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Post-termination ribosome interactions with the 5′UTR modulate yeast mRNA stability

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A novel form of post-transcriptional control is described. The 5′ untranslated region (5′UTR) of the *Saccharomyces cerevisiae* gene encoding the AP1-like transcription factor Yap2 contains two upstream open reading frames (uORF1 and uORF2). The *YAP2*-type of uORF functions as a *cis*-acting element that attenuates gene expression at the level of mRNA turnover via termination-dependent decay. Release of post-termination ribosomes from the *YAP2* 5′UTR causes accelerated decay which is largely independent of the termination modulator gene *UPF1*. Both of the *YAP2* uORFs contribute to the destabilization effect. A G/C-rich stop codon context, which seems to promote ribosome release, allows an uORF to act as a transferable 5′UTR-destabilizing element. Moreover, termination-dependent destabilization is potentiated by stable secondary structure 3′ of the uORF stop codon. The potentiation of uORF-mediated destabilization is eliminated if the secondary structure is located further downstream of the uORF, and is also influenced by a modulatory mechanism involving eIF2. Destabilization is therefore linked to the kinetics of acquisition of reinitiation-competence by post-termination ribosomes in the 5′UTR. Our data explain the destabilizing properties of *YAP2*-type uORFs and also support a more general model for the mode of action of other known uORFs, such as those in the *GCN4* mRNA.

Keywords: eukaryotic initiation factor 2 phosphorylation/ mRNA stability/ribosome–mRNA interactions/translation termination/yeast gene expression

Introduction

The 5′ untranslated region (5′UTR) of eukaryotic mRNA plays a key role in the post-transcriptional regulation of gene expression. Until very recently, attention was focused exclusively on the role of the 5′UTR in controlling translational initiation. Translational initiation exerts strong rate control on gene expression, thereby determining the specific rate of protein synthesis from a given mRNA. Small upstream open reading frames (uORFs) are a feature of at least a few percent of the mRNAs in yeast, plants and mammals (Kozak, 1991; Vilela *et al*., 1998), and can be important players in translational control. The best characterized example is the regulation of *GCN4* translation in *Saccharomyces cerevisiae* mediated via four uORFs in the 591 nucleotide long leader of this gene (Hinnebusch, 1984, 1996, 1997; Thireos, 1984). In the case of the very short *GCN4* uORFs, it is not the encoded product, but rather the nature of the interactions between the mRNA sequence and the translational apparatus, that is relevant for regulation. In contrast, in two other examples of uORF-mediated translational regulation of fungal genes: *CPA1* in *S.cerevisiae* (Werner *et al*., 1987) and *ARG1* in *Neurospora crassa* (Luo and Sachs, 1996), the uORF-encoded peptides are thought to be involved in the regulatory mechanism. Both classes of uORF function are also identifiable in plant and mammalian systems (Geballe, 1996).

A number of studies of heterologous or modified mRNAs in yeast have indicated that uORFs can influence more than translational efficiency (Oliveira and McCarthy, 1995; Ruiz-Echevarria *et al*., 1996, 1998a; Linz *et al*., 1997). Moreover, we have estimated that there could be up to a few hundred natural yeast mRNAs containing uORFs (McCarthy, 1998; Vilela *et al*., 1998). Recent work has shown that uORFs can act as naturally occurring modulators of the stability of such mRNAs (Vilela *et al*., 1998). In an initial study of the post-transcriptional control of the uORF-containing mRNAs of *YAP1* and *YAP2*, it was determined that the *YAP2*-type uORFs destabilize the mRNA by a factor of five, which constitutes a major suppressive effect on gene expression. *YAP1* and *YAP2* encode proteins showing strong homology to AP1-like factors in higher eukaryotes and to Gcn4p in *S.cerevisiae* (Harshman *et al*., 1988; Moye-Rowley *et al*., 1989; Bossier *et al*., 1993; Wu *et al*., 1993). *YAP1* and *YAP2* are also regulatory genes involved in the mechanisms used by the yeast cell to protect itself in situations of stress. For example, overexpression of the two related *YAP1* and *YAP2* genes confers general stress resistance to a variety of unrelated compounds, including metal ions and various inhibitors and drugs (Hertle *et al*., 1991; Schnell and Entian, 1991; Haase *et al*., 1992; Bossier *et al*., 1993; Wu *et al*., 1993; Hirata *et al*., 1994; Lesuisse and Labbe, 1995; Turton *et al*., 1997).

The *YAP2* leader has one 6-codon uORF (uORF1) and an overlapping short reading frame (uORF2) of 23 codons (Vilela *et al*., 1998), while the *YAP1* 5′UTR has one 7-codon uORF (Moye-Rowley *et al*., 1989). Previous results (Vilela *et al*., 1998) indicated the existence of two types of functional influence exerted by the respective *YAP* uORFs. The *YAP2* uORFs act to block ribosomal scanning and also to accelerate mRNA decay, whereas the *YAP1* uORF has only a negligibly small inhibitory influence on downstream translation and is not destabilizing. Strikingly, the accelerated decay imposed by the *YAP2* uORFs was

found to be largely *upf1*-independent, thus contrasting with the *upf*-dependent decay seen in aberrant mRNAs containing premature nonsense codons (Jacobson and Peltz, 1996).

Here we investigate the mechanistic principles underlying mRNA destabilization by the respective *YAP2* uORFs and examine why these uORFs are functionally so different to the *YAP1* type of (non-destabilizing) uORF. The results uncover a causal link between the ribosome–mRNA interactions in the 5′UTR and the novel form of accelerated (largely *UPF*-independent) decay manifested by *YAP2* mRNA. We also demonstrate a new role for a eukaryotic initiation factor: eIF2 modulates the destabilizing influence of *YAP2* uORF-dependent termination. Moreover, additional experiments with the *GCN4* mRNA suggest a unifying working model which can explain the apparent discrepancies between results obtained with different uORF-containing mRNAs. Termination-dependent mRNA destabilization mediated via the 5′UTR is thus shown to constitute a novel principle of post-transcriptional control acting on non-aberrant mRNAs. This in turn means that translation termination on non-aberrant mRNAs has an additional significance beyond that of generating complete polypeptide chains, namely as a site for modulation of gene expression via the mRNA decay rate.

Results

Two uORFs contribute to the destabilization of YAP2

Earlier work showed that the *YAP2* leader imposes both translational inhibition and reduced stability on the *YAP2* and *LUC* mRNAs (Vilela *et al*., 1998). The first step towards understanding the basis for the destabilization effect is to characterize the roles of the respective uORFs in this 5′UTR. We therefore constructed derivatives of *YAP2* in which each of the uORF start codons was mutated to AAG (Figure 1). Analysis of the decay rates of the mRNAs encoded by these constructs revealed that both uORFs contribute to the overall destabilization effect of the *YAP2* leader. The total effect of the natural 5′UTR therefore constitutes the combination of the destabilizing influence of the two uORFs, whereby uORF2 acts as a slightly more potent destabilizing element.

We next examined whether the uORF-dependent destabilization effect can be transferred to a further, more stable, yeast mRNA. In other studies, *PGK1* has frequently been used as a model of relationships between translation and mRNA stability. One particular deletion derivative, the so-called 'mini-*PGK1*' gene, has been a favoured tool in investigations of the phenomenon of nonsense-dependent decay (Peltz *et al*., 1993a). The mini-*PGK1* sequence is believed not to contain any of the 'downstream elements' (Peltz *et al*., 1993a) that have been proposed to mediate the acceleration of mRNA degradation observed upon the introduction of premature stop codons into the first two-thirds of the *PGK1* reading frame. We found that, as with the *YAP2* ORF itself, the *YAP2* leader acted to destabilize the mini-*PGK1* mRNA (Figure 1).

We explored a further aspect of the destabilization mediated by the *YAP2* ORFs, namely its dependence on Upf1. Since *UPF1* dependence is typical of a number of nonsense-destabilized mRNAs (Peltz *et al*., 1993a,b), the observation that the *YAP2* uORFs act on *YAP2* to a large extent independently of this gene (Figure 1) indicated that they do not force the mRNA to follow the nonsense-dependent decay pathway described in previous investigations of aberrant mRNAs (Jacobson and Peltz, 1996). Interestingly, the *YAP2* leader::mini-*PGK1* mRNA was also destabilized in a *UPF1*-independent fashion (data not shown). In control experiments (data not shown), we found that the *upf1*$^{-}$ strain used in this study did show stabilization of an mRNA (BIA*cat*) shown previously to respond to inactivation of the *UPF1* gene (Linz *et al*., 1997). This confirmed that the strains used here were capable of supporting *upf1*-dependent accelerated decay.

Specific uORF properties contribute additively to destabilization

If specific sequence elements individually or collectively determine translation and mRNA turnover rates, it should be possible to convert one type of uORF into another type by modifying its sequence environment. We therefore investigated what modifications are needed to convert the *YAP1* uORF into an inhibitory, destabilizing type of uORF (Figure 2A). Since we wished to establish the generality of the relationship between strong translational inhibition by an uORF and its ability to destabilize, we used components of *GCN4* uORF4 and its flanking sequences to modify the *YAP1* uORF in its natural leader (see Table I for details). The use of the *cat* (chloramphenicol acetyl transferase) gene enabled us to monitor both the translation and the stability of the mRNA. Like the *YAP1* and *YAP2* mRNAs, this reporter mRNA is one of the more rapidly degraded transcripts in *S.cerevisiae*. The initial change was the substitution of the downstream sequence of *GCN4* uORF4 (pu*Y1*du4*G4*). This was followed by the penultimate codon of *GCN4* uORF4 combined with mutation of the U at –3 to A (pAmu*Y1*du4*G4*), and finally by substitution of the complete *GCN4* uORF4 sequence (pu4*G4*). U is a less favoured nucleotide that lowers the efficiency of AUG recognition by the 40S ribosomal subunit (Cigan and Donahue, 1987; Cavener and Ray, 1991; Yun *et al*., 1996). Its substitution by A therefore increases the efficiency of start-codon recognition. A control construct in which the start codon of the destabilizing uORF was mutated to AAG (pΔAmu*Y1*du4*G4*) served to confirm that the destabilizing effect was specifically associated with translation of the uORF. In conclusion, the experiments in Figure 2A demonstrate how a non-destabilizing type of uORF can be progressively converted to a destabilizing type in a series of small (additive) steps. In further experiments, another reporter mRNA (*LUC*, encoding firefly luciferase) was also found to be subject to the same stepwise translational inhibition and destabilization as *cat* (data not shown), thus confirming the relationship observed.

Taking four of the series of leader sequences shown in Figure 2A, we next investigated whether sequences derived from the *GCN4* leader could be used to destabilize the *YAP1* mRNA, which is not normally destabilized by its own leader (Vilela *et al*., 1998). Progressive increases in the destabilizing potential of the uORF were indeed reflected in reductions in the half-life of *YAP1* mRNA (Figure 2B). As with the *cat* gene, the individual changes associated with the respective steps were relatively small,

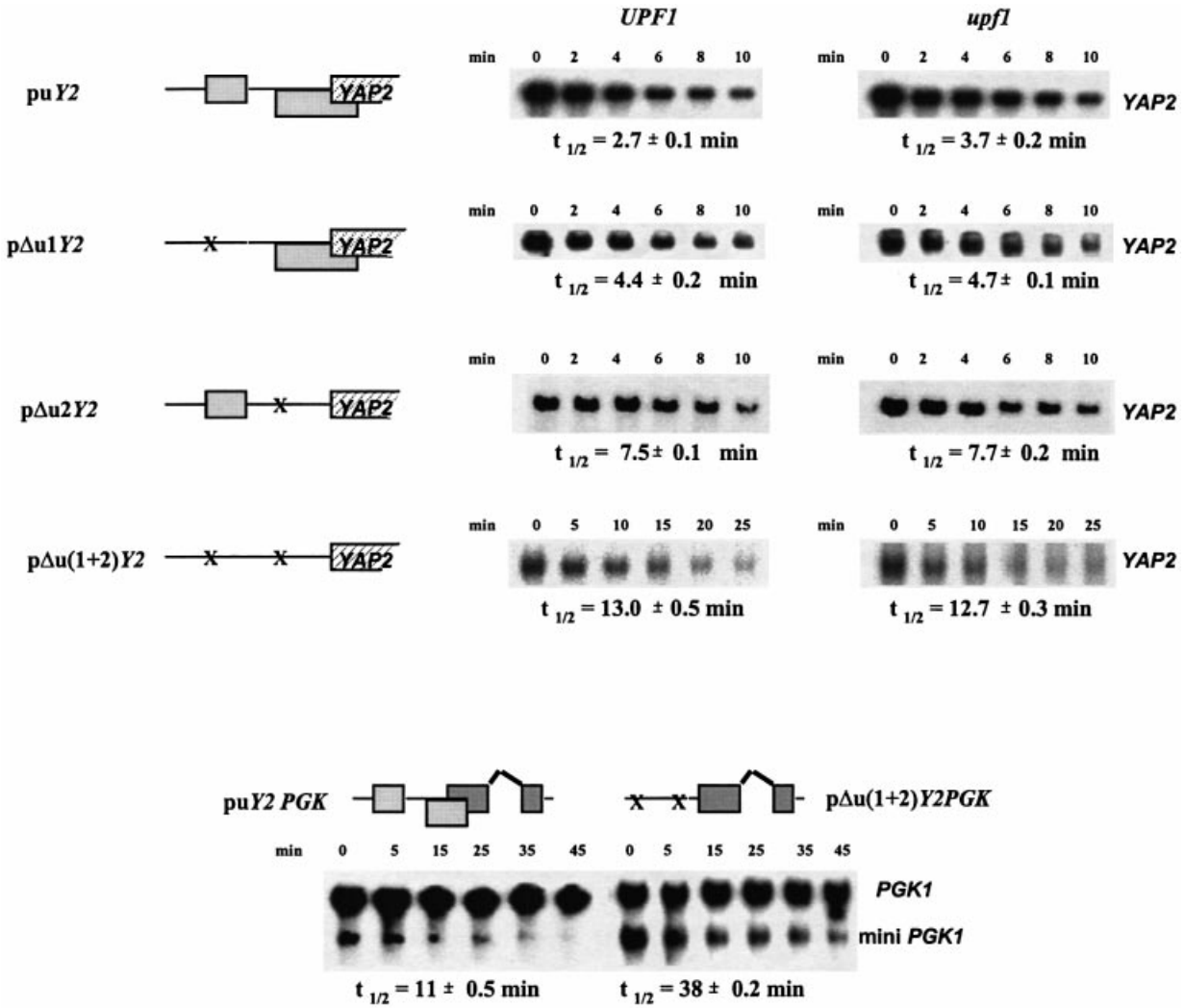

Fig. 1. Both *YAP2* uORFs contribute to mRNA destabilization. Northern blots show the results of hybridization using RNA preparations from strains SWP154 (+)(*UPF1*$^+$) and SWP154 (–)(*upf1*$^-$) taken during half-life determination experiments. The upper part of the figure shows the decay of the *YAP2* mRNA containing either the wild-type 5′UTR or three other derivative leaders. The wild-type endogenous *PGK1* mRNA was used as an internal control. The estimated half-life values represent averages of measurements performed using at least three independent sets of RNA preparations (± SD). The lower part of the figure shows the influence of the *YAP2* leader on the decay of the 'mini'-*PGK1* mRNA (compared with a control construct lacking the uORFs). The light grey boxes preceding the *YAP2* and mini-*PGK1* reading frames represent the *YAP2* uORFs. The inverted 'v', bridging the two dark grey boxes, indicates the region of *PGK1* deleted in the mini-*PGK1* reading frame. The X symbols indicate where AUG start codons have been mutated to AAGs.

but added up to a maximum overall destabilization of ~3-fold, thus showing again how small changes in mRNA sequences can be used to achieve progressive modulation of mRNA function. This also means that uORF-mediated destabilization is not an all-or-nothing effect. It should be pointed out that previous studies have confirmed that small changes in mRNA half-life in this range can be reproducibly measured in *S.cerevisiae* and are significant in terms of cellular decay kinetics (Herrick *et al*., 1990; Cui *et al*., 1995; Hatfield *et al*., 1996; Hennigan and Jacobson, 1996). The *YAP* mRNAs we are studying here belong to the more unstable end of the scale of mRNA stabilities, but we have observed major changes in the decay rate of both *YAP* mRNAs in response to alterations in uORF structure and function. Comparison of the effects of the pAmu*Y1*du4*G4* leader on the stability of *cat* (Figure 2A) and *YAP1* (Figure 2B) mRNAs revealed that the degree of destabilization imposed is very similar. Up to this stage therefore, we had shown that five different mRNAs (*YAP1*, *YAP2*, *cat*, *LUC* and 'mini-*PGK1*') were subject to uORF-dependent destabilization via a largely *upf*-independent pathway.

Manipulation of uORF structure in the YAP leaders modulates the cellular stress response

We examined how uORF structure can influence the physiological function of one of the *YAP* mRNAs, investigating how manipulation of the normal *YAP1* uORF changes the tolerance of *S.cerevisiae* to oxidative stress. Strikingly, the relatively moderate change (compared with, for example, pu4*G4*) in stability and expression caused by the leader pAmu*Y1*du4*G4* was already sufficient to drastically decrease tolerance to H_2O_2 in the plate assay (Figure 2C). This result illustrates how sensitively the stress response can be modulated by uORF-mediated post-transcriptional control of this *YAP* gene.

Overall, the experiments shown in Figure 2 demonstrate the principle that a wide range of post-transcriptional

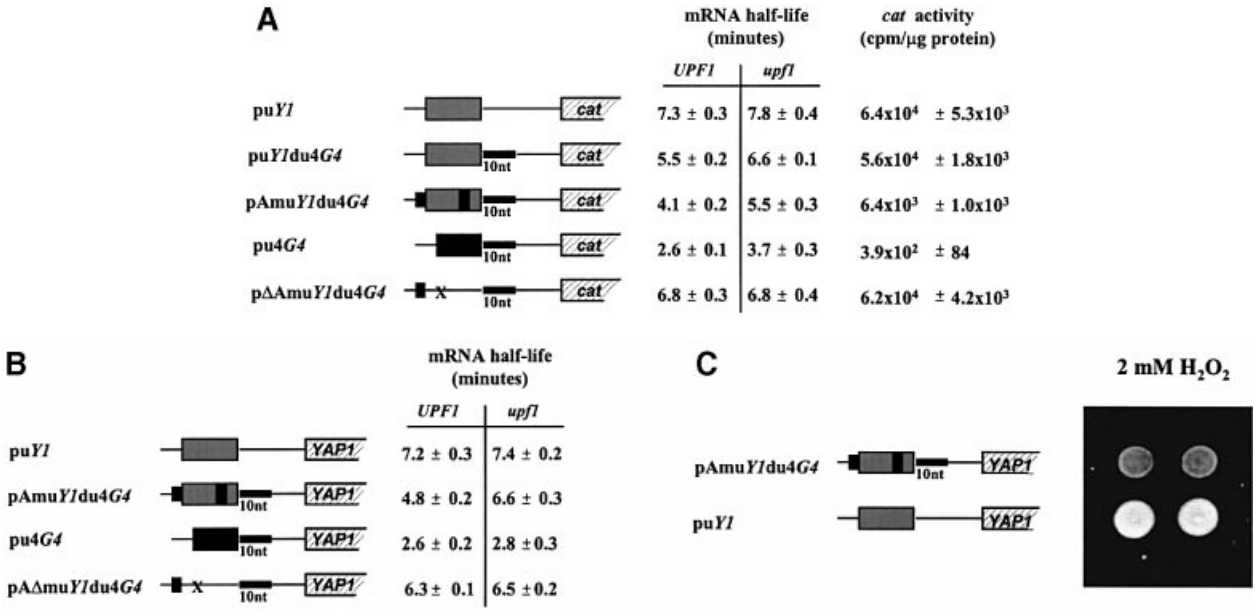

Fig. 2. Progressive conversion of a *YAP1*-type uORF to a *YAP2*-type destabilizing element. Starting from the *YAP1* leader preceding the *cat* gene, the uORF was progressively converted into a destabilizing uORF by introducing internal and flanking elements from the *GCN4* uORF4 (**A**). The final stage is the complete substitution by the *GCN4* uORF4 plus its downstream 10-nucleotide region. Elimination of the start codon of one of the destabilizing uORF constructs by mutation to AAG (pΔAmu*Y1*du4*G4*) reverts the leader to its non-destabilizing status. Sequences derived from the destabilizing type of uORF (*YAP2* uORF1 and *GCN4* uORF4) also induce destabilization of the *YAP1* mRNA (**B**). These changes in uORF structure diminished yeast resistance to H_2O_2 (**C**). The CAT activities are corrected for variations in the *cat* mRNA levels of the respective strains and given to two significant figures. The boxes preceding the *cat* main ORF represent uORFs (grey = *YAP1* uORF; black = *GCN4* uORF4).

Table I. pu*Y1* series of constructs[a]

pu*Y1*	UGCAUGAACACGAGCCAUUUUUAGUUUGUUUAAG
pu*Y1*du4*G4*	UGCAUGAACACGAGCCAUUUUUAG**CGGUUACCUU**
pAmu*Y1*du4*G4*	AGCAUGAACACGAGCCAU**CCG**UAG**CGGUUACCUU**
pu4*G4*	**AAGAUGUUUCCGUAACGGUUACCUU**
pΔAmu*Y1*du4*G4*	AGCAAGAACACGAGCCAU**CCG**UAG**CGGUUACCUU**

[a]These sequences correspond to the segment of the *YAP1* 5′UTR which is modified in Figure 2. The table shows nucleotides 79–112 of the wild-type *YAP1* leader (pu*Y1*) followed by the sequences that are substituted for it in the other constructs. The derivative leaders all contain different portions of the *GCN4* uORF4 and its flanking sequences. The uORF in each vector is shown underlined and the *GCN4* uORF4 sequences are given in bold.

control can be imposed generally on yeast mRNAs via alterations in the structure and immediate environment of short uORFs. The effects can be subjected to fine or coarse control, depending on the combination and number of individual small changes in mRNA structure, and can clearly be of physiological significance.

The post-termination behaviour of ribosomal subunits is linked to mRNA destabilization

We next proceeded to investigate the principles underlying uORF-dependent destabilization. In order to be able to focus on the properties of the individual uORFs, we inserted them into a synthetic leader which supports a translation efficiency that is comparable to the average efficiency of natural yeast mRNAs (Oliveira *et al*., 1993b; Table II; Figure 3). The system chosen for these more detailed studies contained the *cat* gene transcribed from the inducible P_{GPF} promoter (Oliveira *et al*., 1993b; Oliveira and McCarthy, 1995). Our initial results revealed that the destabilization effects of uORFs are independent of the type of promoter used to transcribe the constructs under study. While the absolute half-lives measured using the P_{GPF} promoter (Figure 3A) are somewhat shorter than with the constitutive P_{TEF1} promoter (compare Linz *et al*., 1997), the degree of destabilization measured for a given construct was, within the limits of experimental error, identical in both cases. Since the repressible P_{GPF} promoter offered enhanced accuracy at the fastest degradation rates, this promoter was chosen for the remaining analysis. The changes in turnover rate observed clearly confirm that the functional influence of an uORF can be shifted progressively between the identified two states via changes in small, defined regions within, or flanking, the uORF.

The first set of experiments showed that the *cat* mRNA is subject to destabilization by the *YAP2* type of uORF in the synthetic leader context, but not by the *YAP1*-type (Figure 3A). The uORFs were inserted into the leader together with their respective (10 nucleotide) downstream sequences. Neither the *YAP1* uORF (pu*Y1cat*) nor the *GCN4* uORF1 (pu1*G4cat*) changed *cat* mRNA stability. In contrast, destabilization was caused by *YAP2* uORF1 (pu1*Y2cat*) and was even stronger in the case of *GCN4* uORF4 (pu4*G4cat*). Only partial *upf1*-dependence was evident.

All of the evidence accumulated so far suggested that post-termination ribosomes play a decisive role in the

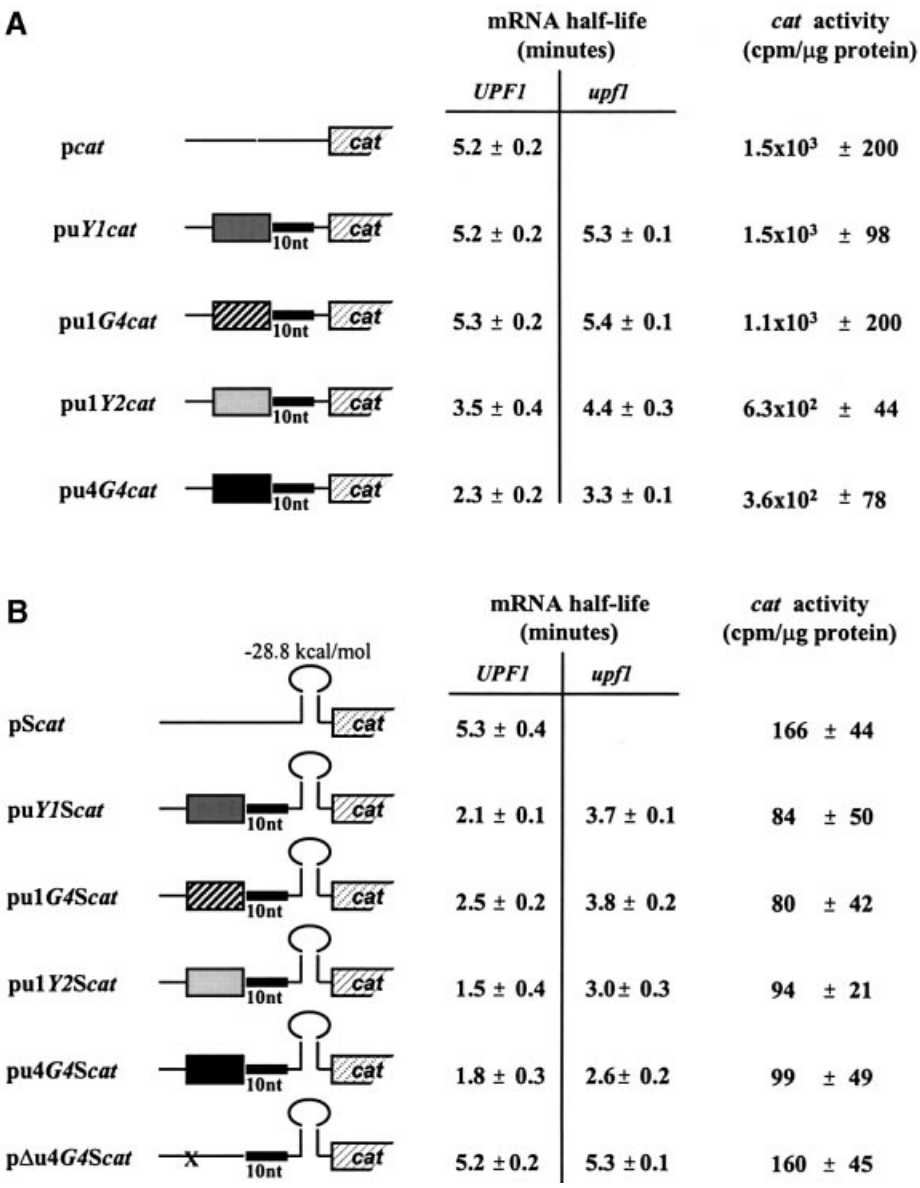

Fig. 3. Post-termination events modulate the destabilization potential of uORFs. Four different uORFs (*YAP1* uORF, *YAP2* uORF1, *GCN4* uORF1 and *GCN4* uORF4) together with their respective downstream sequences were inserted upstream of the *cat* gene in p*cat* (**A**). A stable hairpin loop capable of strongly inhibiting translation (–28.8 kcal/mol) was inserted 5 nucleotides upstream of the *cat* mRNA in each construct (**B**). The presence of the stem–loop decreased, in all cases, both translation and the mRNA half-life. This effect was eliminated when the start codon of the uORF was mutated to AAG (pΔu4*G4*S*cat*).

Table II. Sequences used in the reconstructed 5′UTRs of the puORF*cat* series of constructs[a]

p*cat*	AAGGATCCAATTATCTACTTAAGAACACAAACTCGAGAACATATG
puORF*cat*	AAGGATCCAAAAAAAGATCT....uORF + 10 nt...CTCGAGTAAACATAG
	AAACTTAAGACAAAGTATAGATACACTACGTAAACTACATATG
S: stem–loop	CTCGAGTAAACATAGAAACTTAAGCTCAAGTATAGATACAC
(–28.8 kcal/mol)	CAGCTTACGCCCGCCAAACAGGCGGGCGTAAGCTGCATATG
s: stem–loop	CTCGAGAATTATCTACATAAGAACACAAAA
(–8.6 kcal/mol)	CTCGAAGATACAAAAAAGTATCCTCGAAAACATATG
60 nt spacer	CTCGATATTTATAAAAACAATTACCACAAACAACAATACTTTC
	TTAAAGATCTTAACCTCGAG
30 nt spacer	CTCGATATTTATAAAAACAATTACCACAAACAACCTCGAG

[a]These are the partially synthetic leader sequences shown schematically in Figures 3 and 4. Each uORF (*YAP1*uORF, *YAP2*uORF1, *GCN4*uORF1 and *GCN4*uORF4) and respective downstream sequence (uORF + 10 nt) was inserted in the form of a *Bgl*II/*Xho*I oligodeoxyribonucleotide pair. The two stem–loop sequences were inserted as *Xho*I/*Nde*I oligodeoxyribonucleotide pairs into puORF*cat*, creating the S and s derivatives of this vector. The restriction sites used in the cloning of the different sequences are underlined.

destabilizing mechanism. We therefore decided to introduce a structural 'hurdle' into the mRNA downstream of the uORFs (Figure 3B). By inserting a stem–loop structure of sufficient stability to block the progress of scanning 40S subunits (Kozak, 1986; Oliveira *et al*., 1993b; Vega Laso *et al*., 1993; see Figure 3B), we could expect to achieve at least one of two objectives: first, to prevent reinitiation downstream of the uORFs; second, to induce an enhanced rate of ribosomal release from the mRNA subsequent to termination on the uORFs. The result of this manipulation was striking: all of the uORF-containing mRNAs were destabilized, irrespective of which type of uORF was present (Figure 3B). The insertion of the same stem–loop structure at the equivalent position into a leader that was identical except for the absence of an uORF had no effect on mRNA stability (p*S*c*at*). Moreover, the requirement for recognition of the uORF by ribosomes in order for destabilization to occur was confirmed by a control in which the start codon of *GCN4* uORF4 was mutated to AAG (pΔu4*G4*S*cat*).

Post-termination ribosomes lose the ability to destabilize mRNA during scanning

As indicated above, one possible explanation of the potentiation effect of a stem–loop structure placed downstream of an uORF is that the RNA structure promotes release of terminating ribosomes. This may even occur downstream of an uORF that allows post-termination scanning, since the ribosomal subunits proceeding beyond the stop codon may be devoid of one or more factors required for stable association with the mRNA during the scanning process. Hinnebusch and colleagues have proposed previously that ribosomal subunits which resume scanning subsequent to *GCN4* uORF1 rebind eIF2–Met–$tRNA_i$ (thus becoming re-initiation competent) at a rate that is slow compared with termination (Abastado *et al*., 1991). Accordingly, we suspected that such ribosomal subunits may be particularly sensitive to the presence of a stem–loop structure because their association with the mRNA in the post-termination phase is relatively unstable. We set out to test this hypothesis (Figure 4A). First of all, we decreased the stability of the stem–loop in order to bring it down to a level which is known to cause only partial inhibition of *cat* translation (Vega Laso *et al*., 1993). This showed a slightly reduced destabilization effect (compare pu1*Y2*s*cat*, Figure 3B, with pu1*Y2*S*cat*, Figure 4A), thus confirming that the size of the thermodynamic barrier presented to the post-termination ribosomal subunits on the mRNA controls the degree of disruption of their normal scanning behaviour. Secondly, we increased the distance of the stem–loop from the uORF in order to allow post-termination ribosomes more time to become initiation-competent before reaching the obstruction (compare pu*Y1*60S*cat*, Figure 4A, and pu*Y1*S*cat*, Figure 3B). The result was abrogation of the destabilization effect, indicating that the time/distance between termination and negotiation of the stem–loop enabled the ribosomal subunits to regain their more resistant status. That this was a progressive effect dependent on distance was confirmed by a construct with a spacer of intermediate length between the uORF and the stem–loop (pu*Y1*30S*cat*, Figure 4A).

In the *GCN4* system, the influence of eIF2 activity on the behaviour of ribosomes that have terminated on uORF1 plays an important role in controlling downstream reinitiation events (Hinnebusch, 1997). It was therefore a logical step to investigate whether decreasing the level of active eIF2 affected the decay of the stem–loop-containing mRNAs. We compared the stability of the pu*Y1*60S*cat* mRNA in a *gcd2*$^-$ strain and in an isogenic *GCD2*$^+$ strain (Figure 4B). The *gcd2*$^-$ strain is defective in the δ subunit of eIF2B, and therefore maintains a reduced level of active eIF2. The decay rate was higher in the former strain, suggesting that the activity of eIF2B, and thus of eIF2, plays a role in uORF-dependent decay. No such effect was seen with a control construct in which the AUG of the uORF had been converted to AAG (puΔ*Y1*60S*cat*; Figure 4B).

Re-initiation prevents destabilization caused by post-termination ribosomes

While we report here evidence that translational termination on a natural 5′UTR promotes destabilization, previous work on nonsense-dependent accelerated decay in aberrant mRNAs carrying premature stop codons has stressed the role of reinitiation following termination in promoting the destabilization process (Peltz *et al*., 1993b). The experiments in Figure 3B already indicated that blocking reinitiation did not prevent destabilization. However, we also examined the effect of reinitiation on the destabilizing influence of the *YAP2* uORF1 by inserting the *YAP1* uORF upstream of it (Figure 5A; pu*Y1*u1*Y2*). Since the *YAP1* uORF allows efficient resumption of scanning (Vilela *et al*., 1998), the *YAP2* uORF downstream of it is translated by a mixture of ribosomes that have 'overlooked' the *YAP1* uORF and a number of reinitiating ribosomes. Termination on *YAP2* uORF1 in this construct is accordingly at a level comparable to that seen with *YAP2* uORF1 alone (data not shown), yet the mRNA is no longer destabilized (Figure 6). Re-initiation therefore suppresses the destabilization mechanism.

Further evidence of the role of reinitiation followed from the deletion of a single base between the two uORFs, thus creating a construct in which the two uORFs are out of frame relative to each other (Figure 5A; pu*Y1*fu1*Y2*). This construct yielded the same *LUC* expression as the initial construct, clearly confirming that after translating the *YAP1* uORF, the ribosomes are capable of reinitiating at *YAP2* uORF1. It rules out the possibility that the enhanced stability is attributable to a certain percentage of the ribosomes translating the first uORF which read through the stop codon and terminate on the stop codon of the second uORF. In a control experiment, the nucleotide context of the *YAP1* uORF was subsequently changed in order to create a construct containing two destabilizing uORFs. The stability of this mRNA was significantly reduced (compare pAmu*Y1*du4*G4*u1*Y2* and pu*Y1*u1*Y2* in Figures 5A and 6) via a primarily *upf1*-independent pathway. That *YAP2* uORF1 is efficiently recognized in this leader environment by scanning ribosomes is indicated by the low-level expression obtained when the start codon of *YAP1* uORF in pu*Y1*u1*Y2* is mutated (pΔu*Y1*u1*Y2*). A proportion of the ribosomes in pu*Y1*u1*Y2* bypass *YAP2* uORF1 subsequent to termination on the *YAP1* uORF; these now translate *YAP2* uORF1 in pΔu*Y1*u1*Y2*, causing

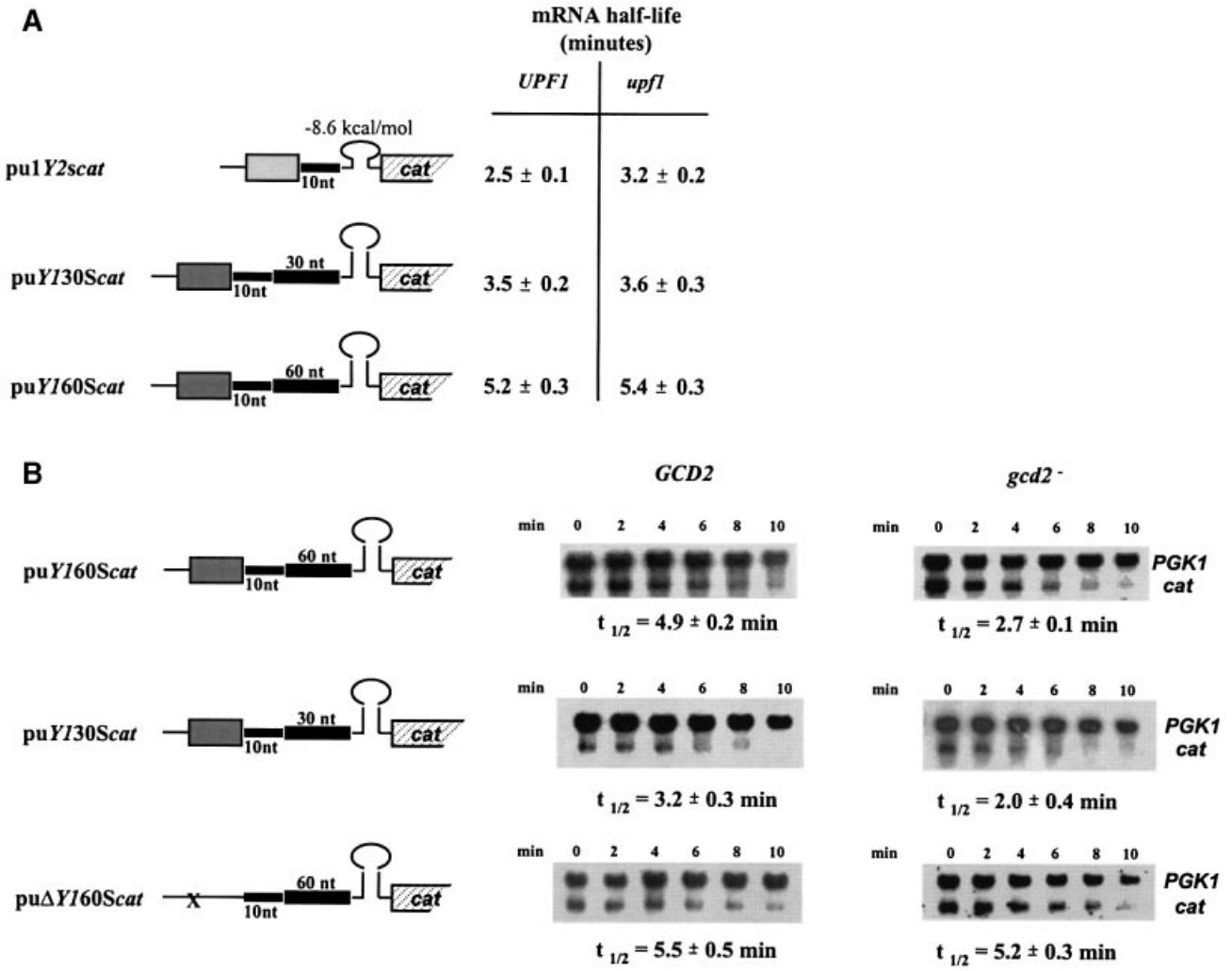

Fig. 4. Re-acquisition of re-initiation competence prevents destabilization. Replacement of the –28.8 kcal/mol secondary structure in pu1*Y2*S*cat* by a less stable one (–8.6 kcal/mol) in pu1*Y2*s*cat* resulted in higher stability (**A**). Complete abrogation of the destabilization effect was achieved by increasing the distance of the stem–loop from the uORF (pu*Y1*60S*cat*). Partial destabilization was measured with an intermediate construct, pu*Y1*30S*cat*. In a *gcd2*$^{-}$strain, spacers between the uORF and the stem–loop were no longer sufficient to prevent destabilization (**B**). Both *cat* mRNA and endogenous *PGK1* mRNA were labelled by hybridization in the Northern blots.

higher overall levels of termination on the leader, and partial destabilization (Figure 6).

Functional characteristics of the uORFs in the YAP1/2 and GCN4 mRNAs

The results of the analysis of *YAP* uORF function up to this point convinced us that a closer comparison with the intensively studied *GCN4* uORFs would be valuable. The first issue to be addressed was related to the fact that functional parallels apparently exist between the *YAP1* uORF and *GCN4* uORF1, and between the *YAP2* uORFs and *GCN4* uORF4. Does this mean that the *YAP* uORFs can be combined in such a way as to create a system that behaves like a *GCN4* mRNA, even so far as to be inducible via attenuation of eIF2 activity? Starting from pu*Y1*u1*Y2*, we constructed a set of leaders in which the inter-uORF distances were extended in order to approach the distances present in the *GCN4* leader (Figure 5B). This was achieved by inserting a 60-nucleotide spacer between the *YAP1* uORF and *YAP2* uORF1 (pu*Y1*60u1*Y2*), between the *YAP2* uORF1 and the main ORF (pu*Y1*u1*Y2*60), or into both regions (pu*Y1*60u1*Y2*60). Since these constructs contained the *YAP1* uORF upstream of *YAP2* uORF1, none of these leaders acted to destabilize the mRNA. There were, however, clearly identifiable changes in the translation rate of the *LUC* gene which were fully explicable in terms of the working model of kinetic control applied to the *GCN4* leader (Hinnebusch, 1996). Extension of the distance between the uORFs led to reduced translation, as would be expected if a greater proportion of the ribosomal subunits that had terminated on the *YAP1* uORF was able to become competent to reinitiate on the *YAP2* uORF and thus be released from the mRNA after termination on the second uORF. Extension of the distance between the *YAP2* uORF and the main ORF, on the other hand, would be expected to allow more ribosomal subunits to become re-initiation-competent before reaching the *LUC* AUG. The former effect partially nullifies the latter effect if both increased distances are combined (pu*Y1*60u1*Y2*60). For comparison, we included a control construct (p*GCN4*) containing a derivative of the *GCN4* leader in which uORF2 and uORF3 had been eliminated (Abastado *et al*., 1991).

Having adjusted the *YAP1* uORF/*YAP2* uORF combina-

tion leader to resemble more closely the dimensions of the *GCN4* leader (pu*Y1*60u1*Y2*60; Figure 5B), we then investigated whether we had also achieved inducibility. The results obtained in *gcd2*$^-$ and *gcn2*$^-$ strains confirmed that this was indeed the case (Figure 5C). While not reaching the equivalent degree of inducibility as the *GCN4* leader (which has different inter-uORF spacing), these results nevertheless demonstrate that the *YAP* uORFs can fulfil analogous functions at the translational level. This is also fully consistent with our earlier suggestion that the *YAP1* uORF/*GCN4* uORF1 and *YAP2* uORFs/*GCN4* uORF4 represent examples of two functional classes

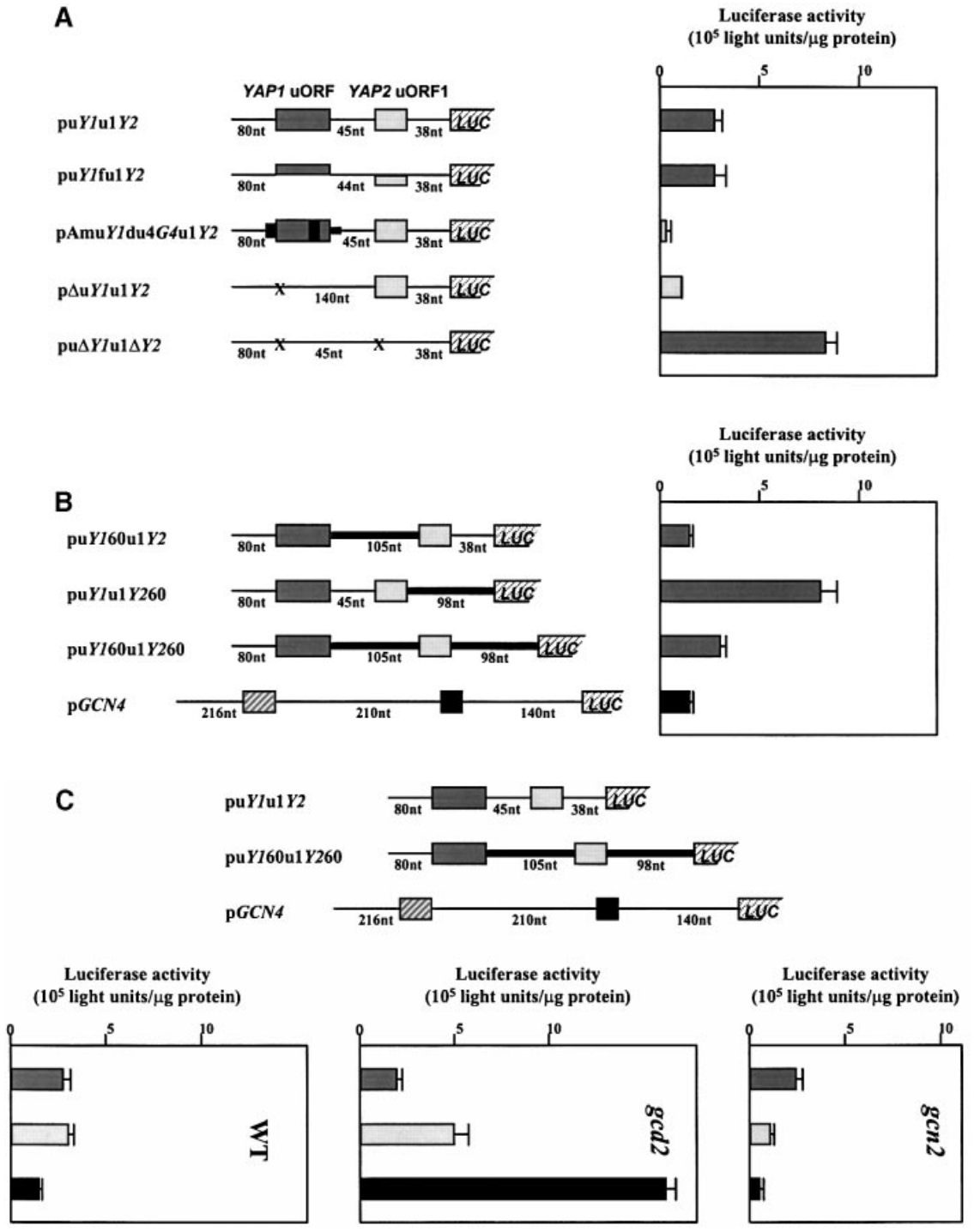

Fig. 5. Combinations of the *YAP1* and *YAP2* uORFs. *YAP1*-type (dark grey) and *YAP2*-type (light grey) uORFs were combined preceding the *LUC* reporter gene. Removal of one nucleotide from the inter-uORF region (pu*Y1*fu1*Y2*) had no effect on expression (**A**). Modification of the *YAP1* uORF using internal and flanking components from *GCN4* uORF4 (as in pAmu*Y1*du4*G4*, Figure 2) created a leader with two inhibitory uORFs (pAmu*Y1*du4*G4*u1*Y2*). In two control constructs, either one (pΔu*Y1*u1*Y2*) or both (puΔ*Y1*u1Δ*Y2*) of the uORF start codons were mutated to AAG. In further experiments, the inter-uORF distances were extended (thicker lines) to approach the lengths of those found in the *GCN4* uORF1/uORF4 leader (**B**). The expression of three of the constructs was then examined in *gcd2*$^-$ and *gcn2*$^-$ strains (**C**). The luciferase activities were corrected for the steady-state mRNA levels of the respective constructs. The values given are averages of at least three independent experiments, and error bars indicate the standard deviations.

(Vilela *et al*., 1998). The stability of the mRNA encoded by the inducible pu*Y1*60u1*Y2*60 was not affected by the *gcd2* mutation (data not shown).

Destabilization of the GCN4 mRNA

Work from another laboratory has indicated that *GCN4* uORF4 differs from the *YAP2* uORFs in that it is not capable of destabilizing the *GCN4* mRNA in the context of the natural *GCN4* leader sequence (Ruiz-Echevarria and Peltz, 1996). We therefore sought to resolve what appears, at least at first sight, to be a discrepancy in the observations made of *GCN4* uORF4 function. We combined the *GCN4* reading frame with a series of 5′UTRs (Figure 7). Fusion with the *YAP2* leader (pu*Y2*) destabilized *GCN4* mRNA, whereas a control construct [pΔu(1+2)*Y2*] was used to show that mutation of the *YAP2* uORF AUGs to AAGs nullified the effect, thus demonstrating the essential role of uORF translation in the destabilization effect. Elimination of only the second *YAP2* uORF (pΔu2*Y2*) partially eliminated the destabilization effect. The most striking observation was that the combination of *GCN4* uORF4 and a stem–loop of relatively low predicted stability [–8.7 kcal/mol (Abastado *et al*., 1991)] also led to destabilization of the *GCN4* mRNA (see pu4sl). A further control (pSL) revealed that even strong translational inhibition by a stem–loop structure of considerably greater stability (–25 kcal/mol) than that of the structure used in pu4sl had a relatively small effect on *GCN4* mRNA stability in the absence of an uORF. These results contrast with the observations that neither the *YAP1* uORF (data not shown) nor *GCN4* uORF4 alone (Ruiz-Echevarria *et al*., 1996, and our own data, not shown) destabilize the *GCN4* mRNA. This means that the context of the termination event in the 5′UTR is particularly critical in the case of this mRNA.

Discussion

A non-aberrant mRNA is destabilized by an uORF-mediated pathway

In this study we have investigated the phenomenon of uORF-mediated destabilization of a natural mRNA. The *YAP2* mRNA is subject to an accelerated decay mechanism that is dependent on translational termination on uORFs in the 5′UTR. Moreover, the destabilization effect was found to be transferable to five other mRNAs. Both of the *YAP2* uORFs contribute to the overall destabilizing influence of the leader, giving a combined destabilization factor of ~5-fold. It is, therefore, evident that uORF-mediated destabilization could play a role in the post-transcriptional control of an as yet undetermined proportion of the >100 other uORF-containing mRNAs predicted to be present in *S.cerevisiae* (Vilela *et al*., 1998). We have also found that just one short, non-overlapping uORF plus its immediate flanking sequences is capable of destabilizing a range of mRNAs. This has therefore provided us with an opportunity to explore the principles of action of a defined translation-linked stability element that is located 5′ of at least one natural main reading frame in yeast. The *YAP2*-type uORF imposes accelerated decay even on mRNAs that, unlike for example *PGK1* (Linz *et al*., 1997), are not directly sensitive to changes in translation rate per se, achieving this via its influence on the post-termination behaviour of ribosomes.

A mechanism of mRNA decay modulation mediated by post-termination ribosomes

The data presented here can be interpreted and discussed in terms of a testable working model (Figure 8) in which uORF-dependent modulation of mRNA decay is a function of three properties of the uORF region of the 5′UTR: firstly, the ability of the termination codon context and downstream sequence to direct either the efficient release of terminating ribosomal subunits or the resumption of post-termination scanning; secondly, the efficiency of recognition of the destabilizing uORF's start codon by scanning ribosomes; thirdly, the suppressive effect of re-initiation on mRNA destabilization. This model links the post-termination behaviour of ribosomal subunits to accelerated decay, predicting that ribosomal subunit release triggers a destabilization pathway, whereas re-initiation suppresses it. The working model also constitutes a unifying hypothesis because it relates the characteristics of uORFs in modulating translation to their potential role as stability elements, and also explains what might otherwise have been regarded as discrepancies between observations made using the *YAP* mRNAs (this study and Vilela *et al*., 1998) and reports on studies of *GCN4* mRNA decay (Ruiz-Echevarria and Peltz, 1996; Ruiz-Echevarria *et al*., 1998a). The termination codon context, therefore, both controls the efficiency of termination and acts as a modulator of gene expression at the level of mRNA decay. In the following, the key aspects of this model are discussed.

Structural features within and flanking uORFs act as stability determinants

The *YAP1* type of uORF (Figure 8A) shows similar characteristics to uORF1 of *GCN4*. It allows relatively efficient reinitiation and is not an effective barrier to scanning ribosomal subunits. Conversion of this class of uORF to an inhibitory type capable of destabilizing mRNA (Figure 8B) can be achieved progressively via the substitution of sequences 5′ and 3′ of the uORF, and of the internal penultimate codon, leading finally to complete replacement by an inhibitory, destabilizing uORF such as *YAP2* uORF1 or *GCN4* uORF4. The stability of a natural mRNA can therefore be varied progressively over at least a 5-fold range by relatively small changes within the coding sequence or flanking regions of an uORF. Moreover, since a destabilizing uORF can be created by as little as a one-nucleotide substitution (see, e.g. Oliveira and McCarthy, 1995), this shows us that the 5′ UTR provides a flexible means of controlling mRNA decay rates over a considerable range. The full extent to which this potential for stability control is realized within the group of uORF-containing eukaryotic mRNAs will now need to be investigated (McCarthy, 1998). This study already illustrates how significant such control can be for cellular responses to external stimuli or stress, since manipulation of the uORF in the 5′UTR has been shown to allow modulation of the tolerance of yeast to a potentially harmful oxidizing compound.

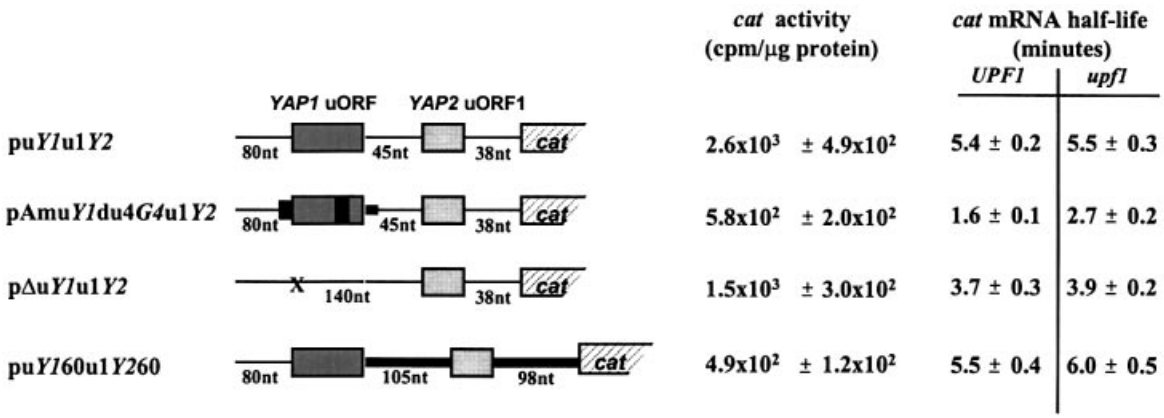

Fig. 6. Combinations of uORFs and mRNA turnover. The levels of expression and mRNA turnover of *cat* mRNAs containing combinations of uORFs (compare with Figure 5) were compared. The CAT activities are corrected for mRNA levels and are given to two significant figures.

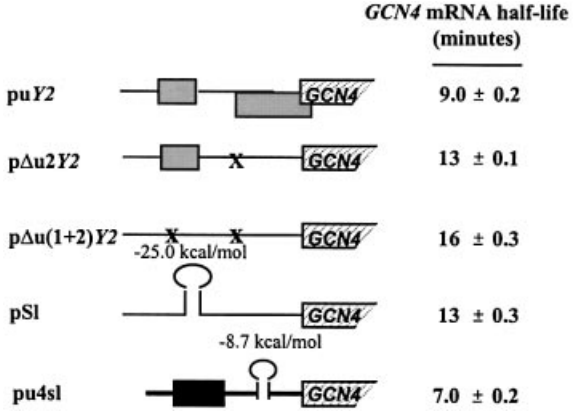

Fig. 7. *GCN4* mRNA can be destabilized by *YAP2*-type uORFs. Measurement of the mRNA half-lives of a series of constructs containing the *GCN4* main ORF revealed which type of leader is capable of destabilizing this mRNA.

Destabilization is coupled to the post-termination behaviour of ribosomes

Insertion of a stable stem–loop structure downstream of an uORF (Figure 8C) converted a normally non-destabilizing uORF of the *YAP1*-type to a destabilizing type, and enhanced the destabilizing nature of an uORF of the *YAP2/GCN4*-uORF4-type. Significantly, although the inhibitory effect on yeast translation of a stem–loop is hardly affected by changes in its position in the 5′UTR (Oliveira *et al*., 1993b; Vega Laso *et al*., 1993), extending the distance between the stop codon of the uORF and the stem–loop up to 60 nucleotides almost completely eliminated the enhancement of uORF-mediated destabilization (Figure 8D). Thus the sensitivity of post-termination ribosomal subunits remaining on the mRNA to disruption of further scanning by secondary structure is affected by the distance they traverse before being blocked. Previous data have indicated that a scanning ribosome can be forced to queue by a stem–loop structure in the 5′UTR (Kozak, 1986). In other words, the stem–loop located after the longer spacer in the leader of pu*Y1*60S*cat* (Figure 4A) is negotiated by ribosomes that have regained the normal capacity to scan along the mRNA.

The foregoing considerations fit with a model in which the reacquisition of fully stable binding to the mRNA is time/distance dependent. Since the mechanism of achieving this status may involve the binding of initiation factors, there is a parallel here to the known dependence of *GCN4* induction on the maintenance of minimum gaps between uORFs and the main reading frame on the mRNA (Hinnebusch, 1996). The observed sensitivity of the decay rate to a mutation in *gcd2* indicates the key significance of the phosphorylation status of eIF2 in the destabilization mechanism. We therefore propose that the same change in status of post-termination ribosomal subunits that is intrinsic to *GCN4* translational regulation is reflected in modulation of the mechanism and rate of mRNA decay, adding a new dimension to the significance of the interactions of translation factors with mRNA-bound ribosomes.

The stop codon sequence environment may influence the partitioning between direct subunit release and resumed scanning by controlling the length of pausing of ribosomal subunits at or near the termination codon. This, in turn, could be a critical factor in controlling the binding of a release factor which then triggers the dissociation of one or both of the ribosomal subunits (compare Grant and Hinnebusch, 1994). As in the case of *GCN4*, it is not possible to identify a specific consensus environment for a stop codon which promotes resumption of scanning. Rather, it seems likely that a range of nucleotide combinations (generally G/C-rich) can delay the movement of the ribosomal subunit beyond the stop codon and/or enhance release factor binding. Moreover, secondary structure in this region is also likely to influence the same process. This means there are many options for subtle control of this phenomenon in different mRNAs. It has recently been argued that eRF3 is the most likely candidate for the yeast release factor (Buckingham *et al*., 1997). If so, it may be possible to analyse this operational crossroads in the fate of ribosomes and mRNA using appropriate mutations in the gene (*SUP35*) encoding this protein. The functions of the release factors can be subject to modulation via other factors such as Upf1 (Czaplinski *et al*., 1998).

The re-initiation competence of post-termination ribosomes on the 5′ UTR

The results obtained with combinations of uORFs indicate that the competence of ribosomal subunits to re-initiate is

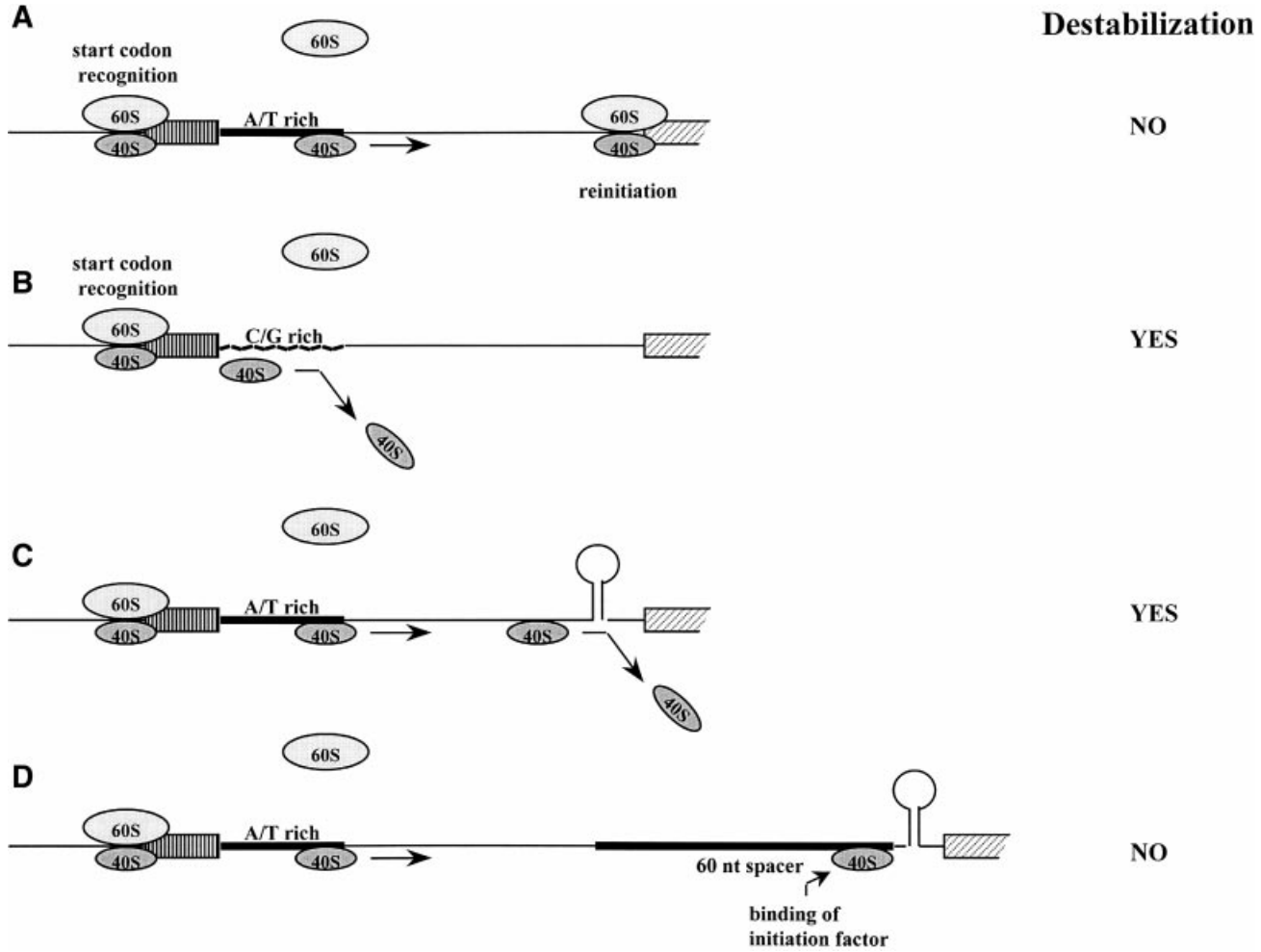

Fig. 8. Principles of uORF-mediated destabilization. uORF-dependent modulation of mRNA decay is a function of three properties of the uORF region of the 5′UTR: firstly, the ability of the termination codon context and downstream sequence to direct either the resumption of post-termination scanning (**A**) or the efficient release of terminating ribosomal subunits (**B**); secondly, the efficiency of recognition of the destabilizing uORF's start codon by scanning ribosomes and thus the number of ribosomes terminating on the uORF; thirdly, the suppressive effect of re-initiation (A) on mRNA destabilization. Improving start codon recognition of the destabilizing-type of uORF increases the degree of destabilization (compare A and B). The post-termination release of ribosomal subunits from the mRNA can be enhanced by placing a stem–loop structure downstream of the uORF (**C**). This accelerates decay even when the stop codon context is not of the destabilizing type. Extending the distance between the uORF and the stem–loop allows the ribosomal subunits to acquire a status that is less sensitive to the blocking of scanning by secondary structure (**D**). This could be attributable to the (re-)binding of an initiation factor, such as eIF2, which primes the 40S subunit for re-initiation. Thus, acquisition of re-initiation competence, or the re-initiation process itself, stabilizes the mRNA. Upf proteins are envisaged to influence mRNA decay by virtue of their influence on the release mechanism and/or kinetics of terminating ribosomes.

a further key factor in determining the destabilization potential of termination within the 5′UTR (Figures 6 and 8A). There are potentially two ways of explaining the role of re-initiation in our model. Firstly, the presence of a re-initiation-promoting uORF (*YAP1*-type) upstream of a destabilizing uORF (*YAP2*-type) can increase the number of scanning ribosomes that bypass the AUG of the second uORF, thus reducing the frequency of termination at its stop codon. It might therefore be this latter termination frequency on the destabilizing type of uORF that dictates the destabilization effect. Secondly, the re-initiation rate per se, on either the downstream uORF or the main ORF, could determine whether destabilization is suppressed. The observation that reducing the activity of eIF2 suppresses re-initiation on the downstream uORF in the pu*Y1*60u1*Y2*60 leader yet has no effect on mRNA stability (Figure 6) is consistent with the second view of the role of re-initiation in mRNA decay; the re-initiation process itself seems to suppress destabilization.

The role of UPF1 in accelerated decay

Any model of uORF-dependent accelerated decay must provide an explanation for the poor *UPF1*-dependence of destabilization linked to the *YAP2* uORFs. Recent evidence has shown that Upf1 can interact with eRF1 and eRF3, possibly acting as a modulator of translational termination (Czaplinski *et al*., 1998). The Upf proteins are clearly not essential for normal termination to occur, but their presence acts to enhance the function of eRFs in translational termination on at least some mRNAs (Czaplinski *et al*., 1998), and they can also influence translational fidelity (Ruiz-Echevarria and Peltz, 1996; Ruiz-Echevarria *et al*., 1998b). While it is known that stop sites can vary significantly in their ability to promote efficient peptide chain termination, it is also possible that they can differ to at least as great an extent in their requirements for Upf proteins in order to achieve rapid termination kinetics. We therefore propose that the ability of stop codons to promote efficient termination, ribosome release and accelerated

decay may be subject to variable degrees of Upf-dependence. This predicts that the Upf-dependence of termination-related accelerated decay can vary from mRNA to mRNA because of variation in the kinetic contributions of the Upfs to the decay process. This may explain, for example, the partial, and variable, *Upf1*-dependence of the decay of our *cat* mRNAs (Figure 3). On the other hand, premature termination on an aberrant mRNA may dictate an extreme dependence on Upf activities. As an addendum to our unifying working hypothesis (Figure 8) we suggest that the Upf proteins may achieve their effects on termination-dependent mRNA decay by influencing the kinetics of ribosome release.

The generality of uORF-mediated destabilization

So far, we have found that destabilization mediated by *YAP2*-type uORFs can be imposed on a number of mRNAs, including *YAP1*, *YAP2*, *cat*, *LUC*, 'mini'-*PGK1* and *GCN4* (this work and Vilela *et al*., 1998), and full-length *PGK1* (Linz *et al*., 1997). We have also observed that *GCN4* uORF4 can act as a potent destabilizing element. However, another laboratory has reported that *GCN4* uORF4 is normally incapable of destabilizing the *GCN4* mRNA either because it is lacking a downstream element of the type proposed to promote nonsense-dependent decay (Ruiz-Echevarria and Peltz, 1996, 1998a) or because a stabilizing element within the *GCN4* leader suppresses the destabilizing potential of this uORF (Ruiz-Echevarria *et al*., 1998a). Examination of our constructs reveals that a *YAP1*-derived leader in which the *YAP1* uORF has been exchanged for *GCN4* uORF4 is capable of destabilizing the *YAP1*, *cat* and *LUC* mRNAs, but not the *GCN4* mRNA. On the other hand, the complete *YAP2* leader destabilizes the *GCN4* mRNA, while the *YAP2* uORF1 alone imposes partial destabilization on this mRNA. The decay rate of *GCN4* is accelerated to a lesser degree by the *YAP2* leader than is that of *YAP2* itself, indicating that the *GCN4* mRNA is less susceptible to this type of destabilization by virtue of its structure. At the same time, the half-life of the *YAP2* mRNA lacking uORFs is very similar to that of the wild-type *GCN4* mRNA. Overall, we conclude that yeast mRNAs seem to vary in their sensitivity to uORF-mediated destabilization. We have not been able to identify either the previously proposed types of destabilizing element (Jacobson and Peltz, 1996) or stabilizing element (Ruiz-Echevarria *et al*., 1998a) in the *YAP1/GCN4* uORF4 hybrid leader. Indeed, as discussed elsewhere (McCarthy, 1998), the 105-nucleotide *PGK1* downstream element used previously (Peltz *et al*., 1993a) has the potential to form stable secondary structure which, according to our experiments, might itself be expected to potentiate the destabilizing action of uORFs.

In conclusion, we have shown that post-termination events occurring even before initiation on a gene's main ORF act to modulate mRNA stability. As a consequence, it turns out that eIF2 is a potential modulator of the stability of a subpopulation of yeast mRNAs. Further predictions of the proposed model are also readily testable, so that future work can examine the exact relationship between the *YAP2* uORF destabilization pathway and the other known mRNA decay pathways. Moreover, we are now in a position to investigate the mechanism that couples the termination event on the *YAP2* type of uORF to accelerated decay.

Materials and methods

Yeast strains, growth conditions and transformation procedures

The following yeast strains were used in this study: SWP154 (–)(*MAT*a *trp1-Δ1 upf1::URA3 leu2-1 his4-38 ura3-52 rpb1-1*; Peltz *et al*., 1993a), SWP154 (+)(*MAT*a *trp1-Δ1 upf1::URA3 leu2-1 his4-38 ura3-52 rpb1-1*<*UPF1 TRP1 CEN*>; Peltz *et al*., 1993b), H952 (*MAT*α *gcd2-1, ura3-52, leu2-3, leu2-112*), H1453 (*MAT*α *GCD2, ura3-52, leu2-3, leu2-112*), H2511 (*MAT*a *ino1, ura3-52, gcn2*Δ), F113 (*MAT*a *ino1, ura3-52, GCN2*) (Müller and Hinnebusch, 1986) and yLF41 FT4 (*MAT*a *leu2Δ::PET56 his3-Δ200 trp1-Δ63 ura3-52 Δgcn4Δyap1*; Fernandes *et al*., 1997). The *Escherichia coli* TG2 strain [*supE hsdΔ5 thi* Δ(*lac-pro*) *AB* Δ(*srl-recA*)*306::Tn10* (*tet*^{r}) *F′* (*traD36 proAB*$^+$ *lacI*^{q} *lacZΔM15*); Sambrook *et al*., 1989], was used to amplify DNA. Yeast media were prepared as described previously (Vilela *et al*., 1998). Cells harbouring the *rpb*1-1 allele were grown at 26°C. Yeast transformation was performed according to standard procedures (Schiestl and Gietz, 1989).

DNA preparation

DNA cloning and sequencing were performed using standard methods (Sambrook *et al*., 1989). Oligodeoxyribonucleotides were synthesized using an Applied Biosystems DNA synthesizer.

RNA preparation and analysis

mRNA half-life analysis was performed using yeast transformants harbouring a temperature-sensitive allele of RNA polymerase II (*rpb*1-1) grown in selective media. The mRNA decay rates were determined as described previously (Linz *et al*., 1997). The results of these experiments were quantified on a Molecular Dynamics PhosphorImager using the ImageQuant software, version 3.3. or, alternatively, the resulting labelled bands were excised from the blotting membranes and used for scintillation counting. The mRNA abundance was normalized using the *PGK1* mRNA as a standard, correcting for the kinetics of *PGK1* mRNA decay (Oliveira *et al*., 1993a; Linz *et al*., 1997).

CAT and Luc assay

Fresh cultures of the yeast transformants were grown in the appropriate selective media to A_{600} = 0.8–1.0. The CAT and luciferase assays were performed as described previously (Oliveira *et al*., 1993a).

Plasmid construction

The vectors were constructed using recombinant DNA fragments generated via PCR using oligodeoxyribonucleotide primers specific for *YAP* sequences as well as synthetic DNA as building blocks. The plasmids have been named according to a systematic nomenclature described previously (Vilela *et al*., 1998). All sequences were inserted into YCpSUPEX1 (*GPF* promoter; Oliveira *et al*., 1993b) and/or YCp22FL (*TEF1* promoter; Oliveira *et al*., 1993a) and verified by means of DNA sequencing. Six genes were used: the genes encoding firefly luciferase (*LUC*) and bacterial chloramphenicol acetyl transferase (*cat*), and *S.cerevisiae YAP1* and *YAP2*, *GCN4* and the modified version of the *PGK1* gene, pRIPPGKH2(3)Δ1. The latter gene was kindly donated by Dr Alan Jacobson (Peltz *et al*., 1993a). The yeast genes were inserted into the YCp22FL vector after introduction of the *Nde*I and *Xba*I sites at the 5′ and 3′ ends of the genes' main ORFs. Constructs p*Y1*, p*Y2*, pΔu*Y1*, pΔu1*Y2*, pΔu2*Y2*, pΔu(1+2)*Y2*, pu*Y1*du4*G4*, pmu*Y1*Δdu4*G4*, pAu*Y1*du4*G4*, pAmu*Y1*du4*G4*, pu4*G4* were described previously (Vilela *et al*., 1998; Table I). pΔAmu*Y1*Δdu4*G4* was used as a control for pAmu*Y1*Δdu4*G4* and was constructed from pAmu*Y1*Δdu4*G4* by mutation of the uORF start codon by a single base change (AUG→AAG). pu*Y1*u1*Y2* resulted from the insertion at position +112 nt in pu*Y1* of the *YAP2* uORF1 and the 38 nt downstream of this element, resulting in a construct containing both the *YAP1* uORF and *YAP2* uORF1. By means of a single base deletion, the *YAP2* uORF was placed out of frame relative to the *YAP1* uORF1 creating construct pu*Y1*fu1*Y2*. pΔu*Y1*u1*Y2* was generated by mutation of the *YAP1* uORF start codon (AUG→AAG) in pu*Y1*u1*Y2*. As a control, the *YAP2* uORF start codon was subsequently mutated (AUG→AAG), generating pΔu*Y1*Δu1*Y2*. In a further control construct, the nucleotide context of the *YAP1* uORF was changed, generating pAmu*Y1*du4*G4*u1*Y2* (pAmu*Y1*du4*G4* is described above; Vilela *et al*., 1998). Constructs pu*Y1*60u1*Y2* and pu*Y1*u1*Y2*60

were derived from pu*Y1*u1*Y2* by inserting, respectively, a 60 nucleotide spacer (Table II) between the restriction sites *Xho*I site and *Nde*I. Similarly, pu*Y1*60u1*Y2*60 resulted from the insertion of both spacers at the *Xho*I and *Nde*I sites of pu*Y1*u1*Y2* (Figure 5A). pGCN4 contains the wild-type *GCN4* leader in which the start codons of uORF2 and uORF3 were mutated (Abastado *et al*., 1991). The vectors described in Figure 3 refer to the combination of different synthetic fragments introduced into YCpSUPEX: the first module, cloned between the *Bam*HI and *Xho*I sites, contains an uORF (*YAP1* uORF, *YAP2* uORF1, *GCN4* uORF1 or *GCN4* uORF4) as well as the 10 nucleotides downstream of each uORF, creating constructs pu*Y1*, pu1*Y2*, pu1*G4* and pu4*G4*. The various uORFs were cloned between the restriction sites *Bgl*II and *Xho*I. The second module was cloned between the restriction sites *Xho*I and *Nde*I and contains either an unstructured leader (p*cat*; Figure 3A) or a stem–loop structure with a predicted stability of –28.8 kcal/mol, 5 nucleotides upstream of the *cat* start codon (pS*cat*; Figure 3B). pΔu4*G4*S*cat* results from mutation of the *GCN4* uORF4 start codon in pu4*G4*S*cat* (AUG→AAG) (Figure 3B). Constructs pu*Y1*60S*cat* and pu*Y1*30S*cat* were made from pu*Y1*S*cat* by inserting, respectively, a 60 nucleotide spacer in the *Xho*I site (Figure 4). As a control, the –28.8 kcal/mol stem–loop was replaced by a stem–loop of a stability of 8.6 kcal/mol, generating construct pu*Y2*s*cat* (Figure 4A). All of the leader sequences based on synthetic DNA fragments are listed in Table II. The constructs depicted in Figure 7 represent combinations of the leaders p*Y1*, p*Y2*, pu4*G4*, pΔu2*Y2* and pΔu(1+2)*Y2* (described above) with the *GCN4* reading frame. The pS leader contains a stem–loop with a predicted stability of –25.5 kcal/mol introduced upstream of the *GCN4* ORF. Finally, construct pu4S refers to the *GCN4* mRNA containing only uORF4 combined with a stem–loop structure with a predicted stability of –8.7 kcal/mol. This plasmid was generated using constructs pA50 and p237, kindly given to us by Dr Alan Hinnebusch (Abastado *et al*., 1991).

Spot test assay

The *yap1* transformants expressing different levels of the *YAP* genes (pu*Y1* and pAmu*Y1*du4*G4*) were grown to late log phase. This strain was chosen because the chromosomal copy of the *YAP2* gene is not sufficient to give a significant resistance phenotype to Cd^{2+} and therefore does not interfere with the results obtained in this study (Hirata *et al*., 1994). Appropriate dilutions were prepared and equal number of cells were spotted in minimal medium with appropriate supplements and containing H_2O_2. The spots were allowed to dry and subsequently incubated at 30°C for the length of time required to enable visualization of phenotypic differences.

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