The yeast transcription factor genes YAP1 and YAP2 are subject to differential control at the levels of both translation and mRNA stability

Cristina Vilela1,2, Bodo Linz1, Claudina Rodrigues-Pousada2 and John E. G. McCarthy1,*

1Posttranscriptional Control Group, Department of Biomolecular Sciences, University of Manchester Institute of Science and Technology (UMIST), PO Box 88, Manchester M60 1QD, UK and 2Instituto Gulbenkian de Ciência, Laboratorio de Genética Molecular, 2780 Oeiras, Portugal

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ABSTRACT

Two forms of post-transcriptional control direct differential expression of the Saccharomyces cerevisiae genes encoding the AP1-like transcription factors Yap1p and Yap2p. The mRNAs of these genes contain respectively one (YAP1 uORF) and two (YAP2 uORF1 and uORF2) upstream open reading frames. uORF-mediated modulation of post-termination events on the 5'-untranslated region (5'-UTR) directs differential control not only of translation but also of mRNA decay. Translational control is defined by two types of uORF function. The YAP1-type uORF allows scanning 40S subunits to proceed via leaky scanning and re-initiation to the major ORF, whereas the YAP2-type acts to block ribosomal scanning by promoting efficient termination. At the same time, the YAP2 uORFs define a new type of mRNA destabilizing element. Both post-termination ribosome scanning behaviour and mRNA decay are influenced by the coding sequence and mRNA context of the respective uORFs, including downstream elements. Our data indicate that release of post-termination ribosomes promotes largely upf-independent accelerated decay. It follows that translational termination on the 5'-UTR of a mature, non-aberrant yeast mRNA can trigger destabilization via a different pathway to that used to rid the cell of mRNAs containing premature stop codons. This route of control of non-aberrant mRNA decay involves the stress response in yeast. It is also potentially relevant to expression of the sizable number of eukaryotic mRNAs that are now recognized to contain uORFs.

INTRODUCTION

It is becoming increasingly clear that the 5'-untranslated region (5'-UTR) of eukaryotic mRNA is a key site of multiple forms of post-transcriptional regulation of gene expression. Until recently attention was focused 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. According to the scanning model, which is thought to apply to the vast majority of cellular mRNAs, the 43S pre-initiation complex binds to the 5'-cap region of the mRNA and then migrates progressively in a 5'→3' direction until it recognizes an AUG start codon in the leader sequence (1,2). Recognition of a potential start codon by the scanning ribosome is influenced by a number of factors, including the distance of the AUG from the 5'-end and its sequence context (1,3,4). However, in eukaryotic genes the first AUG in the mRNA sequence is not the translational start site of the major reading frame. For example, a considerable number of mammalian mRNAs encoding proteins with a proposed function in cell growth and differentiation have one or more AUGs or small upstream open reading frames (uORFs) that precede the major open reading frame (5,6). These AUGs or uORFs usually inhibit downstream translation (6), although some cases have been described where the upstream regulatory sequences stimulated translation of the major ORF (11,12).

A major paradigm of eukaryotic translational regulation via uORFs is the GCN4 system of Saccharomyces cerevisiae (7). Studies of the functional role of the four uORFs in the GCN4 5'-UTR have revealed the existence of a regulatory mechanism apparently based on kinetic control of ribosomal AUG recognition (8,9). This control mechanism operates at the level of translational initiation and is mediated by the four uORFs, although wild-type regulation is approximated by a 5'-UTR containing only uORF1 and uORF4. Each of these two uORFs has a distinct effect on ribosome behaviour: whereas uORF1 allows efficient resumption of scanning of the ribosomes following termination of translation, uORF4 acts as a strong translational barrier (10).

Moreover, recent work has demonstrated that, at least under certain circumstances, uORFs influence more than translation alone. In a study of the expression of CYC1 mutants Pinto and colleagues found that the presence of a uORF led to reduced steady-state mRNA levels, but were uncertain whether this was caused by transcriptional or post-transcriptional effects (11). Subsequently it was shown that an mRNA encoding chloramphenicol acetyltransferase can be rapidly degraded if a uORF is inserted into its 5'-UTR (12). Later work then revealed that both heterologous and homologous mRNAs are destabilized by uORFs in yeast (13,14). This destabilization involves the UPF-dependent degradation
The yeast strains used in this study were SWP154 (MATa trp1-Δ1 upf1::URA3 leu2-1 his4-38 ura3-52 rpb1-1 <UPF1 TRP1 CEN>势); 34) and yLf4 [F4 (MATa leu2::PET56 his3-Δ200 trp1-Δ63 ura3-52 Δgcn4Δ yap1; 35]. The Escherichia coli TG2 strain [supE hisd58 thi Δlac-pro AB Δ(srl- recA)306::Tn10 (terF) F’ (traD36 proAB+ lacI8 lacZAM15)] was used to amplify DNA. Yeast media were prepared as described (36). Cells were cultured on media lacking uracil and tryptophan, to select and maintain the plasmids used in these studies, and containing either 2% glucose (for the TEF1 promoter constructs; see below) or 2% galactose (for the GPF promoter constructs; see below). Induction of the GPF promoter was performed as described previously (37). Cells harbouring the rpb1-1 allele were grown at 26°C. Yeast transformation was performed according to standard procedures (38).

**DNA preparation**

DNA cloning and sequencing were performed using standard methods (39). 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 (rpb1–1) grown in selective media. The mRNA decay rates were determined as described previously (13). The results of these experiments were quantified on a Molecular Dynamics Phosphorimager using the ImageQuant software v.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 standard, correcting for the kinetics of PGK1 mRNA decay (13).

**Luc assays**

Fresh cultures of the yeast transformants were grown in the appropriate selective medium to A600 = 0.8–1.0. The luciferase assays were performed as described previously (40,41).

**Plasmid construction**

The vectors were constructed using recombinant DNA fragments generated via PCR using oligonucleotides specific for YAP sequences as well as synthetic DNA as building blocks. The plasmids have been named according to the systematic nomenclature explained in Table 1. All sequences were inserted into YCpSUP6X1 (GPF promoter; 37) and/or YCP22FL (TEF1 promoter; 41) and verified by means of DNA sequencing. Four genes were used: the genes encoding firefly luciferase (LUC) and bacterial chloramphenicol acetyltransferase (cat) and S. cerevisiae YAP1 and YAP2. The yeast genes were inserted into the YCp22FL vector after introduction of NdeI and XbaI sites at the 5′- and 3′-ends of the YAP genes main ORFs. The leader sequences inserted are schematically represented in Figure 2. Also given is the sequence context of the uORFs. The restriction sites BamHI and NdeI were introduced by PCR at the 5′- and 3′-ends of the YAP genes main ORFs. The leader sequences inserted are schematically represented in Figure 2. Also given is the sequence context of the uORFs. The restriction sites BamHI and NdeI were introduced by PCR at the 5′- and 3′-ends of the YAP genes main ORFs. The leader sequences inserted are schematically represented in Figure 2. Also given is the sequence context of the uORFs. The restriction sites BamHI and NdeI were introduced by PCR at the 5′- and 3′-ends of the YAP genes main ORFs. The leader sequences inserted are schematically represented in Figure 2. Also given is the sequence context of the uORFs.
Table 1. Key to the plasmid nomenclature

<table>
<thead>
<tr>
<th>Plasmid designation</th>
<th>Description</th>
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<tbody>
<tr>
<td>puY1</td>
<td>YAP1 leader: wt YAP1 uORF</td>
</tr>
<tr>
<td>pΔuY1</td>
<td>YAP1 leader: elimination (Δ) of YAP1 uORF (AUG → AAG)</td>
</tr>
<tr>
<td>puY2</td>
<td>YAP2 leader: wt YAP2 ORF1 and uORF2</td>
</tr>
<tr>
<td>puΔuY2</td>
<td>YAP2 leader: wt YAP2 uORF2; elimination (Δ) of YAP2 uORF1 (AUG → AAG)</td>
</tr>
<tr>
<td>puΔuY2Δdu4G4</td>
<td>YAP2 leader: elimination (Δ) of YAP2 uORF1 and uORF2 (AUG → AAG)</td>
</tr>
<tr>
<td>puY1du4G4</td>
<td>YAPI leader: YAP1 uORF; 10 nt downstream sequence of GCN4 uORF2</td>
</tr>
<tr>
<td>puY1du4G4Δ</td>
<td>YAPI leader: YAP1 uORF; position changed from T to Δ; 10 nt downstream sequence of GCN4 uORF2</td>
</tr>
<tr>
<td>puAuY1du4G4</td>
<td>YAPI leader: YAP1 uORF; position changed from T to Δ; mutant YAP1 uORF; codon 6 of YAP1 uORF → codon 2 of GCN4 uORF2; 10 nt downstream sequence of GCN4 uORF2</td>
</tr>
<tr>
<td>puAuY1Δdu4G4</td>
<td>YAPI leader: YAP1 uORF; position changed from T to Δ</td>
</tr>
<tr>
<td>pu4G4</td>
<td>YAPI leader: wt GCN4 uORF2</td>
</tr>
<tr>
<td>puAuY1Δdu4G4</td>
<td>YAPI leader: wt YAP1 uORF; 10 nt downstream sequence of YAP2 uORF1</td>
</tr>
<tr>
<td>puY1Δdu4G4</td>
<td>YAPI leader containing mutations of in-frame stop codons; YAPI uORF overlapping (−1) with LUC</td>
</tr>
<tr>
<td>puY1Δdu4G4LUC</td>
<td>YAPI leader containing mutations of in-frame stop codons; YAPI uORF overlapping (−1) with LUC; mutation of YAPI uORF start codon</td>
</tr>
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All plasmids used in this study are given in the above list.

The YAPI uORF in order to facilitate cassette mutagenesis of the uORF and surrounding sequences. In the case of the YAP2 uORFs the latter purpose was achieved by insertion of a unique BglII site at position +93 of the YAP2 leader. puY1du4G4 (Fig. 5A) contains the 10 nt sequence immediately 3′ of the GCN4 uORF stop codon downstream of the YAPI uORF. A derivative of puY1du4G4 was generated by replacing the last sense codon of the YAP1 uORF by the corresponding codon of GCN4 uORF4, creating pmuY1du4G4 and pAmuY1du4G4 are identical to puY1du4G4 and pmuY1du4G4 respectively except that the codon immediately upstream of the YAPI uORF start codon was replaced by AGC, creating a favourable context. pAuY1 was used as a control for pAuY1du4G4 and pAmuY1Δdu4G4 and was constructed from pY1 by insertion of the same AGC codon immediately upstream of the YAPI uORF. Also, as a control, the YAPI uORF was replaced by GCN4 uORF4, generating p4G4. The 10 nt downstream of the YAP2 uORF stop codon were also inserted 3′ of the YAPI uORF in puY1, creating puY1du1Y2. The YAPI uORF–LUC overlap construct puY1::LUC (Fig. 5B) was derived from construct puY1, in which the uORF TAA stop codon, as well as two downstream TAA codons, at positions +88 and +118, were each mutated by a single base change (TAG → AAG and TAA → AAA respectively). puY1Δ::LUC is identical to puY1::LUC except for a T→A substitution which changes the ATG codons of the YAPI uORF to AAG.

Spot test assay

The yap1 transformants expressing different levels of the YAP genes [puY1, puY1Δ, pAuY1Δdu4G4, puY2 and puY2Δ(1+2)] 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 Cd2+ and therefore does not interfere with the results obtained in this study (25). Appropriate dilutions were prepared and equal numbers of cells were spotted in minimal medium with appropriate supplements and containing the indicated toxic compounds. The spots were allowed to dry and subsequently incubated at 30°C for the length of time required to enable visualization of phenotypic differences.

RESULTS

Expression of YAPI and YAP2 is differentially attenuated by their respective uORFs

Examination of the YAP sequences reveals the presence of uORFs in their respective 5′-UTRs (Fig. 1). The YAPI leader has one 7 codon uORF, whereas the YAP2 leader has one 6 codon uORF (uORF1) and an overlapping short reading frame (uORF2) of 23 codons which is position −1 with respect to the main reading frame. The chromosomally encoded YAP mRNAs are of extremely low abundance in the cell and thus not reliably quantifiable via
Figure 1. The 5′-UTRs of the YAP1 and YAP2 mRNAs contain uORFs. The 5′-UTRs of YAP1 and YAP2 contain one and two upstream open reading frames (solid boxes) respectively. The YAP1 leader has one 7 codon uORF, whereas the YAP2 leader has one 6 codon uORF (uORF1) and an overlapping reading frame (uORF2) of 23 codons. The transcription start sites are indicated by arrows and the initiator codons of the main reading frames are underlined.

Figure 2. Differential control of gene expression by the YAP uORFs. Schematic representations indicate how the YAP leaders and their derivatives were combined with the LUC gene. The plasmid designs employed throughout this paper conform to a systematic terminology that reflects the identities and order of the components used in their construction (see Table 1). The restriction sites BamHI (B) and NdeI (N) were used in cloning of the 5′-UTRs. The YAP1 uORF and YAP2 uORFs are shown as grey and light grey boxes respectively. Crosses in the leader region indicate point mutations in the ATG codons (creating AAGs) of the various uORFs. The total lengths of the respective 5′-UTRs and of the sequences between the 5′-end and the uORFs, as well as between the uORFs and the reporter coding region, are indicated [as nucleotide (nt) values] at the very left and above the 5′-UTRs respectively. The lengths of the uORFs are also indicated below the boxes. The sequences of the YAP1 uORF and of YAP2 uORF1 in each construct (in bold italic) plus their respective 5′ and 3′ context sequences are shown on the right side of the panel. Each sequence change in the different leaders is underlined.

The luciferase activities for the YAP–LUC constructs are the averages derived from measurements made on at least three independent transformants, the standard deviations being indicated by error bars. The right hand side of the figure also shows the relative mRNA abundance and luciferase activity corrected for mRNA abundance for each construct. Each value represents the average of three independent determinations and is normalized to puY1.

YAP1 leader seq.

YAP2 leader seq.

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uORF has little effect on the resistance of yeast to H₂O₂, whereas elimination of the YAP2 uORF start codons greatly enhances cellular resistance to heavy metals (Fig. 3). Thus the uORF-bearing leaders exercise differential control on expression of the YAP genes, whereby uORF1 and uORF2 of YAP2 act additively to strongly attenuate this gene’s expression. The YAP2 uORFs could also be shown to strongly affect steady-state mRNA abundance. The effects of the respective leaders on steady-state levels of LUC and YAP2 mRNAs (Figs 2 and 3, compare pu2 and pΔu(1+2)2) were similar, if not identical. This confirmed the generality of the effects of the uORFs, while indicating that the main open reading frame can modulate their influence to a limited extent. In the remainder of this paper we explore the causes of these differences in uORF function between the YAP genes. Given that both translational and mRNA decay effects can contribute to changes in post-transcriptional gene expression, we have analysed the influence of the YAP leaders on both translation and mRNA half-lives throughout. The impact of the YAP2 uORFs is at this stage more evident than that of the YAP1 uORF, at least in terms of the cellular response to heavy metal stress. However, it will become evident that the comparatively small influence of the uORF of YAP1 on this gene’s expression by no means disqualifies it as a regulatory element. The effect seen is likely to be essential to the fine tuning of the yeast stress response in ways that we have not investigated in this study.

The uORF sequence context modulates post-transcriptional control

The very different types of effect of the uORFs on overall YAP expression described above are reminiscent of the effects on translation of the uORFs in the mRNA encoding the Yap homologue Gcn4p. This constituted an initial indication that investigations of the YAP genes would shed light on principles of control that are of wide relevance. Hinnebusch and colleagues have shown that the first uORF of the GCN4 5’-UTR (uORF1) allows efficient downstream re-initiation, whereas GCN4 uORF4 is responsible for strong termination and a high level of ribosomal release (18,44). This suggested to us that there are functional similarities between the YAP1 uORF and GCN4 uORF1 and between the YAP2 uORFs and GCN4 uORF4. Indeed, our examination of the downstream context sequences of these respective uORFs leads to the suggestion that there are two basic types of uORF (Fig. 4). The most striking correlation is seen in the high A/T contents of the downstream sequences immediately 3’ of the non-inhibitory uORFs (YAP1 uORF and GCN4 uORF1) and the high C/G contents of the inhibitory uORF downstream sequences (YAP2 uORF1 and GCN4 uORF4). Given that the downstream sequences of GCN4 uORF4 were shown previously to influence the scanning process (44), we subsequently changed the sequence and nucleotide context of the YAP uORFs to test the hypothesis that these determine the degree of inhibition by the uORFs (Fig. 5).

Insertion of the GCN4 uORF4 downstream sequence immediately 3’ of the YAP1 uORF in puY1du4G4 reduced the levels of LUC (Fig. 5A). Moreover, the last sense codon of the YAP1 uORF was also replaced by the corresponding codon of GCN4 uORF4, which resulted in a further decrease in expression of the reporter gene (puY1du4G4). Furthermore, when the recognition context of the uORF start codon was improved in these constructs by changing the T at –3 to A (puY1du4G4), there was a significant decrease in the expression values of LUC (compared with LUC values obtained from improving recognition of the wild-type YAP1 uORF in pAuY1). In a further comparative experiment the YAP1 uORF was replaced entirely by GCN4 uORF4 (puG4), which resulted in very strong inhibition of LUC expression. Overall, the above results show that the YAP1 uORF could be progressively converted into a YAP2-type uORF by substituting the individual sequence elements of GCN4 uORF4 that are known to render this latter uORF inhibitory (44). This conversion could also be achieved by exchanging elements of the YAP1 uORF region with corresponding sequences associated with YAP2 uORF1. For example, substitution of the YAP1 uORF downstream sequence by the corresponding sequence from YAP2 uORF1 (puY1du12) resulted in an inhibitory effect equivalent to that obtained using the GCN4 uORF4 downstream sequence (puY1du4G4).

The initial study of the YAP1 uORF (Fig. 5A) left unresolved the question whether the minimal effect of the YAP1 uORF on expression is simply due to its poor recognition by scanning ribosomes. In order to investigate the relationship between termination on the YAP1 uORF and (re-)initiation on the main ORF we mutated the uORF stop codon UAG to AAG (Fig. 5B). Moreover, mutation of two further stop codons located in the
whose decay is essentially uORF AUGs yields an mRNA that is five times more stable and the start codon of the uORF start codon and terminating at position +80 with respect to frame generated a –1 overlapping reading frame initiating at the natural destabilizing effect in the case of codon was mutated to AAG (pu

We discovered that translational control is only one component of the functional influence of the YAP2-type uORF on gene expression. Analysis of the mRNA degradation behaviour of mRNAs carrying the YAP leaders revealed that the YAP2-type uORF acts as a destabilizing element (Fig. 6). The overall destabilizing effect in the case of YAP2 is large: mutation of the uORF AUGs yields an mRNA that is five times more stable and whose decay is essentially UPF1 independent (Fig. 6E and F). The results in Figure 6 therefore demonstrate that a normal cellular mRNA containing short uORFs is subject to down-regulation via a destabilization pathway.

In order to perform a more complete study of the influence of the respective YAP uORFs on mRNA degradation we investigated both the full-length YAP mRNAs as well as hybrid mRNAs in which the YAP leaders have been fused to other reading frames. These experiments were performed using centromeric plasmids and promoters that are stronger than the YAP promoters, since the cellular levels of the YAP mRNAs encoded by the chromosomal genes are extremely weak. It was shown in previous work that the reporter genes we have used in this paper (LUC and cat) are not destabilized by inhibition of translation rates per se, thus indicating that their respective mRNA decay behaviour is comparable with that of natural yeast mRNAs such as MF2A2 (13,41,45). Moreover, the half-lives of the cat and LUC mRNAs (respectively 7.5 and 6 min under the conditions of Fig. 6) are in a similarly short time range to those of the YAP mRNAs. Thus, whereas a stem–loop structure placed in the 5′-UTR of LUC or cat is known not to accelerate decay of its mRNA (13,45), we have now found that the YAP2-type uORF does exert a strong destabilizing effect on cat (Fig. 6C and D) and on LUC (data not shown). This means that the inhibitory effects of the YAP2 uORFs on gene expression, which we have quantitated using LUC (see Figs 2 and 5), are attributable to both translational inhibition and mRNA destabilization. Indeed, the YAP2 uORFs destabilize both the YAP2 (Fig. 6E and F) and the LUC mRNAs. We found that the equivalent half-lives for LUC were 5.4 min for the YAP1 leader (puY1::LUC) and 1.7 min for the YAP2 leader (puY2::LUC) (data not shown).

The YAP2 type of uORF accelerates mRNA decay

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Figure 4. Comparison of the uORF sequences of YAP1, YAP2 and GCN4. The uORFs are boxed and the numbers indicate the lengths (in nt) of the segments whose sequences are not shown (A). The sequences of the GCN4 uORFs are either given in full (uORF1 and uORF4) or are represented by filled boxes. Comparison of the nucleotide contents of the uORF downstream sequences (B) suggests a correlation between A/T or G/C bias and uORF function. Whereas a high A/T content immediately 3′ of the upstream coding regions is characteristic of non-inhibiting uORFs (YAP1 uORF and GCN4 uORF1), the presence of a G/C-rich content downstream is a feature of the two inhibitory uORFs (YAP2 uORF1 and GCN4 uORF4).
Figure 5. The sequence and nucleotide context of the YAP uORFs determine their effects on gene expression. (A) Replacement of the YAP1 uORF downstream sequence and last codon with the corresponding G/C-rich sequences of GCN4 uORF4 (solid (10 nt) black line and bar within the uORF respectively) or YAP2 uORF1 (solid downstream black line) results in inhibition of LUC translation (see puY1du4G4, pmuY1du4G4 and puY1du1Y2). This effect is enhanced by improving the upstream context of the uORF start codon (solid upstream bar in pAuY1du4G4 and pAmuY1du4G4). The constructs pAuY1, in which the recognition efficiency of the wild-type YAP1 uORF was improved, and pu4G4, in which the YAP1 uORF was replaced by GCN4 uORF4, were used as controls. (B) Extending the YAP1 uORF to overlap the beginning of the LUC coding region reduced LUC expression. puY1::LUC has a point mutation in the YAP1 uORF stop codon and point mutations in two downstream in-frame termination codons, which together lengthen the uORF so that it overlaps with the beginning of the LUC coding region by 59 nt. puY1Δ::LUC is identical to puY1::LUC except for a point mutation changing the uORF AUG codon to AAG.

or by modifying the non-destabilizing YAP1-type uORF through addition of sequence elements normally associated with the destabilizing class of uORF (Fig. 6C and D). In the latter case we have achieved partial conversion from the YAP1-type to the YAP2-type by modifying the 5' and 3' sequence contexts, as well as the penultimate codon, of the YAP1 uORF. As with the inhibitory influence on translation of the uORFs (Fig. 5A), full conversion is achieved by complete replacement of the uORF itself. In all of these experiments there was a correlation between the half-lives of the mRNAs and their steady-state levels in the cell (compare Fig. 2), thus confirming the critical role of uORF-mediated modulation of stability in controlling mRNA abundance.

**DISCUSSION**

**uORFs mediate post-transcriptional control of the YAP stress-response mRNAs**

The present work has established that the YAP mRNAs are subject to two different kinds of differential control at the post-transcriptional level. First, the YAP1 and YAP2 uORFs represent distinct functional classes, the YAP1-type allowing scanning 40S subunits to proceed via leaky scanning and re-initiation to the major ORF, the YAP2-type acting to block ribosomal scanning by promoting efficient termination. Second, we have found that the overall post-transcriptional control of YAP2 also involves a form of mRNA destabilization which is linked to the fates of post-termination ribosomes that have translated the uORFs. The YAP2 mRNA provides a precedent for destabilization linked to translational termination on the 5'-UTR of a natural mRNA. It also constitutes the first example of how this form of post-transcriptional control can determine the capacity of the yeast cell to respond to stress. The wild-type chromosomal YAP genes are transcribed from weaker promoters than those used in this study. The influence of the post-transcriptional mechanisms we have described will be at least as significant at these low mRNA levels, thus strongly influencing the tuning of the yeast stress response.

In this study we have focused on the short uORFs that lie within the respective YAP leaders. We have demonstrated that the properties of these uORFs and the control elements associated with them are transferable to different genes and can operate in alternative leader environments. Consequently, we have characterized transferable functional elements that can act generally without any requirement...
Figure 6. The uORFs are important determinants of mRNA stability. Northern blots (A, C and E) show the results of hybridization using RNA preparations from strains SWP154 (+) (UPF1+) and SWP154 (−) (upf1−) taken at various time points during half-life determination experiments. The wild-type endogenous PGK1 mRNA was used as an internal control and is shown as an example in (A). The radioactivities of the respective bands obtained with the respective constructs in UPF1+ (full symbols) and upf1− (open symbols) strains were expressed as a ratio to the corresponding PGK1 mRNA values and plotted as logarithm (y-axis) versus time (B, D and F). The plotted data and estimated values represent averages of measurements performed using at least three independent sets of RNA preparations (± standard deviations). The YAP2 uORFs exert a strong destabilizing effect in a largely UPF1-independent fashion (A and B, E and F). Destabilization was also achieved by modifying the non-destabilizing YAP1-type of uORF through the addition of sequence elements associated with the destabilizing class of uORF (YAP2 uORF1 and GCN4 uORF4; see Fig. 4 and C and D). In contrast, the decay rate of the mRNAs containing the YAP1 5′-UTR is the same in UPF1+ or upf1− strains (A and B, C and D). The slower decay of pΔu(1+2)Y2 was assessed using 5 min (E) time points and only three of these points are plotted in (F). The decay rates of pΔu(1+2)Y2 in the upf− and UPF1 strains were so similar as not to be distinguishable in the small scale plot (F). The half-life of the cat mRNA with a leader bearing no uORFs was 7.5 min under these conditions of measurement (data not shown).

for additional elements within the body of the mRNA. In YAP2 the destabilizing effect described here is achieved via a combination of two uORFs. On the basis of the effects of the individual YAP2 uORFs on expression (Fig. 2) it might be expected that both uORF1 and uORF2 contribute to accelerated decay. This will need further investigation. Overall, short uORFs must be recognized as potent transferable agents of multi-level post-transcriptional control.

In emphasizing the destabilizing effects of the YAP2-type uORF it should not be forgotten that the YAP1-type uORF is far from being a passive passenger; its presence in the leader ensures that at least 50% of the ribosomal subunits reaching the main ORF have undergone one cycle of initiation/termination. As seen both in the present work and with the GCN4 system (9), this transition to post-termination status confers properties on these ribosomal subunits that are of critical significance for post-transcriptional control. The downstream elements in the mRNA determine how, and to what extent, these properties are 'harnessed' for the purposes of post-transcriptional regulation.

**Properties of the YAP2 leader mediating accelerated decay**

Our data indicate that the upf-linked decay pathway, which is used to rid the cell of aberrant mRNAs containing premature stop codons (46), is not the major agent in the differential control of these non-aberrant mRNAs in the yeast cell. However, we cannot rule out that there can be partial involvement of a upf-related mechanism. In order to be able to make accurate and simultaneous determinations of both translation and decay we have performed our experiments using both YAP and reporter mRNAs all of which have relatively short half-lives. Further work using other, longer lived transcripts should help establish whether uORF-mediated destabilization is fully independent of the upf-related pathway in a range of different mRNAs. There is a distinct possibility that the
Table 2. Examples of uORF-containing leaders in *S. cerevisiae*

<table>
<thead>
<tr>
<th>Gene</th>
<th>Length of major 5′UTR</th>
<th>No. and size of uORFs (codons)</th>
<th>Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>CBS1</td>
<td>101</td>
<td>uORF (4)</td>
<td>PET gene involved in the 5′ end processing of the cytchrome 6 (51)</td>
</tr>
<tr>
<td>CPA1</td>
<td>244</td>
<td>uORF (26)</td>
<td>Small subunit of cytosolic carbamoyl phosphate synthetase (52)</td>
</tr>
<tr>
<td>DCD1</td>
<td>33</td>
<td>uORF (4)</td>
<td>dCMP deaminase (53)</td>
</tr>
<tr>
<td>GCN4</td>
<td>591</td>
<td>uORF1 (4) uORF2 (3) uORF3 (4) uORF4 (4)</td>
<td>Transcriptional activator of amino acid biosynthetic pathway (18)</td>
</tr>
<tr>
<td>HAP4</td>
<td>−280</td>
<td>uORF1 (10) uORF2 (4)</td>
<td>Subunit of transcriptional activator complex binding CCAAT (54)</td>
</tr>
<tr>
<td>HOL1</td>
<td>−385</td>
<td>uORF (6)</td>
<td>Major facilitator family (drug resistance subfamily) of putative transport proteins (55)</td>
</tr>
<tr>
<td>LEU4</td>
<td>85</td>
<td>uORF1 (13)</td>
<td>a-isopropylmalate synthase (cytoplasmic) (56)</td>
</tr>
<tr>
<td>PET11I</td>
<td>459</td>
<td>uORF1 (6) uORF2 (31) uORF3 (11) uORF4 (30)</td>
<td>Mitochondrial translational activator (57)</td>
</tr>
<tr>
<td>PPR1</td>
<td>50</td>
<td>uORF (6)</td>
<td>Regulatory protein controlling transcription of two genes in pyrimidine biosynthesis pathway (58)</td>
</tr>
<tr>
<td>SCH09</td>
<td>−600</td>
<td>uORF (55)</td>
<td>Protein kinase that positively regulates the progression of yeast through G1 phase (59)</td>
</tr>
<tr>
<td>SCO1</td>
<td>−150</td>
<td>uORF (3)</td>
<td>PET gene involved in the accumulation of cytochrome c oxidase subunits I and II (60)</td>
</tr>
<tr>
<td>TIF 4631</td>
<td>295</td>
<td>uORF1 (12) uORF2 (20) uORF3 (16) uORF4 (8) uORF5 (12) uORF6 (22)</td>
<td>Translation initiator factor p150 (61)</td>
</tr>
<tr>
<td>YAP1</td>
<td>164</td>
<td>uORF (7)</td>
<td>Stress related transcription factor (17)</td>
</tr>
<tr>
<td>YAP2</td>
<td>157</td>
<td>uORF1 (6) uORF2 (23)</td>
<td>Stress related transcription factor (15)</td>
</tr>
</tbody>
</table>

**uORFs are a widespread feature of eukaryotic transcriptomes**

uORFs are a widespread feature of eukaryotic transcriptomes. A pertinent feature of uORFs is the fact that they are readily incorporated into mRNAs via a limited number of nucleotide changes, an aspect that was explored in a recent study using a synthetic 5′-UTR (12). Thus the cell has a flexible regulatory device at its disposal that can evolve to modulate translation and/or mRNA stability to various, potentially regulatable degrees. It is therefore significant that inspection of the characterized *S. cerevisiae* genes reveals the presence of a sizable group of uORF-containing mRNAs (Table 2). Many of these have regulatory functions in the cell. Other analyses suggest that there are likely to be at least 200 uORF-containing mRNAs in the *S. cerevisiae* transcriptome, which comprises a total of ~6000 mRNA species (data not

However, the role of the uORF in the destabilizing potential of the *PPR1* leader is uncertain. There was no change in stability of the *PPR1* mRNA when the two upstream AUGs in its 5′-UTR were mutated to AGGs (50). On the other hand, fusion of this leader with the *PGK1* gene so that the overlapping uORF is preserved in the same configuration was found to generate a highly unstable mRNA (13). It will therefore be necessary to determine the role of the *PPR1* main reading frame in the decay process before the function of this overlapping uORF can be resolved.

**uORF-dependent and non-uORF-dependent pathways can contribute to differing degrees to the degradation of individual mRNAs.**

It has been proposed that destabilization via the nonsense-dependent pathway requires the presence of a specific motif downstream of the stop codon (14,46,47). This sequence motif (TGYYGATGYYYY) has been suggested to support re-initiation (48) and/or pausing of 40S ribosomal subunits (49), thereby triggering accelerated decay via an as yet unknown mechanism. Peltz and colleagues have also proposed that a uORF needs to be followed by such an element in order to destabilize mRNA and that it may act as a binding site for an as yet unknown factor (49). Recent reports have rejected the need for either AUG within the motif or for re-initiation (47,49). We find no evidence that the Peltz type of motif is required for the destabilization effect exercised by the natural *YAP2* uORF1. It is not identifiable 3′ of the *YAP2* uORF1 in its natural leader (Fig. 1). This again suggests that the uORF-dependent destabilization described in this paper is attributable to a different mechanism to that proposed to act in the nonsense-dependent decay of aberrant mRNAs.

It is informative to compare the results of studies with one other yeast 5′-UTR that has been found to have transferable destabilizing properties. This is the leader of the very short-lived *PPR1* mRNA (50). The *PPR1* 5′-UTR has a 6 codon uORF that overlaps +1 at its 3′-end (AUA UGA) with the start codon of the main ORF.
shown). Given the regulatory function of many of these mRNAs, it becomes clear that uORFs are likely to make a major contribution to the post-transcriptional control of the yeast genome.

Finally, the present work has established the basic principles of action of the YAP uORFs. Future work will allow us to explore the mechanistic details of destabilization related to termination in the 5′-UTR and how these are involved in controlling the yeast stress response. In a wider context, it is evident that termination is not simply the end point of polypeptide synthesis. It is also the beginning of a series of post-termination events of general significance to the control of cellular gene expression. Further studies of natural uORF-dependent control should continue to advance our understanding as to how termination can function as a regulatable branch point leading to alternative pathways of translational (re-)initiation and degradative processes involving non-aberrant mRNAs.

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