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 *UPFI*. 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 (Gehalle, 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 (Hurtle 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 (BlAcat) 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 enables 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 Saccharomyces cerevisiae. The initial change was the substitution of the downstream sequence of GCN4 uORF4 (puY1du4G4). This was followed by the penultimate codon of GCN4 uORF4 combined with mutation of the U at –3 to A (pAmuY1du4G4), and finally by substitution of the complete GCN4 uORF4 sequence (pu4G4). 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 (pAmuY1du4G4) 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,
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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 pAmuY1du4G4 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, pu4G4) in stability and expression caused by the leader pAmuY1du4G4 was already sufficient to drastically decrease tolerance to H₂O₂ 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
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 (puY1cat) nor the GCN4 uORF1 (pu1G4cat) changed cat mRNA stability. In contrast, destabilization was caused by YAP2 uORF1 (puY2cat) and was even stronger in the case of GCN4 uORF4 (pu4G4cat). Only partial upf1-dependence was evident.

All of the evidence accumulated so far suggested that post-termination ribosomes play a decisive role in the
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**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 (pΔu4G4Scat).

**Table II.** Sequences used in the reconstructed 5’UTRs of the puORF*cat* series of constructs*

<table>
<thead>
<tr>
<th></th>
<th>mRNA half-life (minutes)</th>
<th>cat activity (cpm/μg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>UPF1</td>
<td>upf1</td>
</tr>
<tr>
<td>p*cat</td>
<td>5.2 ± 0.2</td>
<td>1.5x10^3 ± 200</td>
</tr>
<tr>
<td>puY1*cat</td>
<td>5.2 ± 0.2</td>
<td>1.5x10^3 ± 98</td>
</tr>
<tr>
<td>pu1G4*cat</td>
<td>5.3 ± 0.2</td>
<td>1.1x10^3 ± 88</td>
</tr>
<tr>
<td>pu1Y2*cat</td>
<td>3.5 ± 0.4</td>
<td>6.3x10^2 ± 44</td>
</tr>
<tr>
<td>pu4G4*cat</td>
<td>2.3 ± 0.2</td>
<td>3.6x10^2 ± 78</td>
</tr>
</tbody>
</table>

**B**

<table>
<thead>
<tr>
<th></th>
<th>mRNA half-life (minutes)</th>
<th>cat activity (cpm/μg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>UPF1</td>
<td>upf1</td>
</tr>
<tr>
<td>pScat</td>
<td>5.3 ± 0.4</td>
<td>166 ± 44</td>
</tr>
<tr>
<td>puY1Scat</td>
<td>2.1 ± 0.1</td>
<td>84 ± 50</td>
</tr>
<tr>
<td>pu1G4Scat</td>
<td>2.5 ± 0.2</td>
<td>80 ± 42</td>
</tr>
<tr>
<td>pu1Y2Scat</td>
<td>1.5 ± 0.4</td>
<td>94 ± 21</td>
</tr>
<tr>
<td>pu4G4Scat</td>
<td>1.8 ± 0.3</td>
<td>99 ± 49</td>
</tr>
<tr>
<td>pΔu4G4Scat</td>
<td>5.2 ± 0.2</td>
<td>160 ± 45</td>
</tr>
</tbody>
</table>

*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 BglII/XhoI oligodeoxyribonucleotide pair. The two stem–loop sequences were inserted as XhoI/NdeI 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 (pScat). 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 (pΔu4G4Scat).

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, (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 puY12Scat, Figure 3B, with puY12Scat, 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 puY160Scat, Figure 4A, and puY18Scat, 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 (puY130Scat, 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 puY160Scat 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 (pΔuY160Scat; 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; puY1u1Y2). Since the YAPI 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 YAPI 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; puY1u1Y2). This construct yielded the same LUC expression as the initial construct, clearly confirming that after translating the YAPI 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 YAPI uORF was subsequently changed in order to create a construct containing two destabilizing uORFs. The stability of this mRNA was significantly reduced (compare pAmuY1du4G4u1Y2 and puY1u1Y2 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 YAPI uORF in puY1u1Y2 is mutated (pΔuY1u1Y2). A proportion of the ribosomes in puY1u1Y2 bypass YAP2 uORF1 subsequent to termination on the YAPI uORF; these now translate YAP2 uORF1 in pΔuY1u1Y2, causing
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 puY1u1Y2, 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 (puY160u1Y2), between the YAP2 uORF1 and the main ORF (puY1u1Y260), or into both regions (puY160u1Y260). 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 reinitiation-competent before reaching the LUC AUG. The former effect partially nullifies the latter effect if both increased distances are combined (puY160u1Y260). For comparison, we included a control construct (pGCN4) containing a derivative of the GCN4 leader in which uORF2 and uORF3 had been eliminated (Abastado et al., 1991).

Having adjusted the YAPI uORF/YAP2 uORF combina-
tion leader to resemble more closely the dimensions of the GCN4 leader (puY160u1Y260; Figure 5B), we then investigated whether we had also achieved inducibility. The results obtained in gec2– 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 fulfill 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 (puY1fu1Y2) had no effect on expression (A). Modification of the YAP1 uORF using internal and flanking components from GCN4 uORF4 (as in pAmuY1 du4G4uY2, Figure 2) created a leader with two inhibitory uORFs (pAmuY1 du4G4u1Y2). In two control constructs, either one (pΔuY1u1Y2) or both (pΔuY1u1ΔY2) 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 gec2– 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.
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 (pu4sl) 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 (pΔu2Y2) 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 puY160Scat (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 (Czapinski 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
Post-termination ribosomes modulate mRNA stability

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.

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 (Czapinski 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 (Czapinski 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 Upf/τ 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 elf2 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 (+)MATα trplΔ1 upf1::URA3 leu2-1 his4-38 ura3-52 rpdi1-1; Peltz et al., 1993a), SWP154 (+)MATα trplΔ1 upfl1::URA3 leu2-1 his4-38 ura3-52 rpdi1-1; Peltz et al., 1993b), H952 (MATα trplΔ1; ura3-52, leu2-3, leu2-12), H1453 (MATα GCD2, ura3-52, leu-3; leu2-12), H2511 (MATα ino1, ura3-52, gcn2Δ), F113 (MATα ino1, ura3-52, GCN2) (Müller and Hinnebusch, 1986) and YL424 FT4 (MATα leu2Δ::PET56 his3Δ200 trpl1Δ63 ura3-52 gcn4Δ4ap11; Fernandes et al., 1997). The Escherichia coli TG2 strain [supE hsdS3/Δ thi (lac-pro) AB Δ(srl-recA3)3066-Tn10 (terf) F' (traD36 proAB lacI lacZYA15)], Sambrook et al., 1989], was used to amplify DNA. Yeast media were prepared as described previously (Vilela et al., 1998). Cells harbouring the rpdi1-1 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 (rpdi1-1) 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 ribosomal RNA 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 A600 = 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 YCPGSEX1 (GPP 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). 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 NdeI and XbaI sites at the 5’ and 3’ ends of the genes’ main ORFs. Constructs pY1, pY2, pΔuY1, pΔu1Y2, pΔu2Y2, pΔu1(1+2)Y2, pY1atuδ4G4, pmuY1atuδG4, pAatuδ4G4, pAatuδ4G4, pAatuδ4G4, pAmuY1atuδG4, pAmuY1atuδG4 were described previously (Vilela et al., 1998; Table 1). pAmuY1atuδG4 was used as a control for pAmuY1atuδG4 and was constructed from pAmuY1atuδG4 by mutation of the ura3 ORF start codon by a single base change (AUG→AAG). pY1atuΔ2 resulted from the insertion at position +112 nt in puY1 of the YAP2 uORF1 and the 38 nt downstream of this element, resulting in a construct containing both the YAP1 uORF and YAP2 uORF. By means of a single base deletion, the YAP2 uORF was placed out of frame relative to the YAP1 uORF creating construct puY1atuΔ2. puY1atuΔ2 was generated by mutation of the YAP1 uORF start codon (AUG→AAG) in puY1atuΔ2. As a control, the YAP2 uORF start codon was subsequently mutated (AUG→AAG), generating puY1atuΔ2. In a further control construct, the nucleotide context of the YAP1 uORF was changed, generating pAmuY1atuδ4G4atuΔ2 (pAmuY1atuδ4G4 is described above; Vilela et al., 1998). Constructs puY1atuΔ2 and puY1atuΔ20
were derived from puY1u12 by inserting, respectively, a 60 nucleotide spacer (Table II) between the restriction sites XhoI site and NdeI. Similarly, puY6u12 and puY6u1260 resulted from the insertion of both spacers at the XhoI and NdeI sites of puY6u12 (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 BamHI and XhoI sites, contains an uORF (YAP1 uORF, YAP2 uORF, GCN4 uORF1 or GCN4 uORF4) as well as the 10 nucleotides downstream of each uORF, constructing constructs puY1, puY12, puYG4 and puG4G4. The various uORFs were cloned between the restriction sites BglI and XhoI. The second module was cloned between the restriction sites XhoI and NdeI and contains either an unstructured leader (pC; Figure 3A) or a stem–loop structure with a predicted stability of −28.8 kcal/mol, 5 nucleotides upstream of the start codon (pC; Figure 3B), pA4G6Scat results from mutation of the GCN4 uORF4 start codon in p4G6Scat (AUG→AAAG) (Figure 3B). Constructs puY160Scat and puY130Scat were made from puY1Scat by inserting, respectively, a 60 nucleotide spacer in the XhoI 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 pu5Scat (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 pY1, pY2, puYG4, pA2u12 and pA1(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 puS4 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 Jacobson for the plasmid pRIPPGKH2(3)

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