Figure 3B). To test whether other residues in the 99–106 sequence are required for Gcn4 degradation, we mutated each of these eight residues to alanine and tested the effect on transcriptional activity of Gcn4, as reflected in the expression of one of its target genes, *HIS4* (Lucchini *et al.*, 1984).

The Gcn4-stimulated expression of *HIS4* was greatly increased in cells expressing the more stable Gcn4 derivative, Thr105→Ala. A similar increase was seen with Ser101→Ala and Pro106→Ala, suggesting that these residues are also required for Gcn4 degradation. Deletion of the entire sequence (Δ99–106) resulted in an even greater increase in the transcriptional activity of Gcn4

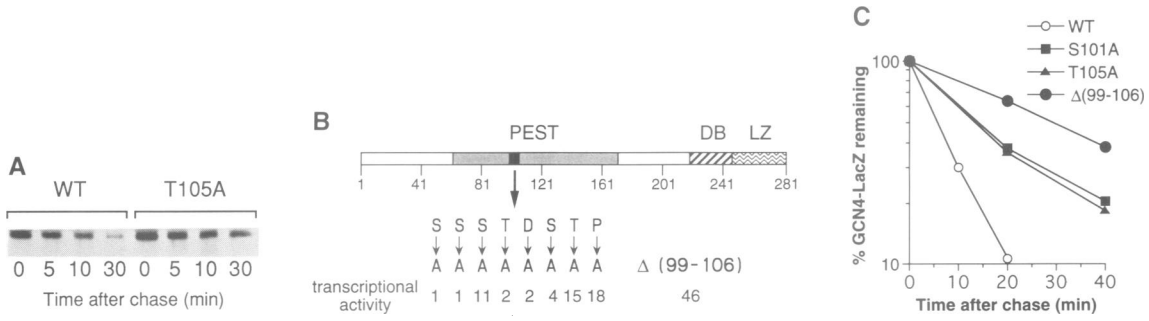

Fig. 3. Effect of point mutations on the stability and activity of Gcn4. (**A**) Effect of mutation Thr105→Ala on the stability of Gcn4, using constructs carrying a single myc epitope after residue 192. (**B**) Schematic depiction of Gcn4. The sequence of residues 99–106, the longest contiguous sequence of PEST residues, is shaded in black and indicated in one letter code. The other sequence elements are as in Figure 2. The relative transcriptional activity of Gcn4 mutants in which each of the eight indicated PEST residues were substituted with alanine (A), as well as that of the deletion of these eight residues, is indicated (activity of wild-type = 1). (**C**) Effect of various mutations on the stability of the Gcn4−LacZ fusion protein.

(Figure 3B). Direct measurement of the stability of Ser101→Ala (S101A) and of Δ99–106 in a Gcn4−LacZ fusion confirmed that these mutations stabilize Gcn4 (Figure 3C).

Gcn4 is degraded through the ubiquitin pathway

Long exposure of the Gcn4−LacZ pulse−chase analysis gels revealed a series of slower migrating species above the main protein band. This pattern is characteristic of proteins covalently linked to ubiquitin on lysine residues (Dohmen *et al.*, 1991; Hochstrasser *et al.*, 1991). To test whether these species represent ubiquitinated forms of the protein, we overexpressed a myc epitope-tagged version of ubiquitin (Ellison and Hochstrasser, 1991). This tagged ubiquitin is larger than ubiquitin itself and, therefore, decreases the rate of migration of ubiquitinated proteins (Ellison and Hochstrasser, 1991). The pattern of bands above Gcn4−LacZ was shifted up in the presence of overexpressed myc−ubiquitin (Figure 4A), indicating that these bands represent ubiquitinated forms of Gcn4. Strikingly, the amount of ubiquitination was greatly reduced in the more stable Gcn4 derivative T105A (Figure 4B); quantitation of the amount of ubiquitinated protein bands relative to the main GCN4−LacZ band showed a 5- to 10-fold reduction in the T105A mutant, suggesting a causal link between ubiquitination and the degradation of Gcn4.

If linkage of ubiquitin to lysine residue(s) on Gcn4 were a prerequisite for degradation, then mutations that alter the critical lysine residues should affect the stability. Indeed, a Gcn4 derivative in which the 11 lysines between positions 81 and 211 were changed to arginine was markedly stabilized; constructs containing some subsets of these lysine substitutions had a milder effect on degradation, whereas other subsets had no effect at all (Figure 4C). From this analysis, it appears that no single lysine substitution is sufficient to affect Gcn4 degradation. Presumably, multiple substitutions are necessary to observe an effect, because any one of a number of lysines sterically available for ubiquitination can serve as an acceptor. The Gcn4 derivatives that were stabilized also displayed increased transcriptional activity, probably because of the increased steady-state levels of these proteins (Figure 4C). The retention of biological activity in these mutants suggests that the increased stabilization does not result from gross alterations in the conformation of the protein, but rather from the specific requirement of these lysine residues for degradation.

Since ubiquitinated proteins are thought to be degraded through the 26S proteasome, we tested the effect of a proteasome mutant on Gcn4 degradation. The half-life of Gcn4 was extended in a temperature-sensitive mutant of *CIM5* (which encodes a regulatory subunit of the 26S proteasome; Ghislain *et al.*, 1993) at the non-permissive temperature (Figure 4D), consistent with a requirement of ubiquitination for Gcn4 degradation.

Cdc34 and Rad6 participate in Gcn4 degradation in vivo

The finding that Gcn4 is ubiquitinated suggested that enzymes of the ubiquitin pathway might be involved in the degradation of this transcription factor. To test this we assayed the stability of Gcn4 in strains carrying mutations in *UBC* genes. Gcn4 degradation is unaffected in *ubc1*, *ubc4 ubc5*, *ubc6 ubc7* and *ubc8* mutants (not shown). In contrast, Gcn4 degradation is strongly inhibited in strains with a temperature-sensitive allele of *CDC34* (*UBC3*) and mildly inhibited in strains deleted for *RAD6* (*UBC2*). In the *cdc34ts* strain, the Gcn4 half-life was increased 1.5- to 2-fold at the permissive temperature of 30°C (not shown) and 4-fold at the restrictive temperature of 36°C (Figure 5A and C). Degradation kinetics in the *rad6*Δ strain was biphasic, with a slower rate of degradation in the first 10 min of the chase, followed by a second phase in which the degradation rate was the same as in wild-type cells (Figure 5C). Surprisingly, we found that the effect of the *rad6*Δ mutation was more pronounced when cycloheximide was added at the time of chase: under these conditions, Gcn4 degradation was further stabilized in the second phase (see Figure 5D).

Upon extended electrophoresis in SDS−PAGE, Gcn4 can be separated into three distinct species, **a**−**c** (fastest−slowest) (Figure 5B). Strikingly, different forms accumulate in different mutants: in the *cdc34ts* mutant, forms **b** and **c** accumulate, whereas in the *rad6*Δ mutant, form **a** is predominant. In the double mutant, all three forms accumulate. These results suggest that Cdc34 and Rad6 are involved in two distinct pathways, Cdc34 recognizing the **b** and **c** species and Rad6 recognizing **a**. However, no additional stabilization was detected in the double *cdc34ts rad6*Δ mutant as compared with the single *cdc34ts* mutant

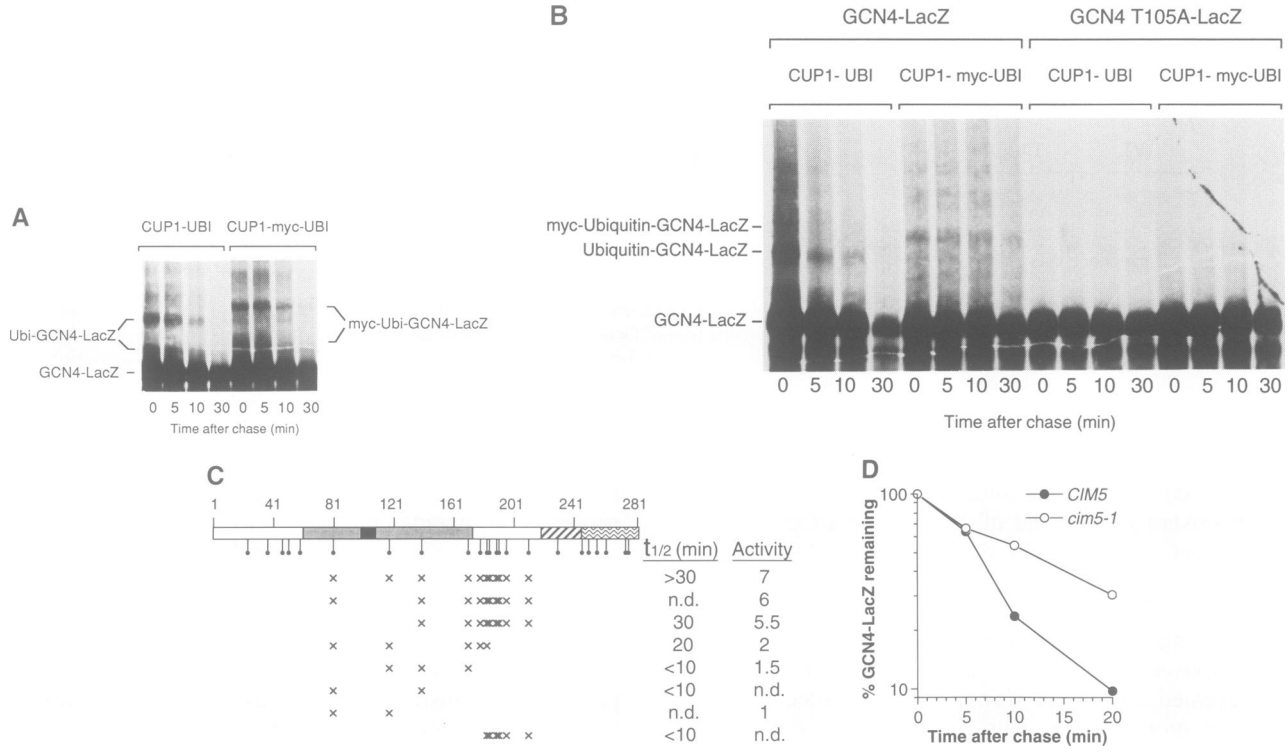

Fig. 4. (**A**) Gcn4 is ubiquitinated. Pulse−chase analysis was performed on cells expressing the GCN4−LacZ fusion from the *GAL1* promoter and either ubiquitin or myc-tagged ubiquitin from the strong *CUP1* promoter of plasmids YEp96 and YEp105 respectively (Ellison and Hochstrasser, 1991). In order to visualize the ubiquitinated bands, the gel was overexposed for 2 months. (**B**) Gcn4 T105A is less ubiquitinated than the wild-type. The experiment was performed as in (A). (**C**) Effect of multiple lysine substitutions on the stability and activity of Gcn4. The various sequence elements are as in Figure 2B. The 23 lysines of Gcn4 are each depicted by (●). In each mutant, represented on a separate line, the lysines substituted with arginine are indicated by (x). The stability was measured using a GCN4−LacZ fusion. Transcriptional activity was measured using *ade8−GCN4* constructs. The activity is normalized to wild-type (= 1). (**D**) Gcn4−LacZ degradation is retarded in the *cim5-1* mutant. Pulse−chase analysis was performed at the non-permissive temperature of 37°C.

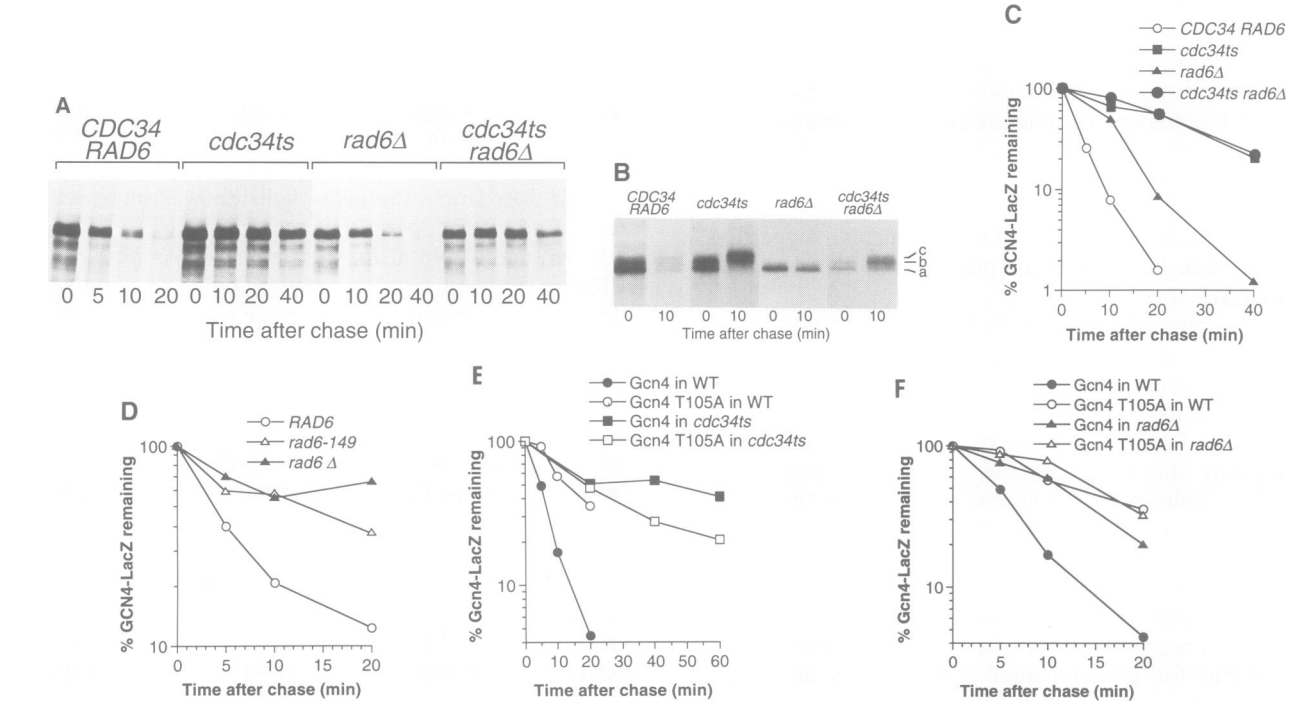

Fig. 5. Degradation of Gcn4 is inhibited in *cdc34* and *rad6* mutants. (**A**) Pulse−chase experiment using GCN4−LacZ in a *cdc34ts* mutant, in a *rad6* deletion mutant and in a strain containing both mutations at 36°C. Note the difference in timescale between the wild-type and mutants. (**B**) Pulse−chase experiment using GCN4−myc in the indicated strains at 36°C. The three electrophoretic forms of Gcn4 are indicated on the right. (**C**) Quantitation of experiment shown in (A). (**D**) Degradation of GCN4−LacZ in *rad6Δ* and *rad6-149* cells in the presence of cycloheximide. (**E, F**) Degradation of GCN4 (T105A)−LacZ versus GCN4−LacZ in *cdc34ts* cells at 37°C (E) and in *rad6Δ* cells at 30°C (F). The rate of degradation of these constructs in the wild-type cells at 30°C is shown as a comparison.

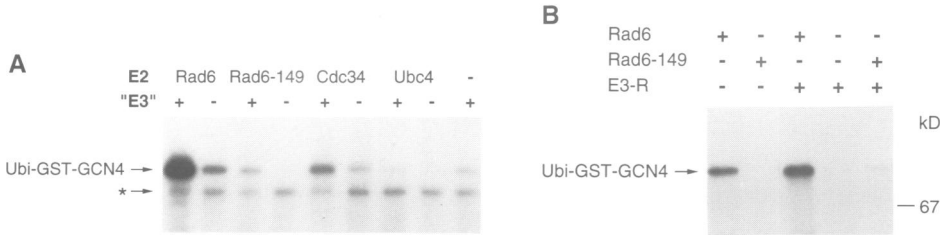

Fig. 6. Rad6 and Cdc34 ubiquitinate Gcn4 *in vitro*. (**A**) Activity of the indicated purified E2 enzymes was tested in the presence (+) or absence (−) of a partially purified yeast cytosolic fraction containing E3 activity ('E3'). Note that the 'E3' fraction by itself has some conjugating activity. The migration of the 67 kDa molecular weight marker is indicated on the right; the position of the ubiquitinated GST−GCN4 band (Ubi-GST-GCN4) and of a contaminating band (*) are indicated on the left. (**B**) Activity of Rad6 and Rad6-149 in the presence of a different E3 fraction, E3-R.

(Figure 5C), even when the chase was performed in the presence of cycloheximide (not shown).

The Rad6 protein carries a 23 residue long C-terminal domain which is required for some, but not all, of its functions: a mutant deleted for these residues (*rad6-149*) is nearly wild-type with regard to its DNA repair and cell proliferation phenotypes, but it is still unable to sporulate or to function in the N-end rule pathway (Morrison *et al.*, 1988; Madura *et al.*, 1993). We found that *rad6-149* cells display kinetics of Gcn4 degradation similar to that of *rad6Δ* cells (Figure 5D). These results suggest that the polyacidic tail of Rad6 is required for its function in Gcn4 degradation. Since Rad6 acts in conjunction with Ubr1, a yeast E3 protein, in the N-end rule pathway (Dohmen *et al.*, 1991; Sung *et al.*, 1991), we asked whether Gcn4 degradation is affected in a *ubr1* null mutant. We found that degradation of Gcn4 is identical in a *ubr1Δ* versus a *UBR1* strain (data not shown), suggesting that Rad6 acts on Gcn4 independently of the N-end rule pathway.

Mutational analysis of Gcn4 indicated that residue Thr105 plays a role in Gcn4 degradation (Figure 3). It is possible that this residue is involved in the recognition of Gcn4 by Cdc34 or Rad6. If so, one would expect little or no difference in degradation of Gcn4 wild-type versus Gcn4 T105A in the *cdc34ts* and *rad6Δ* mutants. Alternatively, if the degradation element defined by T105A is independent of Cdc34 or Rad6, an additive effect would be expected. In fact, degradation of Gcn4 wild-type and Gcn4 T105A is very similar in both *cdc34ts* cells (Figure 5E) and *rad6Δ* cells (Figure 5F), suggesting that Thr105 is part of the sequence recognized by both Cdc34 and Rad6.

Rad6 and Cdc34 ubiquitinate Gcn4 in vitro

Full understanding of the degradation pathway of Gcn4 requires reconstitution of the pathway *in vitro*. As a first step in this direction, we asked whether Gcn4 could be ubiquitinated by the purified Cdc34 and Rad6 proteins. The ubiquitin conjugating system and a GST−Gcn4 fusion protein were prepared as described in Materials and methods. Rad6 and, to a smaller extent, Cdc34 were both able to ubiquitinate the GST−Gcn4 fusion protein (but not the GST protein by itself; data not shown), albeit inefficiently (Figure 6A). The Rad6-149 derivative was unable to ubiquitinate Gcn4 *in vitro*, in agreement with its inability to complement the Gcn4 degradation defect of a *rad6* deletion *in vivo*. Similarly, the Ubc4 protein, which is not involved in degradation of Gcn4 *in vivo*, is unable to ubiquitinate Gcn4 *in vitro*. These results indicate that the purified system maintains the specificity of the *in vivo* reaction. Addition of a partially purified yeast cytosolic fraction ('E3'; see Materials and methods) to the *in vitro* ubiquitination reaction greatly increased ubiquitination by both Rad6 and Cdc34 (Figure 6A). Addition of a different fraction containing E3 activity, previously characterized as E3-R (Sharon *et al.*, 1991), also enhanced Rad6 activity on Gcn4 (Figure 6B).

Amino acid starvation stabilizes Gcn4

Gcn4 synthesis is induced by severe amino acid limitation, usually achieved using amino acid analogs or auxotrophic strains (Hinnebush, 1992). Given the short half-life of the protein, stabilization of Gcn4 could constitute another way of increasing its steady-state levels. Therefore, we assayed Gcn4 turnover in a *leu2* auxotrophic mutant after the strain was shifted from synthetic complete medium containing all amino acids (SC) to synthetic minimal medium (SD). This starvation protocol stabilized Gcn4 when the cells were transferred either to SD medium (Figure 7A and B) or SC medium lacking leucine (not shown), but not when the cells were shifted to SD medium plus leucine (Figure 7A and B), indicating that removal of the required amino acid is necessary. A time-course analysis of the response indicated that the full extent of the stabilization was reached within 30 min after the shift (not shown). The specificity of the Gcn4 stabilization was tested by assaying, under identical conditions, the turnover of two other previously characterized substrates of the ubiquitin pathway, Matα2(deg1)−LacZ (Hochstrasser and Varshavsky, 1990) and the N-end rule substrate Arg−LacZ (Bachmair *et al.*, 1986). Starvation had almost no effect on the proteolysis of Matα2(deg1)−LacZ and actually increased the rate of Arg−LacZ degradation (Figure 7C and D).

Regulation of Gcn4 by amino acid starvation is thought to be mediated by an increased concentration of uncharged tRNA, which activates the kinase Gcn2 (Hinnebush, 1992). Indeed, strains carrying a temperature-sensitive mutation in the $tRNA^{Ile}$ synthetase gene *ILS1*, which results in increased levels of uncharged $tRNA^{Ile}$, display increased expression of *GCN4* (Niederberger *et al.*, 1983). We tested whether Gcn4 protein stability was increased in the *ils1-1* mutant. Our results show that Gcn4 is stabilized in the *ils1-1* mutant at the non-permissive temperature (Figure 7E), indicating that uncharged tRNA probably constitutes the primary signal for the stabilization of Gcn4 as well. However, Gcn2 does not appear to be involved in the stabilization of Gcn4, as Gcn4 is stable in a *gcn2* (null) strain starved for an amino acid (data not shown).

Discussion

The stabilization of Gcn4 by starvation constitutes an example of regulated degradation as a mechanism for

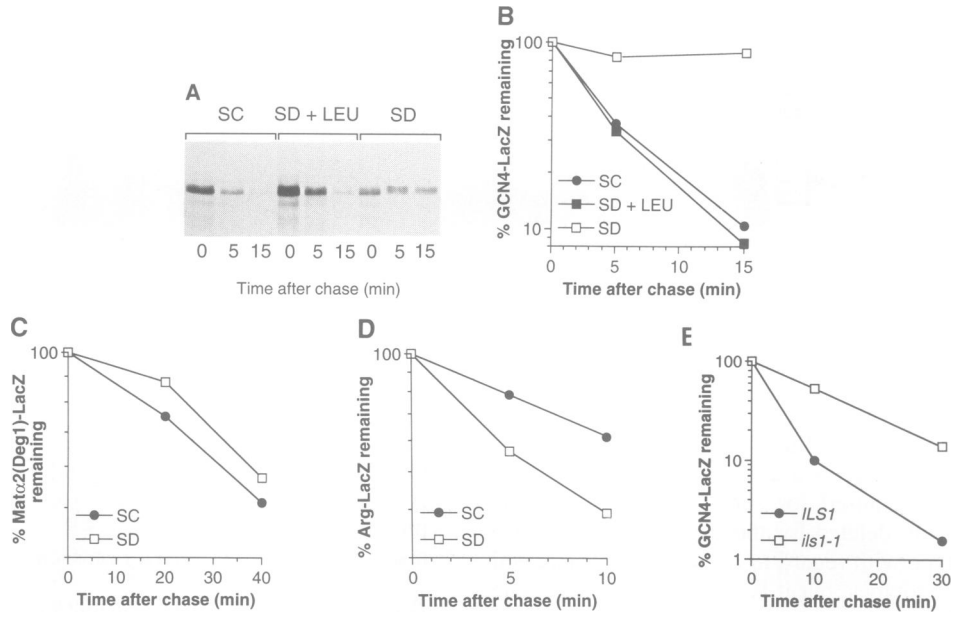

Fig. 7. Gcn4 is stabilized under amino acid starvation. (**A**) Pulse–chase experiment with GCN4–LacZ in minimal (SD) versus complete (SC) medium or in minimal medium + leucine (SD + LEU). (**B**) Quantitation of the experiment shown in (A). (**C**) Degradation of Matα2(deg1)–LacZ under starvation conditions. (**D**) Degradation of Arg–LacZ under starvation conditions. (**E**) Degradation of GCN4–LacZ in an *ils1-1* strain at 37°C.

controlling the activity of a eukaryotic transcription factor. To understand the mechanism of this regulation, we searched for *cis*- and *trans*-acting factors responsible for Gcn4 degradation in rich medium. Two specific ubiquitin conjugating enzymes, Cdc34 and Rad6, are required for Gcn4 degradation *in vivo* and are able to ubiquitinate Gcn4 *in vitro*. Our finding that a sequence in Gcn4 rich in 'PEST' residues is required for its rapid turnover supports the importance of these residues for protein degradation. Our results suggest that this sequence is required for the ubiquitination of Gcn4 by Cdc34 and Rad6, thus providing a link between the ubiquitin system and the degradation of 'PEST'-type proteins.

Sequences in Gcn4 required for degradation

A random screen for mutations that stabilize Gcn4 repeatedly uncovered mutations of residue Thr105. This residue is embedded in a stretch of eight 'PEST'-type residues extending from positions 99 to 106 (SSSTDSTP). Since sequences rich in these residues have been suggested to function as degradation signals (Rogers *et al.*, 1986; Rechsteiner, 1988), we tested the importance of these residues for the degradation of Gcn4 by converting each of them to alanine. Some of the residues were clearly important (SSATDSTP and SSSTDSAP were much more stable than the wild-type); other mutations in the same sequence did not affect degradation (e.g. SASTDSTP or SSSTASTP). These results, while not excluding the importance of a high proportion of 'PEST' residues for the destabilizing activity of a sequence, suggest the existence of sequence specificity in the recognition of 'PEST' regions by the degradation apparatus.

The individual residues identified genetically as being important for degradation, at positions 101, 105 and 106, are immediately adjacent to the core of the Gcn4 activation domain (Hope *et al.*, 1988; Van Hoy *et al.*, 1993). Our findings therefore suggest that, in addition to its function in the activation of transcription, this segment is also involved in the regulation of the stability of Gcn4. Furthermore, point mutations in this segment not only stabilize the protein, but also lead to dramatic increases in *HIS4* transcription (Figure 3B). This increased transcription is at least in part a consequence of the stabilization of Gcn4, although we cannot exclude the possibility that these mutations also increase the specific transcriptional activity of Gcn4 because of their proximity to the activation domain.

The ubiquitin system ligates ubiquitin via an isopeptide bond to an internal lysine residue on the target protein. Since we had evidence that ubiquitination was involved in Gcn4 degradation, we attempted to identify the ubiquitinated lysine by site-directed mutagenesis. However, no single lysine was found to be essential for degradation and even a number of multiple substitutions had no effect on degradation. Only when five or more lysine residues in the vicinity of the 99–106 sequence required for degradation were mutated could a significant increase in half-life be detected. These results suggest that a number of lysines in that region of Gcn4 can function as alternative ubiquitin acceptors. In two other instances where similar analyses were performed, the N-end rule substrate X-LacZ (Chau *et al.*, 1989) and the proto-oncogene product Mos (Nishizawa *et al.*, 1993), two and one lysine respectively were required for ubiquitination. In both cases these residues were the only lysines in the proximity of the degradation signal (at least at the primary sequence level). Taken together, these observations support the suggestion that the ubiquitin accepting residue is stochastically selected and therefore the relative importance of a particular lysine increases with proximity to the primary signal recognized by the ubiquitination machinery (Varshavsky, 1992).

The degradation pathway of Gcn4

The finding that both Cdc34 and Rad6 are required for normal Gcn4 degradation *in vivo*, taken together with the

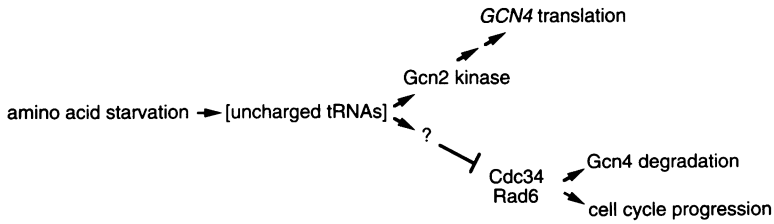

Fig. 8. Working model of the signal transduction pathway leading from amino acid starvation to Gcn4 stabilization and G_1 arrest.

observation that these enzymes are able to ubiquitinate Gcn4 *in vitro*, provides strong evidence for the direct role of these proteins in ubiquitination of Gcn4. The accumulation of different species of Gcn4 in the *cdc34ts* and *rad6*Δ mutants suggests that these two enzymes define two independent pathways of Gcn4 degradation (Figure 5B). However, a few additional observations need to be reconciled with this simple model. First, no further stabilization was observed in the double *cdc34ts rad6*Δ mutant as compared with the single *cdc34ts* mutant (Figure 5C). Second, the kinetics of degradation of Gcn4 in the *rad6*Δ mutant are unusual: in the absence of Rad6, newly synthesized Gcn4 is initially stabilized, but in a second phase (after about 10 min), degradation reaches wild-type levels again. In the presence of cycloheximide, in contrast, degradation is further inhibited in this second phase. One possible explanation for the biphasic degradation in *rad6*Δ cells is that in this mutant, Gcn4 needs to be transported to a different compartment in order for degradation to proceed. Either degradation in that compartment, or the transport itself, might be cycloheximide-sensitive. This cycloheximide-sensitive step is only detectable in *rad6*Δ cells and does not necessarily reflect the normal degradation pathway in wild-type cells. Indeed, *rad6*Δ cells are known to have increased activity of at least one other ubiquitin conjugating enzyme, Ubc4 (Dohmen *et al.*, 1991). Alternative degradation pathways or ubiquitin conjugating enzymes induced in the *rad6*Δ cells could be responsible for the second phase of Gcn4 degradation in these mutant cells. Similarly, induction of alternate degradation pathways by the *rad6* deletion could be responsible for the lack of an additive effect observed in the *cdc34ts rad6*Δ double mutant.

Our results demonstrate that the ubiquitin pathway is required for the degradation of a 'PEST'-containing protein, Gcn4. Matα2, the only other naturally occuring protein where specific Ubcs required for degradation have been identified, requires Ubc4, Ubc5, Ubc6 and Ubc7, but not Rad6 or Cdc34 (Chen *et al.*, 1993). Matα2 contains two short 'PEST' sequences, but it is not known whether these sequences are required for the degradation of the protein (Hochstrasser and Varshavsky, 1990). Furthermore, many of the substrates ubiquitinated *in vitro* by Cdc34 and Rad6 in the presence of E3-R have 'PEST' sequences (Sharon *et al.*, 1991; Raboy and Kulka, 1994). Taken together with our work on Gcn4, these data raise the possibility that the Ubcs Rad6 and Cdc34 are specific for 'PEST'-type proteins.

The absence of known physiological substrates of the Cdc34 and Rad6 enzymes had until now restricted the study of their activity to model substrates *in vitro* (Haas *et al.*, 1991; Sharon *et al.*, 1991). The availability of Gcn4 as a physiological substrate of these enzymes allowed us to examine their requirement for substrate recognition in a purified system. We found that the *in vitro* system consisting only of the substrate Gcn4, the E1 activating enzyme and the E2 conjugating enzymes Cdc34 or Rad6 is sufficient to achieve specific ubiquitination. The *in vivo* requirement of the polyacidic tail of Rad6 is maintained in this system. This demonstrates that at least part of the specificity of these enzymes towards Gcn4 does not require any additional factors.

The activity of these enzymes towards Gcn4 can be enhanced in the presence of two different yeast cytosolic fractions, E3-R and 'E3'. It remains to be established whether the same factor is responsible for the activity of the E3-R fraction on model substrates (Sharon *et al.*, 1991) and on Gcn4 and for the activity of the 'E3' fraction on Gcn4. Furthermore, since Cdc34 is less active *in vitro* than Rad6 (Figure 6), whereas *in vivo* its activity appears to be more important (Figure 5), it is likely that Cdc34-specific factors exist that remain to be identified.

Even in the presence of the enhancing fractions, only mono-ubiquitinated Gcn4 was detected, although poly-ubiquitinated Gcn4 is detectable *in vivo* (Figure 4A) and multi-ubiquitin chains are thought to be required for the function of ubiquitin in protein degradation (Chau *et al.*, 1989; Deveraux *et al.*, 1994). The factor(s) responsible for the polyubiquitination of Gcn4 (possibly a Ubc specific for ubiquitin or ubiquitinated substrates rather than for Gcn4) therefore remains to be identified.

Regulation of the degradation of Gcn4

The finding that Cdc34 and Rad6 are involved in the degradation of a regulator of amino acid biosynthesis is surprising, because *CDC34* was first identified as a gene required for cell cycle progression (Goebl *et al.*, 1988) and *RAD6* was mainly characterized as a gene required for DNA repair (Haynes and Kunz, 1981) (although *rad6*Δ cells have many phenotypes, including a cell cycle defect under certain conditions; Ellison *et al.*, 1991). We also noted that the physiological conditions found to stabilize Gcn4 (amino acid starvation) have the effect of arresting the cell cycle in G_1 (Unger and Hartwell, 1976). Therefore, one hypothesis to account for these observations is that Gcn4 is degraded in a cell cycle-dependent fashion and its stabilization in the *cdc34* and *rad6* mutants and under amino acid starvation is an indirect consequence of cell cycle arrest. However, we believe this to be unlikely because: (i) stabilization of Gcn4 under all the conditions observed occurs well before cell cycle arrest; (ii) Gcn4 degradation in wild-type and *cdc34ts* cells is unaffected by prior cell cycle arrest with mating pheromone (D.Kornitzer and G.R.Fink, results not shown); and (iii)

Table I. List of yeast strains

Name	Genotype	Source
L4210	*MATa ura352 leu2-Δ2 bas1-2 bas2-2*	our collection
KY26	L4210 *gcn4-Δ1*	this work
KY130	L4210 *gcn4-Δ1 ade8-GCN4*	this work
KY203	KY130 *cdc34-2*	this work
KY206	KY130 *rad6-Δ1*	this work
KY211	KY130 *cdc34-2 rad6-Δ1*	this work
KY218	KY130 *ubr1-Δ1*	this work
KY228	*MATa ura3-52 ILS1*	Our collection
KY240	*MATα ura3-52 ils1-1*	this work
YPH500	*MATα ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1*	P.Hieter
cim5	YPH500 *cim5-1*	(Ghislain *et al.*, 1993)
BBY62	*MATa ura3-52 leu2-3,112 his3-Δ200 lys2-801 trp1-1 pep4::HIS3 ubr1-Δ1::LEU2*	B.Bartel

ubiquitination of Gcn4 by Cdc34 and Rad6 *in vitro* (Figure 6) suggests a direct involvement of these ubiquitin conjugating enzymes in Gcn4 degradation. We therefore favor the hypothesis that Gcn4 stabilization and cell cycle arrest are two parallel consequences of amino acid starvation: Cdc34 and Rad6 may be involved not only in the signal transduction pathway leading from amino acid starvation to Gcn4 stabilization, but could also mediate the effects of amino acid starvation on progression of the cell cycle (Figure 8).

Materials and methods

Plasmids and strains

GCN4 was placed under control of the *GAL1* promoter by cloning the *Hin*dIII–*Eco*RI fragment of YCp88-GCN4 (Hope and Struhl, 1986) in vector pDAD1 (kindly provided by D.Miller and D.Pellman), to generate pGAL-GCN4. The two single myc epitopes of plasmid KB62 were introduced by site-directed mutagenesis. For the GCN4–LacZ fusion, PCR methods were used (Ausubel *et al.*, 1989) to clone a DNA fragment carrying the complete *GCN4* coding sequence under the *GAL1,10* promoter from plasmid pGAL-GCN4 in vector YEp357R (Myers *et al.*, 1986), generating KB64. Plasmids KB73, KB77, KB78, KB92, KB94 were built by introducing the appropriate *GCN4* deletions described by Hope and Struhl (1986) into KB64; KB80, KB296 were independently constructed by PCR cloning using appropriate primers, as were KB430 and KB441. KB129 was constructed by introducing the appropriate *Bam*HI–*Bgl*II *GCN4* fragment generated by PCR into the *Bam*HI site of pLGSD5. To construct KB105, PCR methods were used to fuse the promoter fragment of *ADE8* (White *et al.*, 1985) from the *Bgl*II site, up to the third codon of the coding sequence, to a *GCN4* fragment extending from the second codon up to the 197th nucleotide 3′ to the coding sequence. This gene fusion was cloned in plasmid pRS315 (Sikorski and Hieter, 1989). Oligonucleotide-directed mutagenesis (Ausubel *et al.*, 1989) was used to create the point mutations in plasmids KB117, KB196-KB203, KB341-KB344, KB357, KB358 and the short deletion in KB162 and KB195.

Isogenic *cdc34ts* and *rad6Δ* strains were constructed by introducing the *cdc34-2* allele and the *rad6* deletion into strain KY130 by homologous recombination. The *cdc34-2* allele was cloned from a mutant strain into an integrative vector, sequenced and found to carry a Gly→Ala substitution at codon 58 (D.Kornitzer and G.R.Fink, unpublished results). Congenic *ILS1* and *ils1-1* strains were constructed by back-crossing the *ils1-1* allele three times to strain KY228, generating KY240.

Selection of GCN4 stability mutants

A *GCN4* construct carrying a mutation in the leucine zipper which reduces the transcriptional activity of the protein without reducing its stability (N264L; D.Kornitzer and G.R.Fink, unpublished observations) was placed under control of the weak *ADE8* promoter of plasmid KB117. Since KY26 cells carrying this plasmid are phenotypically His^- at 36°C, second-site revertants of KB117 to His^+ could be isolated at that temperature. To prevent the isolation of true revertants, a DNA fragment encoding only the first 246 amino acids of Gcn4 was isolated and mutagenized by PCR amplification in the presence of 0.5 mM $MnCl_2$ (Leung *et al.*, 1989). Sequencing of seven revertants showed that the heavy mutagenesis had produced from two to eight changes in each of these Gcn4 derivatives. Five revertants had a mutation at Thr105; this single mutation, when isolated from the other changes by cloning and site-directed mutagenesis, fully accounts for the phenotype of these mutants. One revertant had mutations in both Ser99 and Ser104, which probably account for its phenotype. The seventh revertant carried eight mutations spread over the length of the protein; this mutant was not analyzed further. The effects of these second-site mutations on the activity and stability of Gcn4 were further tested in constructs lacking the original leucine zipper mutation and were found to be independent of that mutation.

HIS4 expression assay

Transcriptional activity of GCN4 was measured by assaying β-galactosidase activity, as described (Daignan-Fornier and Fink, 1992), in strain KY26 containing a *HIS4–LacZ* fusion under the control of the *HIS4* promoter (plasmid pFN6; Nagawa and Fink, 1985) and the GCN4 constructs expressed as a fusion to the *ADE8* promoter on plasmid KB105. In strain KY26, which lacks Bas1–Bas2 and Gcn4 activity, *HIS4* transcription is entirely dependent on the plasmid-borne *GCN4* allele (Arndt *et al.*, 1987).

Pulse–chase assays

SC and SD medium were as described (Sherman *et al.*, 1986). Overnight cultures grown in SC − uracil, − methionine, + raffinose medium were diluted and grown for 6 h in SC − uracil, − methionine, + galactose, concentrated by centrifugation, pulse-labeled for 5 min with 500–700 μCi Expre^{35}s^{35}s (NEN), pelleted again and chased in SC + galactose containing 10 mM methionine (and 0.5 mg/ml cycloheximide where indicated). For the experiments involving temperature-sensitive mutants, the cells were shifted from 30 to 37°C 30–60 min before labeling. Similarly, for the starvation studies, cells were harvested by centrifugation and grown in SD medium (+ adenine where required) for 30–60 min before labeling and chased in the same medium + 10 mg/ml methionine. For *CUP1* promoter induction, 0.1 mM $CuSO_4$ was added 6–8 h before labeling. At various times of the chase, an aliquot of the culture was removed and incubated for 15 min on ice with 0.35 M NaOH, 1.5% 2-mercaptoethanol, followed by precipitation with 6% trichloroacetic acid. The protein precipitate was resuspended by boiling in 2.5% SDS and equal amounts of TCA-precipitable radioactivity were immunoprecipitated using either anti-β-galactosidase antiserum (Cappel) or anti-myc epitope monoclonal antibodies (Kolodziej and Young, 1991) in at least 10 volumes of buffer A (Hochstrasser and Varshavsky, 1990) containing protein A–Sepharose (Pharmacia). The immunoprecipitates were run on SDS–polyacrylamide gels and the protein bands were quantitated using a Fujix Bas 2000 bio-image analyzer (Fuji).

Preparation of GST–GCN4, E1, E2's, E3-R and 'E3' fraction

E1 and E3-R were prepared according to Sharon *et al.* (1991). Rad6, Rad6-149 and Cdc34 were prepared as described previously (Raboy and Kulka, 1994). GST–GCN4 was prepared from *Escherichia coli* cells transformed with plasmid KB430 using the Pharmacia GST protein fusion system according to the manufacturer's instructions.

To prepare the 'E3' fraction, BBY62 cells grown in YEPD were harvested at mid-log phase. The pellet was suspended in an equal volume of buffer containing 50 mM Tris–HCl pH 7.5, 1 mM DTT, 5 mM EDTA and 0.5 mM PMSF and the cells were broken with glass beads

Table II. List of plasmids

Name	Markers	Source
pDAD1	2 micron, *URA3, GAL1-10* promoter	D.Pellman
pGAL-GCN4	*GCN4* under *GAL1-10* promoter in pDAD1	this work
pLGSD5	2 micron, *URA3*, lacZ under *CYC1* promoter	L.Guarente
KB62	pGAL-GCN4 with single myc epitopes after residues 4 and 192 of GCN4	this work
KB 183	pGAL-GCN4 with single myc epitope after residue 192 of GCN4	this work
KB184	*GCN4(T105A)* in KB183	this work
KB64	full-length *GCN4-LacZ* under *GAL1-10* promoter	this work
KB73	*GCN4(95-281)* in KB64	this work
KB77	*GCN4(141-281)* in KB64	this work
KB78	*GCN4(118-281)* in KB64	this work
KB80	*GCN4(1-221)* in KB64	this work
KB92	*GCN4(Δ93-140)* in KB64	this work
KB94	*GCN4(Δ118-140)* in KB64	this work
KB162	*GCN4(Δ 99–106)* in KB64	this work
KB296	*GCN4(1-157)* in KB64	this work
KB129	*GCN4(81-172)* in pLGSD5	this work
KB343	*GCN4(K140, 172, 178, 180, 181, 187, 188, 194, 211 to R)* in KB64	this work
KB344	*GCN4(K 81, 118, 140, 172, 178, 180, 181, 187, 188, 194, 211 to R)* in KB64	this work
KB357	*GCN4(S101A)* in KB64	this work
KB358	*GCN4(T105A)* in KB64	this work
KB105	*ade8-GCN4* in *LEU2, CEN* vector	this work
KB117	*GCN4 (N264L)* in KB105	this work
KB195	*GCN4(Δ99-106)* in KB105	this work
KB196–203	*GCN4(N99-106A)* in KB105	this work
KB341	*GCN4(K140, 172, 178, 180, 181, 187, 188, 194, 211 to R)* in KB105	this work
KB342	*GCN4(K 81, 118, 140, 172, 178, 180, 181, 187, 188, 194, 211 to R)* in KB105	this work
KB227	Ub–ARG–LacZ	(Bachmair *et al.*, 1986)
KB441	*Matα2(1-72)–LacZ* under *GAL1-10* promoter	this work
yEp96Δ	2 micron *TRP1 CUP1* promoter	(Ellison and Hochstrasser, 1991)
yEp96	2 micron *TRP1, UBI* under *CUP1* promoter	(Ellison and Hochstrasser, 1991)
yEp105	2 micron *TRP1, myc-UBI* under *CUP1* promoter	(Ellison and Hochstrasser, 1991)
pR67	*RAD6* in yCp50	(Morrison *et al.*, 1988)
pR615	*rad6-149* in yCp50	(Morrison *et al.*, 1988)
KB452	*RAD6* in *LEU2 CEN* vector	(Morrison *et al.*, 1988)
KB453	*rad6–149* in *LEU2 CEN* vector	this work
KB430	*GCN4* fused to *GST* in pGEX-2TK vector (pharmacia)	this work
pFN6	*HIS4–LacZ* in *URA3 CEN* vector	(Nagawa and Fink, 1985)

by vortexing. The extract was centrifuged for 30 min at 20 000 *g* and fractionated by ammonium sulfate precipitation. A fraction precipitating at a 50–60% saturated ammonium cut-off was further fractionated by chromatography on a DEAE cellulose (Whatman DE52) column equilibrated with 3 mM phosphate buffer, pH 7, containing 1 mM DTT and 0.5 mM PMSF. Proteins were eluted with successive steps of 1.5 column volumes each of 100, 200, 300, 400 and 500 mM KCl in 20 mM Tris–HCl buffer, pH 7.2, containing 1 mM DTT and 0.5 mM PMSF. 'E3' activity was eluted with 300 mM KCl.

Ubiquitin conjugation assay

The standard assay system for ^{125}I-labeled ubiquitin conjugation contained (in 12.5 μl) 50 mM Tris–HCl, pH 7.5, 5 mM $MgCl_2$, 2 mM DTT, 2 mM ATP, 10 mM creatine phosphate, 2.5 μg creatine kinase, 0.25 mM PMSF, 10 μg each of the following protease inhibitors, leupeptin, pepstatin, chymostatin, bestatin and antipain, 7 pmol ^{125}I-labeled ubiquitin (2×10^6 c.p.m.), 0.2 pmol E1, 0.2 pmol E2, 5 μg GST–Gcn4 and the E3-containing fraction as indicated. After incubation for 30 min at 30°C, 20 μl of 50% (v/v) glutathione–agarose beads (Sigma; G-4510) were added. The reaction mixture was incubated at room temperature for 10 min with periodic mixing. The beads were pelleted by centrifugation, washed twice with 10 volumes of phosphate-buffered saline and the GST–Gcn4 protein was eluted by heating in a boiling water bath for 5 min with 20 μl of SDS electrophoresis sample buffer. The ^{125}I-labeled ubiquitin-conjugated GST–Gcn4 was detected by SDS–PAGE on 10% Laemmli gels followed by autoradiography.

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