

A single amino acid substitution in yeast eIF-5A results in mRNA stabilization

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Most factors known to function in mRNA turnover are not essential for cell viability. To identify essential factors, ~4000 temperature-sensitive yeast strains were screened for an increase in the level of the unstable *CYH2* pre-mRNA. At the non-permissive temperature, five mutants exhibited decreased decay rates of the *CYH2* pre-mRNA and mRNA, and the *STE2*, *URA5* and *PAB1* mRNAs. Of these, the mutant *ts1159* had the most extensive phenotype. Expression of the *TIF51A* gene (encoding eIF-5A) complemented the temperature-sensitive growth and mRNA decay phenotypes of *ts1159*. The *tif51A* allele was rescued from these cells and shown to encode a serine to proline change within a predicted α -helical segment of the protein. *ts1159* also exhibited an ~30% decrease in protein synthesis at the restrictive temperature. Measurement of amino acid incorporation in wild-type cells incubated with increasing amounts of cycloheximide demonstrated that a decrease in protein synthesis of this magnitude could not account for the full extent of the mRNA decay defects observed in *ts1159*. Interestingly, the *ts1159* cells accumulated uncapped mRNAs at the non-permissive temperature. These results suggest that eIF-5A plays a role in mRNA turnover, perhaps acting downstream of decapping.

Keywords: eIF-5A/mRNA decapping/mRNA turnover/protein synthesis/temperature-sensitive growth

Introduction

Differences in mRNA turnover rates can have significant effects on the expression of specific genes and provide a cell with flexibility in bringing about rapid changes in protein levels. The structures and mechanisms involved in the determination of individual mRNA decay rates have begun to be elucidated, particularly in yeast (Caponigro and Parker, 1996; Jacobson and Peltz, 1996). It is now well established that mRNA decay is a precise process dependent on a variety of specific *cis*-acting sequences and *trans*-acting factors. In addition, there is extensive evidence for an important role for translation in mRNA decay, including experiments demonstrating that: (i) inhibition of translational elongation can reduce mRNA decay rates (Peltz *et al.*, 1992; Beelman and Parker, 1994); (ii) instability elements can be localized to mRNA coding regions (Parker and Jacobson, 1990; Caponigro *et al.*,

1993; Hennigan and Jacobson, 1996; Jacobson and Peltz, 1996); (iii) the activity of some instability elements depends on ribosome translocation up to, or through the element (Parker and Jacobson, 1990; Peltz *et al.*, 1993a; Hennigan and Jacobson, 1996); (iv) some 3'-untranslated region (UTR) instability elements can influence mRNA translational activity (Kruys *et al.*, 1989; Marinx *et al.*, 1993); (v) factors involved in decay and the decay process are polysome-associated (Peltz *et al.*, 1993b; Caruccio and Ross, 1994; Atkin *et al.*, 1995, 1997; Zhang *et al.*, 1997); (vi) premature translational termination can enhance mRNA decay rates (Peltz *et al.*, 1993a,b); (vii) factors essential for the rapid decay of nonsense-containing mRNAs also regulate translational suppression (Leeds *et al.*, 1992; Cui *et al.*, 1996; Weng *et al.*, 1996); and (viii) metabolism of the poly(A) tail is a rate-limiting step in the decay of several mRNAs yet this structure and its binding protein also have a role in translational initiation (Jacobson, 1996; Sachs *et al.*, 1997).

The general outlines for at least three mRNA decay pathways have been delineated in the yeast *Saccharomyces cerevisiae*. In two of these pathways, mRNA decay proceeds from decapping to 5'→3' exonucleolytic digestion (Decker and Parker, 1993, 1994), whereas, in the third pathway, the predominant event is 3'→5' exonucleolytic digestion (Muhlrad *et al.*, 1995). The initiation of decapping is known to be promoted by shortening of the mRNA poly(A) tail or premature translational termination (Muhlrad and Parker, 1994; Muhlrad *et al.*, 1994). A number of *trans*-acting factors with demonstrated or implied roles in these pathways have been identified and include: a 5'→3' exonuclease (Xrn1p; Hsu and Stevens, 1993), the decapping enzyme (Dcp1p; Stevens, 1988; Beelman *et al.*, 1996; La Grandeur *et al.*, 1998), poly(A)-binding protein (Pab1p; Adam *et al.*, 1986; Sachs *et al.*, 1986), constituents of a poly(A) nuclease complex (Pan2p and Pan3p; Boeck *et al.*, 1996; Brown *et al.*, 1996), two proteins that regulate decapping (Mrt1p and Mrt3p; Hatfield *et al.*, 1996) and a number of factors that are required specifically for the decay of nonsense-containing mRNAs (Upf1p, Nmd2p/Upf2p and Upf3p; Peltz *et al.*, 1994; Cui *et al.*, 1995; He and Jacobson, 1995; Lee and Culbertson, 1995; Lee *et al.*, 1995; He *et al.*, 1997).

Only three of these mRNA decay factors (Pab1p, Mrt1p and Mrt3p) are encoded by essential genes (Adam *et al.*, 1986; Sachs *et al.*, 1986; Hatfield *et al.*, 1996). In contrast, many genes involved in other complex cell processes, such as transcription or translation, are essential. In order to identify other essential factors involved in mRNA decay, we screened a yeast library of ~4000 temperature-sensitive (*ts*) mutants for those that were incapable of degrading either wild-type or nonsense-containing mRNAs at the restrictive temperature. We have identified several mutants in which a shift to the non-permissive temperature

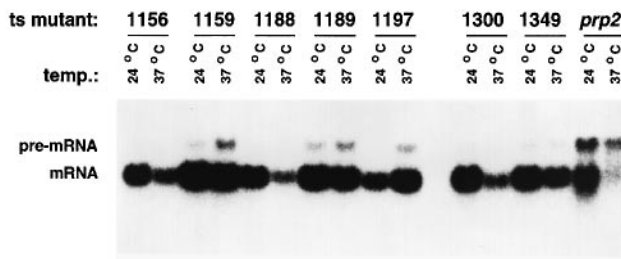


Fig. 1. *CYH2* pre-mRNA levels increase in some mutants grown at 37°C. RNA was prepared from cells of the indicated ts mutant strains grown at 24°C or shifted to 37°C as described in Materials and methods. Northern blots containing 15 µg of RNA per lane were hybridized to a radiolabeled *CYH2* probe. pre-mRNA and mRNA depict the respective *CYH2* transcripts.

promotes mRNA stabilization and report here on the ts strain displaying the most extensive impairment of mRNA decay. That strain, ts1159, was found to harbor a mutation in *TIF51A*, the gene encoding eIF-5A.

Results

Identification of temperature-sensitive strains that selectively accumulate the unstable *CYH2* pre-mRNA

The collection of yeast strains used in this study was generated by J. Abelson and colleagues in a study of ts mutants defective in pre-mRNA splicing. The yeast strain SS330 was treated with ethylmethylsulfonate (EMS), and ~4000 mutants capable of growth at 24°C, but not at 37°C, were isolated (Vijayraghavan *et al.*, 1989; J. Abelson, personal communication). Northern blots of RNA isolated from each mutant after a 2 h shift to the non-permissive temperature were then hybridized to an *ACT1* probe. This led to the identification of a number of new splicing mutants that accumulated the *ACT1* pre-mRNA at 37°C (Ruby and Abelson, 1991).

The original collection of blots was rescreened to identify mutants in which mRNA was stabilized at 37°C. Earlier work had shown that endogenous substrates of the nonsense-mediated mRNA decay pathway include inefficiently spliced pre-mRNAs that enter the cytoplasm (such as the *CYH2* pre-mRNA; He *et al.*, 1993). Mutants incapable of nonsense-mediated mRNA decay, i.e. strains containing deletions of the *UPF1*, *NMD2* or *UPF3* genes, lead to the stabilization and selective accumulation of such RNAs (He *et al.*, 1993; Peltz *et al.*, 1994; He and Jacobson, 1995) and we expected that strains with ts lesions in this pathway would have related phenotypes. The blots were thus hybridized with probes for *CYH2* and also for *ACT1* transcripts (the latter to minimize the chance of isolating splicing mutants). We identified 96 mutants that accumulated the *CYH2* pre-mRNA, but not the *ACT1* pre-mRNA, at the restrictive temperature (data not shown).

The RNA in the original collection of Northern blots had been prepared only from cells that had been shifted to the non-permissive temperature for 2 h. In order to directly compare the levels of the *CYH2* transcripts in these cells before and after the change in temperature, we prepared RNA from aliquots of these strains grown at 24°C or incubated at 37°C for 45 min. Northern blots of these RNA preparations were probed with radiolabeled *CYH2* DNA. Figure 1 shows an example of these blots

including seven ts mutants and a splicing mutant (*prp2*) control. Three mutants, ts1159, ts1189 and ts1197, exhibited an increase in the level of the *CYH2* pre-mRNA after the shift to the non-permissive temperature and were thus chosen for additional testing. Nine other strains (ts817, ts942, ts970, ts1100, ts1387, ts1581, ts3753, ts3766 and ts3771) were chosen from additional blots by the same criteria (data not shown).

Identification of mutants with temperature-sensitive defects in mRNA decay

Increases in *CYH2* pre-mRNA abundance could be attributable to changes in the rates of decay or synthesis of that transcript. To distinguish between these possibilities for the selected mutants, and to characterize their mRNA decay defects further, we analyzed the kinetics of turnover of a number of mRNAs in these strains after a shift to the non-permissive temperature. The cells were grown at 24°C to mid-log phase and shifted to 37°C for 1 h. Transcription was then inhibited by the addition of thiolutin (Jimenez *et al.*, 1973). RNA was isolated 0, 5, 10, 20 and 35 min after inhibition of transcription and analyzed by Northern blotting, using probes for five transcripts (Herrick *et al.*, 1990). These represented different classes of transcripts found in the cell and included the *CYH2* pre-mRNA and the *CYH2*, *STE2*, *PAB1* and *URA5* mRNAs. The *CYH2* pre-mRNA, as noted above, is a substrate of the nonsense-mediated mRNA decay pathway, the *STE2* and *URA5* mRNAs have short half-lives (<7 min) and in this strain, the *PAB1* and *CYH2* mRNAs are moderately stable transcripts (Herrick *et al.*, 1990).

Seven of the mutant strains tested in this manner (ts970, ts1197, ts1387, ts1581, ts3753, ts3766 and ts3771) did not show a defect in the decay rates of the mRNAs tested. They were thus deemed not to be mRNA-turnover mutants and were not characterized further (data not shown). The remaining mutants (ts817, ts942, ts1100, ts1159 and ts1189) exhibited decreased decay rates of some or all of the transcripts tested when compared with the rates measured in the wild-type parental strain, SS330. Of the latter set of mutants, ts1159 exhibited the most substantial effects on mRNA decay, stabilizing some mRNAs up to 5-fold (Figure 2).

Identification of the gene mutated in ts1159

The ts growth phenotypes of the five strains that exhibited mRNA decay defects were complemented by a yeast genomic DNA library (cloned in the centromere vector YCp50; Rose *et al.*, 1987) and plasmids capable of inducing temperature-independent growth in each strain were identified. Transformation of ts1159 cells with the YCp50 library yielded three clones (p1159-1 to p1159-3) capable of growth at the restrictive temperature. Cells from the original ts1159 strain, or from that strain transformed with a complementing plasmid, were patched onto rich, synthetic complete (SC) or SC-ura plates, grown for 3 days at 24°C, and then replica-plated to two plates, one of which was incubated at 24°C and the other at 37°C, each for 3 days. Expression of the relevant YCp50 clones was shown to restore growth at 37°C to the mutant cells (Figure 3A). Moreover, growth at the higher temperature was shown in two ways to be plasmid dependent: (i) by

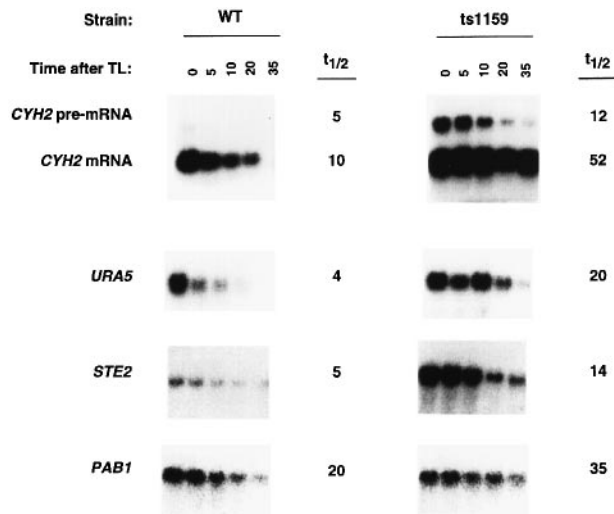


Fig. 2. mRNA half-lives are increased in ts1159 cells shifted to 37°C. Wild-type (SS330) and ts1159 cells were grown at 24°C, shifted to 37°C for 1 h, and then incubated with 4 µg/ml thiolutin (see Materials and methods). Northern blots were prepared from RNAs extracted from aliquots taken at the indicated time points thereafter (Time after TL) and hybridized to a series of radiolabeled probes (indicated to the left of the blots). The half-lives ($t_{1/2}$) of each transcript were calculated and are listed to the right of each panel.

isolation of the respective plasmids, retransformation into fresh mutant cells and verification of the complementation phenotype; and (ii) by growing complemented cells to saturation in non-selective media and then showing that all clones that had lost the plasmid also lost the ability to grow at 37°C (data not shown).

In order to verify that the ts growth phenotype was linked to the mRNA decay defect, ts1159 cells with and without a complementing plasmid were grown at 24°C and shifted to 37°C for 1 h. RNA prepared from these cells was analyzed by Northern blotting, using an *STE2* fragment as a hybridization probe. There is a 3.3-fold increase in the level of the *STE2* mRNA in ts1159 cells after a shift to the non-permissive temperature (Figure 3B), but this increase is not evident in cells harboring the complementing 1159-1 YCp50 plasmid. This indicates that the same plasmid which complemented the ts growth phenotype also complemented the mRNA decay defect.

Initial mapping of the three plasmids rescued from the complemented cells using the restriction enzymes *EcoRI* and *SaII* showed that they comprised two different plasmids (one represented twice, and the other once; data not shown). The vector-insert junctions of the two plasmids (1159-1 and 1159-3) were sequenced, compared with the available databases and found to contain overlapping fragments of chromosome V. The overlapping sequence included three open reading frames (ORFs) of unknown function (yEL033w, yEL035c and yEL038w), a serine tRNA gene, the *TIF51A* (*HYP2*) gene (which encodes the putative translation initiation factor eIF-5A; Kemper *et al.*, 1976; Benne *et al.*, 1978; Schnier *et al.*, 1991), the *ANP1* gene (which encodes an endoplasmic reticulum protein; Chapman and Munro, 1994) and the *RAD23* gene (involved in DNA repair; Miller *et al.*, 1982).

The ORFs contained in the complementing plasmids were then subcloned separately into the centromere vector pRS316 (Sikorski and Hieter, 1989), along with a substan-

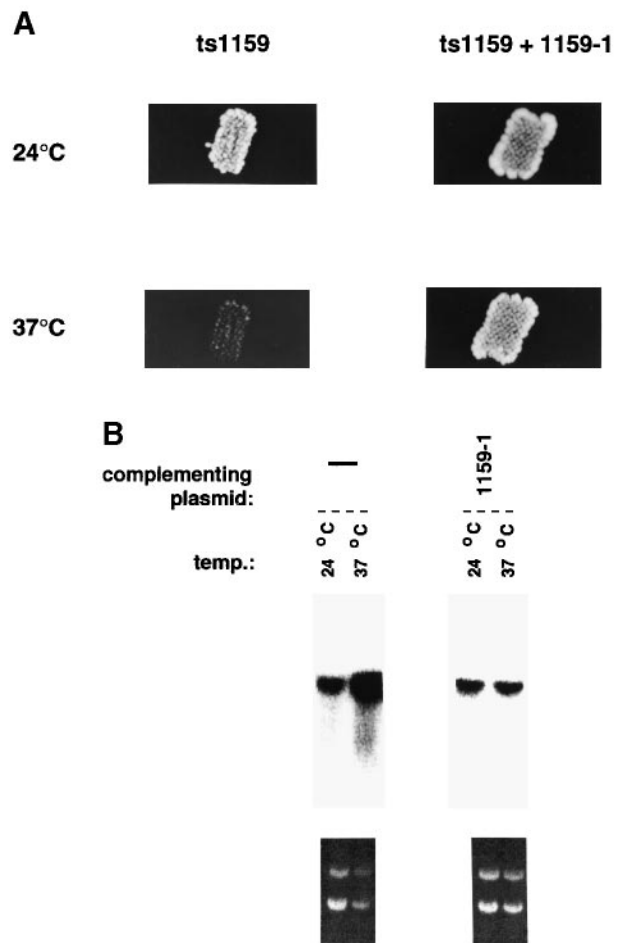


Fig. 3. A YCp50 library clone complements the ts growth and mRNA decay phenotypes of ts1159 at 37°C. (A) ts1159 cells were patched onto SC plates and grown at 24°C for 3 days. These were replica-plated to two SC plates, and grown at 24 and 37°C, respectively (left panels). A similar experiment (on SC-ura plates) was done with ts1159 cells transformed with the YCp50 plasmid 1159-1 (right panels). (B) ts1159 cells, with and without the 1159-1 plasmid, were grown at 24°C or shifted to 37°C for 1 h. RNA was isolated from the respective cells and analyzed by Northern blotting, using a radiolabeled *STE2* probe. The ethidium bromide-stained rRNAs, indicating the amount of RNA in each sample, are shown under each lane.

tial length of 5' and 3' sequences to ensure inclusion of the regulatory sequences flanking each gene. Each ORF construct was transformed into ts1159 cells and tested for complementation of the growth defect at 37°C. Only one plasmid (pDZ4), expressing the *TIF51A* ORF, was capable of complementing the growth of the mutant strain at the non-permissive temperature. ts1159 cells transformed either with the pRS316 vector alone or with the complementing *TIF51A* plasmid were patched onto SC-ura plates, grown for 3 days at 24°C, and then replica-plated to two plates, one of which was incubated at 24°C and the other kept at 37°C, each for 3 days. It is evident from the data shown in Figure 4A that the construct expressing *TIF51A* restored the growth at 37°C, while the vector alone could not complement the growth defect.

In order to ensure that the complementation of the ts growth phenotype and mRNA decay defect resulted from the same ORF on the original YCp50 clone, ts1159 cells transformed with the empty vector and with pDZ4 were grown at 24°C and shifted to 37°C for 1 h. RNA prepared

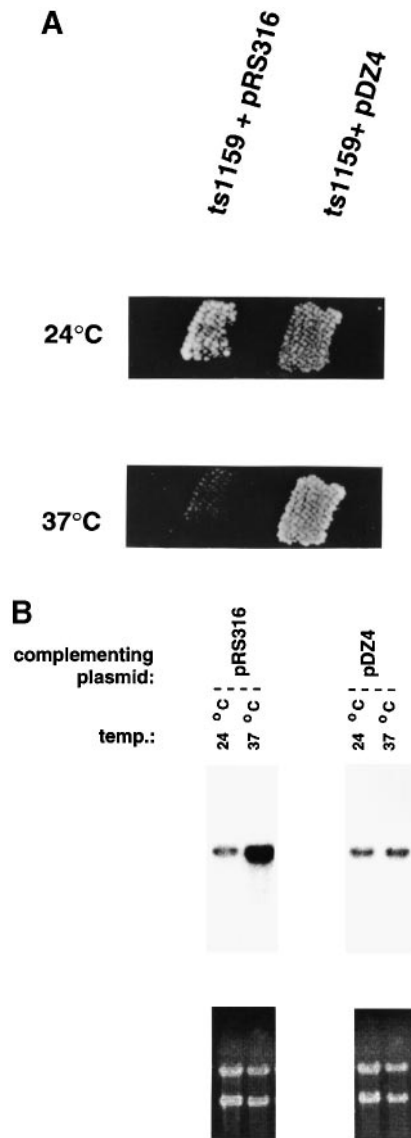


Fig. 4. A plasmid expressing *TIF51A* complements the ts growth and mRNA decay phenotypes of ts1159. Experiments similar to those described in Figure 3 were done with ts1159 cells transformed with pRS316 or with pRS316 containing *TIF51A* (pDZ4). (A) Growth at 24 and 37°C. (B) Northern blots of the cells grown at 24°C, and after a 1 h shift to 37°C, using a *STE2* fragment as a hybridization probe. The ethidium bromide-stained rRNAs, indicating the amount of RNA in each sample, are shown under each lane.

from these cells was again analyzed by Northern blotting. As can be seen in Figure 4B, there is a 3-fold increase in the level of the *STE2* mRNA in the ts1159 cells transformed with pRS316 after a shift to the non-permissive temperature (as was seen for the untransformed ts mutant in Figure 3B), but this increase does not occur in the cells harboring the plasmid expressing *TIF51A*. This indicates that both the growth and mRNA decay defects were complemented by expression of a single gene.

Comparable experiments identified the genes complementing the other four mutants (Table I). *SLA2* encodes a protein containing a talin-like domain, which previously was identified as being involved in membrane cytoskeletal assembly, endocytosis of α -factor and maintenance of the *PMA1* ATPase (Holtzman *et al.*, 1993; Raths *et al.*, 1993; Na *et al.*, 1995). *GRC5* (also called *QSR1* and *QMI*)

Table I. Complementation of ts mutants deficient in mRNA decay

ts mutant	Chromosome	Complementing ORF
ts817	XIV	<i>SLA2</i>
ts 942	XII	<i>GRC5</i>
ts1100	IX	<i>THS1</i>
ts1159	V	<i>TIF51A</i>
ts1189	XI	<i>MRT4</i>

The indicated ORFs plus enough flanking sequence to overlap with neighboring ORFs were cloned into the pRS316 vector and expressed in the relevant strain. Complementing = allowing growth at the non-permissive temperature.

encodes the 60S ribosomal subunit protein L9 (Dick *et al.*, 1997; Nika *et al.*, 1997), and *THS1* encodes the yeast threonyl-tRNA synthetase (Pape and Tzagoloff, 1985). *MRT4* (mRNA turnover; yKLO09w) is a previously uncharacterized gene which encodes a protein similar to the P0/L10 family of 60S ribosomal proteins. Here we report on the initial characterization of the ts1159 mutant and the *tif51A* allele found in that strain. Further studies of the other four mutants will be reported elsewhere.

Isolation of the mutated *tif51A* allele from ts1159

To identify the specific mutation in the ts1159 *tif51A* allele (which we designate *tif51A-1159*), we rescued the mutation by gap repair. The pDZ4 construct contains the 473 nucleotide *TIF51A* ORF plus 193 nucleotide upstream and 105 nucleotide downstream sequences in the *URA3*-based vector, pRS316 (see Materials and methods). We generated two types of pDZ4 plasmids containing 74 or 209 nucleotide gaps at the 3' end of the gene (with the latter encompassing the former). These plasmids were transformed into ts1159 cells, *ura*⁺ colonies were selected, and the repaired plasmids (which now included sequence from the chromosomal copy of *tif51A-1159*) were isolated.

Re-transformation of the wild-type and repaired alleles into fresh ts1159 cells showed that the repaired *tif51A-1159* allele [pDZ4(g-r)] could no longer complement the ts growth phenotype of the ts1159 mutant when compared with the complementation observed in cells transformed with the original *TIF51A* gene (Figure 5A). In order to test for complementation of the mRNA turnover phenotype, cells harboring the pDZ4 wild-type allele, the repaired plasmid or no plasmid were grown at 24°C, shifted to 37°C for 1 h and then tested for levels of *STE2* mRNA by Northern blotting. The results in Figure 5B reiterate those shown in Figure 4B, i.e. the ~3-fold increase in *STE2* mRNA levels in the ts1159 cells at 37°C is abrogated by the expression of the wild-type *TIF51A* allele (pDZ4). However, this increase is not reversed by expression of the gap-repaired pDZ4(g-r) construct.

Sequencing of the gap-repaired plasmids revealed a single T→C change at position 445 of the *TIF51A* ORF (Figure 5C), which resulted in a serine to proline change at position 149 of the eIF-5A protein. The remaining chromosomal sequence in the repaired gap is the same as in the construct expressing the wild-type *TIF51A* allele, as is the sequence in the rest of the pDZ4 insert. Therefore, we conclude that the lack of complementation of both the growth and mRNA decay phenotypes in the ts1159 cells

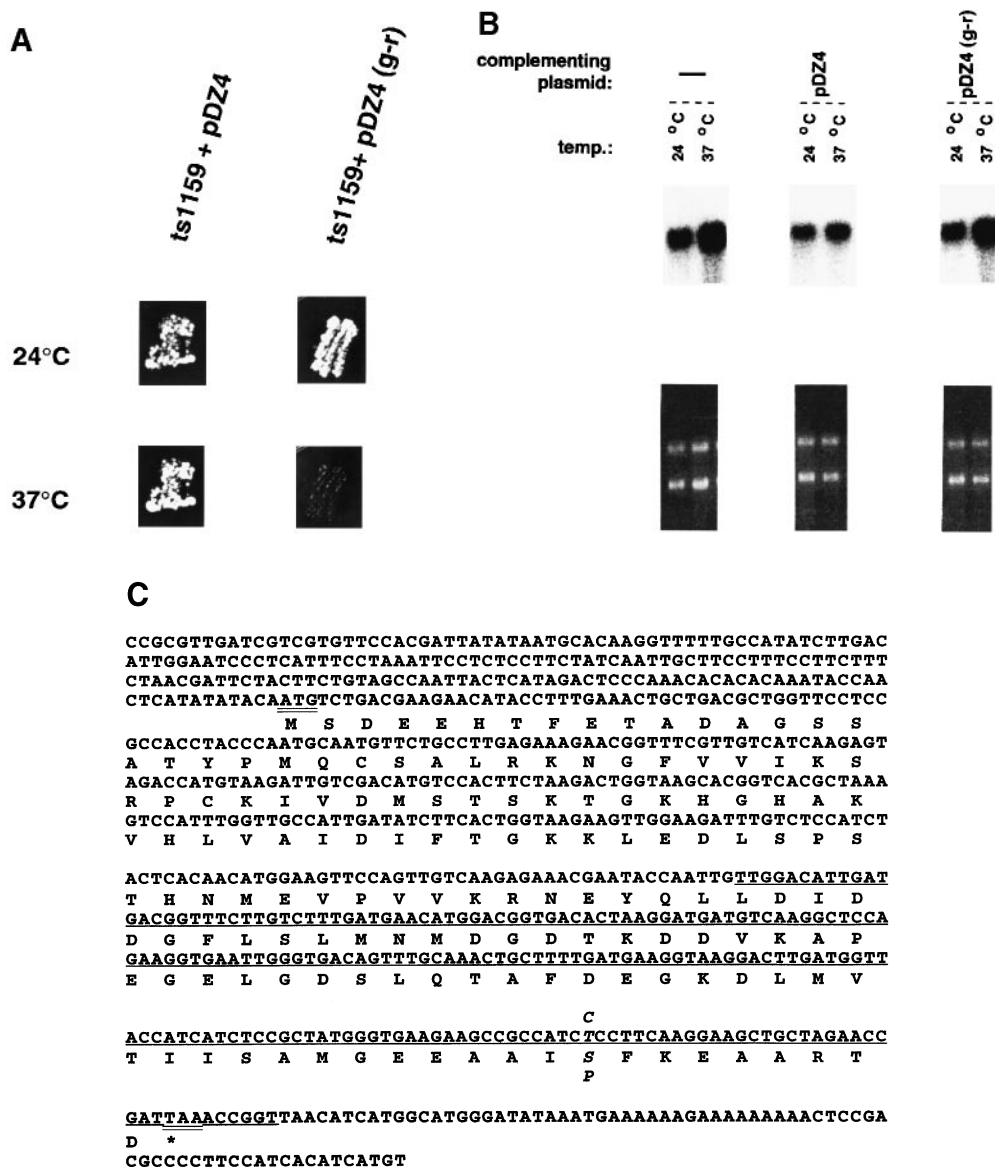


Fig. 5. The *tif51A* allele isolated from *ts1159* cells is unable to complement the *ts* growth and mRNA decay phenotypes of this strain. (A) *ts1159* cells transformed with the construct expressing the wild-type *TIF51A* allele (pDZ4; left panels) or with the large gap-repaired construct expressing the *tif51A* allele rescued from the *ts* mutant [pDZ4(g-r); right panels] were grown on SC-ura plates at 24°C for 3 days. These were replica-plated to two SC plates, and grown at 24 and 37°C, respectively. (B) *ts1159* cells that were untransformed, harboring the pDZ4 construct, or harboring the gap-repaired construct [pDZ4(g-r)] were grown at 24°C or shifted to 37°C for 1 h. RNA was isolated and analyzed by Northern blotting, using a *STE2* probe. The ethidium bromide-stained rRNAs, indicating the amount of RNA in each sample, are shown under each lane. (C) The sequence of the insert in the pDZ4 and pDZ4(g-r) constructs. The sequence of the eIF-5A protein is listed beneath the DNA sequence. The ATG translation start and the TAA translation stop sites are indicated by double underlines. The large gap created in the pDZ4 plasmid (which was then repaired in the *ts1159* cells) is underlined. The T→C (S→P) mutation is indicated above (and below) the sequence and italicized.

expressing the gap-repaired plasmid is due to this single amino acid change.

The results of this experiment and of the complementation experiments shown in Figure 4 support the notion that both the growth and mRNA decay phenotypes are due to the same mutation in the *TIF51A* gene. Additional evidence for mutation of a single gene comes from the fact that tetrads resulting from sporulation of a cross between the *ts1159* strain and the SS328 parent strain segregate 2:2 for *ts* growth (data not shown).

Protein synthesis properties of the mutant strain

Since the eIF-5A protein was isolated initially from a ribosomal fraction of rabbit reticulocytes, it was considered

a translation initiation factor (Kemper *et al.*, 1976; Benne *et al.*, 1978). It was thus important to investigate whether the *ts1159* strain was defective in protein synthesis. Cytoplasmic extracts of *ts1159* and wild-type cells before and after a shift to the non-permissive temperature were fractionated on sucrose gradients and ribosome distribution was examined. The wild-type cells display a polysome peak of approximately eight ribosomes at both 24°C and after a 1 h shift to 37°C (Figure 6A and B, respectively), with a slight increase in the amount of 80S subunits after the shift to the restrictive temperature. The profiles in the *ts1159* mutant at both temperatures look very similar to those seen in the wild-type extracts, except that polysomes now peak at approximately six ribosomes (Figure 6C and D).

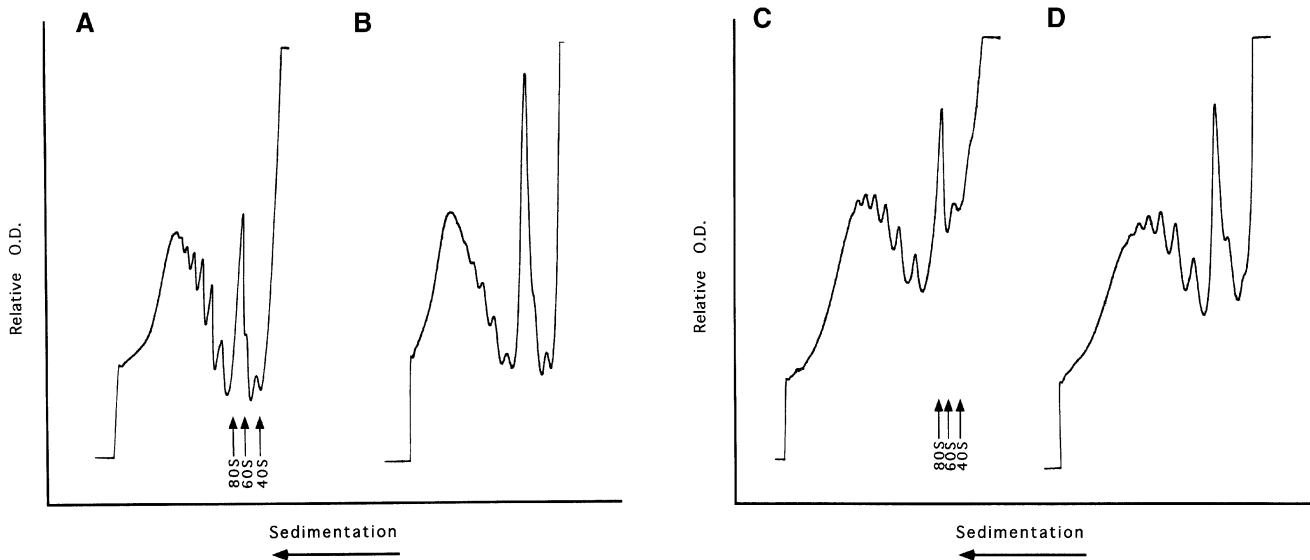


Fig. 6. Polysome profiles are not altered in *ts1159* cells after a shift to 37°C. Cytoplasmic extracts were prepared from wild-type (**A** and **B**) and *ts1159* (**C** and **D**) cells grown at 24°C (**A** and **C**) or after a shift to 37°C for 1 h (**B** and **D**) and fractionated on sucrose gradients as described in Materials and methods. The OD trace of the gradients is shown, with the direction of sedimentation and the 80S, 60S and 40S peaks indicated by arrows.

These experiments suggest that any temperature-induced translation defect in the *ts1159* mutant is not extensive and, because of the absence of a substantial shift to smaller polysomes at 37°C, is not likely to have a significant effect on translation initiation.

In order to study the protein synthesis properties of the *ts1159* cells in a more quantitative manner, we measured the incorporation of ³⁵S-labeled amino acids (methionine and cysteine) into cells after a shift to the non-permissive temperature. Duplicate aliquots of cells were taken at various time points after the shift, incubated with the labeled amino acids, and then precipitated and washed with trichloroacetic acid (TCA). The resulting precipitates were counted and compared with the amount of label incorporated at the time of the shift to 37°C ($t = 0$). The results of these experiments are shown in Figure 7. It is evident that protein synthesis in the wild-type cells increases after 60 min at the higher temperature (after a modest drop at 30 min, probably due to the heat shock of the shift to 37°C). The *ts1159* (*tif51A-1159*) cells display a drop in translation of ~30% after 60 min at the non-permissive temperature (the time at which decay rates were measured; Figure 2).

Effect of general inhibition of translation on the accumulation of mRNAs

While the results of the polysome gradients and the amino acid incorporation assays suggest that any translation defects in *ts1159* are modest (Figures 6 and 7), they nevertheless raise the question of whether the mRNA decay phenotype observed in this strain is an indirect consequence of impaired protein synthesis. Previous work has shown that complete inhibition of protein synthesis results in a general stabilization of all cellular mRNAs (Herrick *et al.*, 1990; Peltz *et al.*, 1992; Beelman and Parker, 1994). However, the effects on mRNA decay rates of a partial deficiency in translation have not been characterized previously.

In order to investigate further the correlation between

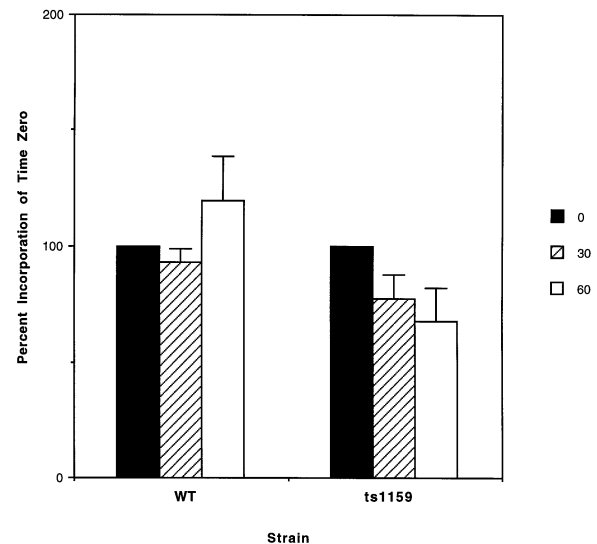


Fig. 7. Amino acid incorporation in *ts1159* and wild-type cells. Incorporation of ³⁵S-labeled amino acids was measured in SS330 wild-type (WT) and *ts1159* cells as described in Materials and methods. The values are given as the percentage incorporation, with time zero taken as 100%, and are averages of duplicate samples. The error bars denote the standard deviations of three separate experiments. Filled bars, time zero after the shift to 37°C; hatched bars, 30 min after the shift; open bars, 60 min after the shift. At $t = 0$, the average c.p.m. for wild-type and mutant cells were 47 970 and 24 092, respectively.

the extent of inhibition of protein synthesis and the resulting stabilization of specific mRNAs, we used the drug cycloheximide as a general inhibitor of translation. The SS330 parental strain was incubated with increasing concentrations of cycloheximide (ranging from 0.05–0.8 µg/ml) for 1 h, and then duplicate aliquots were allowed to incorporate ³⁵S-labeled amino acids. RNA was prepared from the same cultures, and Northern blots of these RNAs were probed with the *CYH2* and *URA5* probes. The results are shown in Figure 8. It is evident that inhibition of protein synthesis by 30% results in a <2-fold

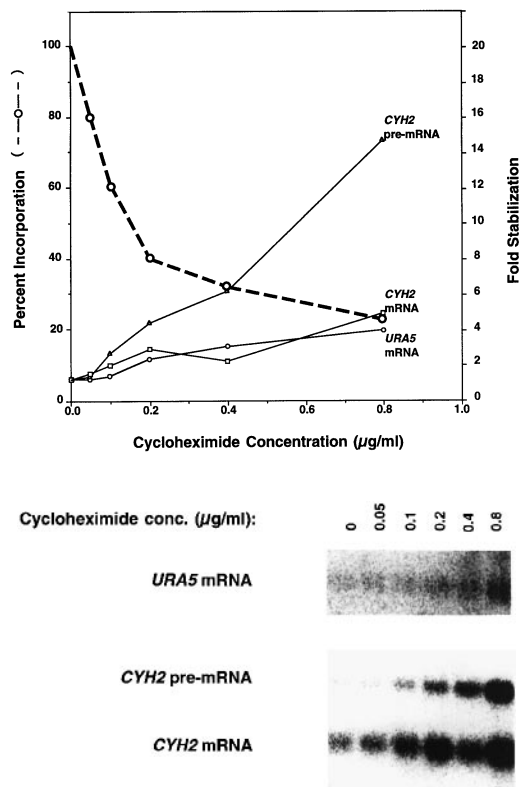


Fig. 8. Extent of mRNA stabilization in cycloheximide-treated cells. Top: SS330 wild-type cells were grown in SC-met and incubated with the indicated concentrations of cycloheximide for 1 h at 30°C. Incorporation of ^{35}S -labeled amino acids was measured in the cells as described in Materials and methods. The values are depicted as the percentage incorporation (thick dashed line, left scale), with the c.p.m. obtained from the culture lacking cycloheximide taken as 100%. RNA was isolated from the same samples, run on Northern blots and hybridized to radiolabeled *URA5* and *CYH2* probes. The values are depicted as fold stabilization (thin lines, right scale), with the amount of RNA in the sample lacking cycloheximide defined as 1. Bottom: Northern blots from which the values in the top panel were derived.

stabilization of the three transcripts tested. The *ts1159* cells, which have a similar defect in protein synthesis, exhibited a 2.4-fold increase in the half-life of the *CYH2* pre-mRNA, a 5-fold increase in that of the *URA5* mRNA and a 5.2-fold increase in the half-life of the *CYH2* mRNA (Figure 2). A similar 4- to 5-fold increase in the abundance of the *CYH2* and *URA5* mRNAs was only observed in the cycloheximide experiments when the inhibition of protein synthesis reached 80% (Figure 8). These results, taken together with the virtually normal polysome profiles of the *ts1159* cells at the non-permissive temperature, suggest that the mRNA decay defects displayed by *ts1159* are at least partially a result of a defect in some metabolic process other than protein synthesis.

The *ts1159* cells accumulate uncapped mRNAs at the non-permissive temperature

The results of the experiments described above suggest that eIF-5A may play a role in mRNA turnover in yeast. Proteins involved in this pathway could function at any of the previously identified stages of this process, i.e. deadenylation, decapping or exonucleolytic decay (Caponigro and Parker, 1996; Jacobson and Peltz, 1996). It has been shown that while inherently unstable mRNAs

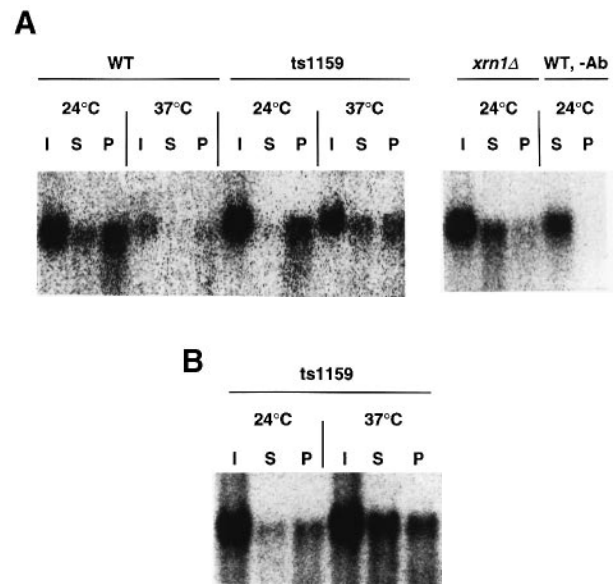


Fig. 9. *ts1159* cells accumulate uncapped mRNAs. RNA (10 µg) prepared from the indicated cell types grown at 24°C, or after a 1 h shift to 37°C, was immunoprecipitated with anti-7mG antisera bound to protein A-Sepharose. The supernatant (S) and pellet (P) fractions, as well as an equal aliquot of input (I) RNA were ethanol precipitated and subjected to Northern analysis. *xrn1Δ* cells were used as a positive control for the specificity of the antisera. As a negative control, an identical aliquot of RNA prepared from wild-type cells grown at 24°C was bound to Sepharose beads lacking the antisera (-Ab). (A) Northern blot of I, S and P fractions hybridized to a radiolabeled *URA5* probe. (B) Fractions from the immunoprecipitation of RNA from the *ts1159* cells hybridized to a radiolabeled *STE2* probe.

decay after degradation of their poly(A) tails (Decker and Parker, 1993), nonsense-containing RNAs decay with long poly(A) tails (Muhlrad and Parker, 1994). The two pathways then converge at the decapping step, since both types of transcripts are substrates for the same decapping enzyme, Dcp1p (Beelman *et al.*, 1996), and exonuclease, Xrn1p (Muhlrad and Parker, 1994; Hagan *et al.*, 1995).

Since the *ts1159* cells exhibited an increase in the stability of both nonsense-containing (the *CYH2* pre-mRNA) and wild-type mRNAs (*CYH2*, *STE2*, *URA5* and *PAB1*), it is reasonable to propose that the mutation in these cells affects steps in mRNA decay at, or downstream of, decapping. In order to determine which step is affected, we examined the cap status of the mRNA in the mutant cells. RNA prepared from wild-type and *ts1159* cells (grown at 24°C or shifted to 37°C for 1 h) was immunoprecipitated with antisera directed against the 5' cap structure. The resulting RNA fractions (input, supernatant and pellet) were subjected to Northern analysis and hybridized to a radiolabeled *URA5* probe.

The results of these experiments demonstrate that in wild-type cells at both temperatures and in *ts1159* cells grown at 24°C, 70–75% of the *URA5* mRNA was precipitated by the anti-cap antibody, indicating that only 25–30% of the mRNA in these cells was uncapped (Figure 9A). However, in *ts1159* cells that had been shifted to the non-permissive temperature, the fraction of uncapped mRNA increased to 50%, suggesting that mRNA was accumulating in the mutant cells in an uncapped form. This effect was consistent ($\pm 2\%$) in two separate experiments. The efficacy of the immunoprecipitation procedure

was demonstrated by the results from *xrn1*Δ cells, in which the majority (78%) of the *URA5* mRNA was shown to be uncapped, a result previously shown for other mRNAs in cells with this mutation (Hsu and Stevens, 1993; Muhlrud *et al.*, 1994).

Similar results were obtained when the blot shown in Figure 9A was reprobbed with a radiolabeled *STE2* probe. The fraction of uncapped *STE2* mRNA in *ts1159* cells increased from 28 to 66% after a shift to the restrictive temperature (Figure 9B). These results indicate that the defect in the *ts1159* cells at the non-permissive temperature generates uncapped mRNAs, suggesting that decapping is occurring in these cells but that the exonucleolytic process may be inhibited.

Discussion

Most of the factors identified to date as having a role in mRNA decay have proved to be non-essential for cell growth. Because of the fundamental nature of this process, we investigated whether essential factors have been overlooked. To identify mutants defective in the turnover of both wild-type mRNAs and those containing premature nonsense codons, we tested a panel of ~4000 yeast strains that are temperature-sensitive for growth for accumulation of the *CYH2* pre-mRNA. (In principle, this panel could also have been tested for mutants that increase turnover rate by seeking strains containing lower levels of the test RNA.)

Of 96 strains that exhibited an increase in the amount of the test RNA, five (*ts817*, *ts942*, *ts1100*, *ts1159* and *ts1189*) proved to have decreased decay rates of several mRNAs (Figure 2; D.Zuk and A.Jacobson, in preparation). The *ts1159* strain had the most extensive overall effect on the decay of the five RNAs tested, and was chosen for further study. The genes complementing the *ts* growth of the other four mutants have been identified (Table I) and the initial characterization of these strains is underway (D.Zuk and A.Jacobson, in preparation).

The growth defect of *ts1159* cells at the non-permissive temperature was complemented by a plasmid from a centromere-based yeast DNA library, and subsequently by expression of the *TIF51A* gene alone. Expression of this gene also complemented the mRNA decay phenotype of *ts1159* cells. The mutation in the *TIF51A* gene harbored by *ts1159* was rescued using a gap repair approach. Subsequent sequencing of the mutant gene showed that it contained a single T→C change at the 3' end of the ORF, resulting in a serine to proline change at position 149 of the eIF-5A protein. Since a plasmid expressing the mutant allele could not complement the growth or mRNA defects of the *ts1159* cells, we conclude that this mutation in eIF-5A is solely responsible for the observed phenotypes.

eIF-5A (originally called eIF-4D) is a highly conserved protein in all eukaryotic cells analyzed (Gordon *et al.*, 1987) that contains a unique spermidine-dependent hypusine modification on a specific lysine residue (Cooper *et al.*, 1983). However, the function of this protein remains elusive, even after a decade of intensive study. Based on its isolation from a rabbit reticulocyte ribosomal high salt wash fraction, and on its activity in an *in vitro* assay for the synthesis of methionyl-puromycin, eIF-5A originally was thought to be a translation initiation factor involved

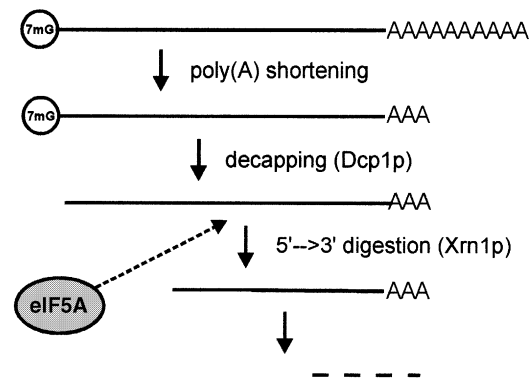


Fig. 10. eIF-5A is involved in a late step in mRNA decay. The general pathway for degradation of poly(A)-containing yeast mRNAs is depicted (Caponigro and Parker, 1996), with the proposed site of action of eIF-5A indicated by a dashed arrow.

in formation of the first peptide bond (Kemper *et al.*, 1976; Benne *et al.*, 1978). Other experiments in mammalian cells are consistent with a role in protein synthesis (Shi *et al.*, 1996, 1997).

However, work by Kang and Hershey (1994) has shown that marked depletion of this protein in yeast cells, while resulting in cell-cycle arrest, only led to a 30% drop in the first round of protein synthesis. The translation assays performed on *ts1159* cells after a shift to the non-permissive temperature showed a comparable drop in incorporation of labeled amino acids. Furthermore, analysis of sucrose gradients of cytoplasmic extracts from these cells shifted to 37°C for 1 h exhibited only modest impairment of polysome profiles. Since the mutation in *ts1159* had an effect comparable with the 'degron' construct of Kang and Hershey (1994), it is likely that the former mutation completely inactivates eIF-5A after a shift to 37°C. In light of the fact that the mutated region of eIF-5A is predicted to be α -helical (by the 'nnpredict' program; Kneller *et al.*, 1990), it is not surprising that a serine to proline substitution should have such a deleterious effect. Collectively, the data obtained in yeast suggest that eIF-5A may not play a major role in protein synthesis, but may be involved in other aspects of cell metabolism, such as mRNA turnover or cellular RNA export, with an indirect effect on translation.

The phenotype of *ts1159* cells at 37°C suggests that eIF-5A may play a general role in the turnover of mRNAs. *ts1159* exhibited decreased decay rates of both wild-type mRNAs (*CYH2*, *STE2*, *URA5* and *PAB1*) and of a transcript containing a premature nonsense codon (the *CYH2* pre-mRNA). Decay of these two types of RNAs is triggered by different events, with wild-type RNAs undergoing shortening of their poly(A) tails before decapping and exonucleolytic cleavage (Decker and Parker, 1993), while the decay of nonsense-containing RNAs is initiated by premature translation termination (Muhlrud and Parker, 1994). However, the two pathways converge at the decapping step, since both types of transcripts are substrates for the same decapping enzyme, Dcp1p (Beelman *et al.*, 1996), and exonuclease, Xrn1p (Muhlrud and Parker, 1994; Hagan *et al.*, 1995). This suggested that the mutation in *ts1159* affects steps involved downstream of decapping (Figure 10).

In order to test this hypothesis, we examined the cap

status of the *URA5* and *STE2* mRNAs in the ts1159 cells after a shift to the restrictive temperature. The fraction of uncapped mRNAs increased 2-fold (from 25 to 50%) in the mutant cells when compared with wild-type cells or with ts1159 cells grown at the permissive temperature (Figure 9). The simplest interpretation of these results is that eIF-5A functions downstream of Dcp1p (since decapping occurs) and upstream of Xrn1p (Figure 10).

The accumulation of uncapped mRNAs in ts1159 cells grown at 37°C not only serves to define the precise step in which eIF-5A functions in mRNA decay, but also supports the conclusion that the stabilization of mRNAs seen in these cells is not attributable to the indirect effects of partially inhibiting translation. Using cycloheximide to inhibit protein synthesis to the same level observed in ts1159 cells at 37°C did not increase mRNA abundance to the same extent seen in the mutant (Figure 8). In addition to this quantitative difference, mRNAs accumulating in ts1159 cells at 37°C, or as a result of cycloheximide treatment, differ qualitatively since those accumulating in the drug-treated cells are predominantly capped (Beelman and Parker, 1994; D.Zuk and A.Jacobson, unpublished observations).

Previous studies have implicated human eIF-5A (heIF-5A) as a cellular co-factor for the HIV protein Rev (Ruhl *et al.*, 1993; Bevec *et al.*, 1996). The latter protein is required for export of the viral transcripts from the nucleus to the cytoplasm (Malim and Cullen, 1993; Fischer *et al.*, 1994). This suggests an alternative, albeit more complex, interpretation of our results, i.e. that there is an increase in the export of uncapped mRNAs from the nucleus after a temperature shift of ts1159 cells. These uncapped transcripts would, however, have to be impervious to the Xrn1p exonuclease (since the ts1159 cells are wild-type for this gene), a result inconsistent with recent experiments demonstrating that uncapped *HIS4* mRNAs are indeed substrates of this exonuclease (Lo *et al.*, 1998).

Materials and methods

Yeast strains and media

The collection of ts mutants used in this study was generated from strain SS330 (*MATa ade2-101 his3-200 tyr1 ura3-52*) by EMS mutagenesis as described in Vijayraghavan *et al.* (1989). A related strain, SS328 (*MAT α ade2-101 his3-200 lys2 ura3-52*), was used for crosses. SS328, SS330 and the mutants derived from the latter strain were all generously provided by John Abelson. The *prp2* ts strain, RL9ZK0, and the W303 *xrn1 Δ* strain were obtained respectively from David Elliot and Robin Ganesan. Cells were grown in rich, synthetic or sporulation media prepared as described in Sherman *et al.* (1986).

Temperature shifts and measurement of mRNA decay rates

For steady-state experiments, 25 ml cultures in rich, synthetic complete (SC) or SC-ura media (as indicated) were grown at 24°C to mid-log phase ($OD_{600} = 0.6-0.8$). The cells were collected by centrifugation, resuspended in 7 ml of fresh medium and shaken at 24°C for 10 min. A 4 ml aliquot to be used for RNA isolation was removed, centrifuged for 10 s, and the pellet frozen quickly in dry ice. The remaining cells were shifted rapidly to 37°C by addition of 3 ml of the same medium pre-warmed to 56°C and then shaken at 37°C for the indicated time periods, after which a 4 ml aliquot was frozen and stored for subsequent RNA isolation.

For measurement of mRNA decay rates (Parker *et al.*, 1991), 100 ml cultures in SC medium were grown to mid-log phase, collected by centrifugation, resuspended in 12 ml of fresh medium and shaken at 24°C for 10 min. An equal volume of medium pre-warmed to 56°C was added and the cultures were shaken at 37°C for 1 h. Transcription was

then inhibited by the addition of thiolutin (Jimenez *et al.*, 1973; Herrick *et al.*, 1990; a generous gift from Pfizer Inc) to a final concentration of 4 μ g/ml, and 4 ml aliquots were taken for RNA extraction at the indicated times thereafter.

RNA extraction and Northern blot analysis

RNA was isolated by the hot phenol method as described previously (Herrick *et al.*, 1990). Aliquots (15 μ g) of each RNA sample were analyzed by Northern blotting, using radiolabeled probes prepared by random priming (Feinberg and Vogelstein, 1983) with a kit from Boehringer Mannheim. mRNA decay rates, expressed as half-lives ($t_{1/2}$), were determined by direct counting of the blots with a Betagen blot analyzer, normalization of the data such that time zero after addition of thiolutin equaled 100%, and plotting the data with respect to time on semi-log axes (Peltz *et al.*, 1992). The transcripts monitored, and the DNA probes used to detect them, included: *CYH2* (a 500 bp *EcoRI-HindIII* fragment from pGEM4ZCYH2 which hybridizes to both the pre-mRNA and the mRNA; Herrick *et al.*, 1990), *STE2* (a 2 kb *SalI* fragment from pDH9; Herrick *et al.*, 1990), *PAB1* (a 3.4 kb *EcoRI-SalI* fragment from pDH2; Herrick *et al.*, 1990) and *URA5* (a 2 kb *KpnI-EcoRI* fragment from FL44; Herrick *et al.*, 1990).

Complementation by the YCp50 library and ORF subclones

The ts1159 strain was electroporated with 1 μ g of the A4 pool of a YCp50 library ($7-10 \times 10^3$ plasmids) prepared by Mark Rose (Rose *et al.*, 1987) using the protocol of Becker and Guarente (1991). The electroporated cells were grown on SC-ura plates containing 1 M sorbitol at 37°C for 5 days. The complementing plasmids were isolated from yeast cells and amplified in *Escherichia coli* using standard procedures (Sambrook *et al.*, 1989). The resulting plasmids were transformed back into ts1159 using the modified PEG-LiAc procedure (Soni *et al.*, 1993) and shown to retain complementation of the growth defect. All the complementing plasmids were cut with *EcoRI* and *SalI* (which flank the insert in the YCp50 vector) to generate a preliminary map of the inserts. Aliquots (30 μ g) of 1159-1 and 1159-3 DNAs were sequenced with the SequenaseII kit (Gibco-BRL) using a primer (made by Operon, CTTCGCTACTTGAGCCACT) complementary to a region 40 nucleotides upstream of the *BamHI* site of YCp50 into which the library inserts had been cloned. The sequence obtained was compared with the *Saccharomyces* Genome Database at Stanford University (<http://genome-www.stanford.edu/Saccharomyces/>) using the GCG BLAST program (Altschul *et al.*, 1990).

The complementing plasmids contained seven ORFs. Using the sequence information, fragments containing a single ORF plus enough sequence of flanking regions to overlap with the neighboring ORFs were cut out of the YCp50 plasmids and cloned into the *URA3*-containing vector pRS316 (Sikorski and Hieter, 1989). Each subclone was transformed into ts1159 cells using the modified PEG-LiAc procedure (Soni *et al.*, 1993). Eight clones from each transformation experiment were patched onto SC-ura plates and grown at 24°C for 3 days. These plates were replica-plated to two SC-ura plates, one of which was kept at 24°C and the other incubated at 37°C for an additional 3 days to test for complementation of the growth defect. As a negative control, cells were transformed with the empty pRS316 vector and analyzed in the same manner. ts1159 was complemented by a plasmid expressing the *TIF51A* ORF (pDZ4). This plasmid contained a 742 bp *RsaI-HaeIII* fragment of 1159-1 cloned into the *SmaI* site of pRS316, including 193 nucleotides upstream of the *TIF51A* translational start site and 76 nucleotides downstream of the translational stop site.

Complementation of the RNA decay defect in cells harboring the different plasmids was done by comparing levels of the *STE2* mRNA in wild-type and ts1159 cells grown at 24°C and after a 1 h shift to 37°C. These experiments utilized Northern blot analyses performed as described above for the temperature shifts.

Identification of the *tif51A* allele in ts1159 by gap repair

The pDZ4 plasmid was cut with *HpaI* (which cuts five nucleotides 3' of the translational stop site of the *TIF51A* ORF) and *BstEII* (which cuts 69 nucleotides 5' of the translational stop), or with *HpaI* and *PflmI* (which cuts 204 nucleotides 5' of the translational stop) to generate plasmids containing one of two overlapping gaps in the *TIF51A* ORF. The gapped plasmids were isolated from a 1% agarose gel and transformed into ts1159 cells using the modified PEG-LiAc procedure (Soni *et al.*, 1993). The repaired plasmids were isolated from the resulting ura⁺ colonies, sequenced with the SequenaseII kit (Gibco-BRL), using the M13pUC forward primer (Gibco-BRL), which hybridizes to sequences in the pRS316 vector, and compared with the sequence of

the original pDZ4 insert. Wild-type and repaired plasmids were re-transformed into the ts1159 cells, and complementation of the ts growth and mRNA decay phenotypes was determined as detailed for the complementation by the original clones.

Polysome analysis

Cells were grown in 200 ml of rich medium at 24°C to an OD₆₀₀ of 0.6–0.8. For the 24°C experiments, cytoplasmic extracts were then prepared as described previously (Peltz *et al.*, 1992). For temperature shifts, duplicate cultures were centrifuged, resuspended in 40 ml of medium, and shaken at 24°C for 10 min. An equal volume of medium pre-warmed to 56°C was added and the cells were shaken at 37°C for 1 h. Extracts were prepared from these cultures in the same manner as for the 24°C cultures. The extracts were fractionated on 15–50% sucrose gradients [buffered with 50 mM Tris–acetate pH 7.4, 50 mM NH₄Cl, 12 mM MgCl₂ and 1 mM dithiothreitol (DTT)] which were centrifuged in a Beckman SW41 rotor at 35 000 r.p.m. for 165 min at 4°C and analyzed by continuous monitoring of A₂₈₀ (Manrow and Jacobson, 1986).

Measurement of amino acid incorporation

Cells were grown in 100 ml of SC-met at 24°C to an OD₆₀₀ of 0.6–0.8, harvested by centrifugation, resuspended in 10 ml of fresh medium, and shaken for 10 min at 24°C. An equal volume of medium pre-heated to 56°C was added and the cells were shaken at 37°C. Duplicate 1 ml aliquots were removed at the indicated time points and incubated with a mixture of 5 µCi of ³⁵S-translabel (ICN; 70% methionine and ~15% cysteine) and 5 µl of 50 mM unlabeled methionine for 4 min at 37°C. Incorporation of the radiolabeled amino acids was monitored by TCA precipitation. TCA (5 ml of a 5% solution) was added to each aliquot, followed by heating at 90°C for 20 min and subsequent incubation on ice. The precipitates were collected on GF/C filters, which were washed with 20 ml of 5% TCA and 20 ml of EtOH, dried under a heat lamp and counted by scintillation spectrometry. Each experiment was repeated at least three times.

Inhibition of translation with cycloheximide

The SS330 wild-type cells were grown in SC-met medium to an OD₆₀₀ of 0.6–0.8, divided in 12 ml cultures containing increasing amounts of cycloheximide (0–0.8 µg/ml) and shaken at 30°C for 1 h. Duplicate 1 ml aliquots from each culture were then incubated with a mixture of 5 µCi of ³⁵S-Translabel (ICN; 70% methionine and ~15% cysteine) and 5 µl of 50 mM unlabeled methionine for 4 min at 30°C. Incorporation of the labeled amino acids was measured as described above. The remaining 10 ml of each culture was centrifuged for 10 s, and the pellet frozen quickly in dry ice. RNA prepared from the pellets was analyzed by Northern blotting and hybridization to the labeled *URA5* and *CYH2* probes (see details in RNA extraction and Northern blot analysis).

Immunoprecipitation of uncapped mRNAs

Immunoprecipitations with anti-cap antibodies were performed essentially as described in Muhrad *et al.* (1994), except that the RNA was released from the protein A–Sepharose beads (Pharmacia) by incubating in 1% SDS for 1 h at 4°C. One µl of the polyclonal anti-7mG antibodies produced by T.W.Munns (Munns *et al.*, 1992) and graciously provided by E.Lund was used to immunoprecipitate 10 µg of RNA prepared as described above. An equal aliquot of the RNA (input) and the supernatant and pellet fractions were ethanol-precipitated in the presence of 10 µg of *E.coli* tRNA (Boehringer Mannheim) and analyzed by Northern blotting (see details above).

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