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

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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 influences 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 many 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 acetyl-transferase 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

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pathway previously shown to be responsible for accelerated decay of effectively aberrant mRNAs containing premature stop codons (13,14). However, none of these studies has concerned fully wild-type mRNAs, thus leaving open the question as to the physiological relevance of uORF-dependent modulation in terms of natural transcripts.

Conventional sequencing of selected genes originally identified a number of *S.cerevisiae* mRNAs that contain uORFs (4,15–19) and the more recent results of the yeast genome sequencing project have increased the number of known cases considerably. Moreover, a large percentage of these genes encode regulatory proteins (6,20), suggesting a possible common mechanism of post-transcriptional control of these genes' expression, mediated by the upstream regulatory sequences. Two striking examples of uORF-containing mRNAs encode Yap1p and Yap2p (15,17,21,22), both of which manifest strong homology to AP1-like factors in complex eukaryotes and to Gcn4p in yeast. Like the *GCN4* gene, *YAP1* and *YAP2* are also regulatory genes involved in the mechanisms used by the yeast cell to protect itself in situations of (severe) stress. Overexpression of the two related *YAP1* and *YAP2* genes confers general stress resistance to a variety of unrelated compounds, from metal ions to different inhibitors and drugs (15,22–28). Loss of function mutations in *YAP1* result in hypersensitivity to hydrogen peroxide, suggesting a role in the cellular response to oxidative stress. The identification of three direct targets of Yap1p, *TRX2* (29), *GSH1* (30) and *GLR1* (31), encoding thioredoxin, γ-glutamylcysteine synthetase and glutathione reductase respectively, might explain the function of this factor in this type of stress response. Another gene, *YCF1* (32), also under the transcriptional activation of only Yap1p, was shown to encode an ATP binding cassette transporter which is involved in cadmium tolerance. In contrast, the exact cellular role of Yap2p is still poorly understood. Although overexpression of *YAP2* also allows cells to grow in the presence of several stress agents, the resistant phenotypes associated with Yap2p are less marked. It is likely that each *YAP* gene is regulated in a distinct and specific manner. In this context, elucidation of the processes that control the activity of these *trans*-acting factors is essential to our understanding of the complex mechanisms underlying this particular stress response.

The present paper describes the forms of post-transcriptional control modulating expression of the *YAP* mRNAs. The uORFs of *YAP1* and *YAP2* mediate differential mechanisms of post-transcriptional control of gene expression: ribosomes on the *YAP1* 5′-UTR can both translate the uORF and proceed to translate the main ORF, whereas the *YAP2* uORFs act as strong translational barriers. Moreover, this work reveals a novel mechanism of post-transcriptional control affecting a physiologically normal mRNA: the *YAP2* uORFs also attenuate this mRNA's expression by acting as mRNA destabilizing elements. We investigate the mechanism of this principle of post-transcriptional control by examining how the sequences of naturally occurring uORFs and their contexts in the 5′-UTR determine their functions in controlling translation and mRNA decay.

MATERIALS AND METHODS

Yeast strains, growth conditions and transformation procedures

The yeast strains used in this study were SWP154 (MAT**a** *trp1-Δ1 upf1*::*URA3 leu2-1 his4-38 ura3-52 rpb1-1*; 33), SWP154 (+) (MAT**a** *trp1-Δ1 upf1*::*URA3 leu2-1 his4-38 ura3-52 rpb1-1*<*UPF1 TRP1 CEN*>; 34) and yLF41 [FT4 (MAT**a** *leu2*Δ::*PET56 his3-Δ200 trp1-Δ63 ura3-52*) Δ*gcn4*Δ*yap1*; 35]. The *Escherichia coli* TG2 strain [*supE hsd*Δ5 *thi* Δ(*lac-pro*) *AB* Δ(*srl-recA*)306::Tn*10* (*tet*^{r}) F′ (*traD36 proAB*$^{+}$ *lacI*^{q} *lac*ZΔM15)] 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 *rpb*1-1 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 (*rpb*1-1) 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 Phosphor-imager 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 A_{600} = 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 YCpSUPEX1 (*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 *Nde*I and *Xba*I 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 *Bam*HI and *Nde*I were introduced by PCR at the 5′- and 3′-ends of *YAP1* and *YAP2* leaders and subsequently cloned into the YCp22FL vector, creating the constructs p*Y1* and p*Y2*. The *YAP* uORFs were mutated by a single base change (AUG→AAG), generating constructs pΔu*Y1*, pΔu1*Y2*, pΔu2*Y2* and pΔu(1+2)*Y2*. In addition, unique *Bgl*II and *Xho*I restriction sites were introduced at positions +72 and +112 of the *YAP1* leader respectively to flank

Table 1. Key to the plasmid nomenclature[a]

Plasmid designation	Description
pu*Y1*	*YAP1* leader: wt *YAP1* uORF
pΔu*Y1*	*YAP1* leader: elimination (Δ) of *YAP1* uORF (AUG → AAG)
pu*Y2*	*YAP2* leader: wt *YAP2* uORF1 and uORF2
pΔu1*Y2*	*YAP2* leader: wt *YAP2* uORF2; elimination (Δ) of *YAP2* uORF1 (AUG → AAG)
pΔu2*Y2*	*YAP2* leader: wt *YAP2* uORF1; elimination (Δ) of *YAP2* uORF2 (AUG → AAG)
pΔu(1+2)*Y2*	*YAP2* leader: elimination (Δ) of *YAP2* uORF1 and uORF2 (AUG → AAG)
pu*Y1*du4*G4*	*YAP1* leader: *YAP1* uORF; 10nt downstream sequence of *GCN4* uORF4
pmu*Y1*du4*G4*	*YAP1* leader: mutant *YAP1* uORF: codon 6 of *YAP1* uORF → codon 2 of *GCN4* uORF4; 10nt downstream sequence of *GCN4* uORF4
pAu*Y1*du4*G4*	*YAP1* leader: *YAP1* uORF -3 position changed from T to A; 10nt downstream sequence of *GCN4* uORF4
pAmu*Y1*du4*G4*	*YAP1* leader: *YAP1* uORF -3 position changed from T to A; mutant *YAP1* uORF: codon 6 of *YAP1* uORF → codon 2 of *GCN4* uORF4; 10nt downstream sequence of *GCN4* uORF4
pAu*Y1*	*YAP1* leader: *YAP1* uORF -3 position changed from T to A
pu4*G4*	*YAP1* leader: wt *GCN4* uORF4
pu*Y1*du1*Y2*	*YAP1* leader: wt *YAP1* uORF; 10nt downstream sequence of *YAP2* uORF1
pu*Y1*:*LUC*	*YAP1* leader containing mutations of 3 in-frame stop codons; *YAP1* uORF overlapping (-1) with *LUC*
pu*Y1*Δ:*LUC*	*YAP1* leader containing mutations of 3 in frame stop codons; *YAP1* uORF overlapping (-1) with *LUC*; mutation of *YAP1* uORF start codon

[a]All plasmids used in this study are given in the above list

the *YAP1* uORF in order to facilitate cassette mutagenesis of the uORFs and surrounding sequences. In the case of the *YAP2* uORFs the latter purpose was achieved by insertion of a unique *Bgl*II site at position +93 of the *YAP2* leader. pu*Y1*du4*G4* (Fig. 5A) contains the 10 nt sequence immediately 3′ of the *GCN4* uORF4 stop codon downstream of the *YAP1* uORF. A derivative of pu*Y1*du4*G4* was generated by replacing the last sense codon of the *YAP1* uORF by the corresponding codon of *GCN4* uORF4, creating pmu*Y1*Δdu4*G4*. pAu*Y1*du4*G4* and pAmu*Y1*du4*G4* are identical to pu*Y1*du4*G4* and pmu*Y1*du4*G4* respectively except that the codon immediately upstream of the *YAP1* uORF start codon was replaced by AGC, creating a favourable context. pAu*Y1* was used as a control for pAu*Y1*du4*G4* and pAmu-*Y1*Δdu4*G4* and was constructed from p*Y1* by insertion of the same AGC codon immediately upstream of the *YAP1* uORF. Also, as a control, the *YAP1* uORF was replaced by *GCN4* uORF4, generating pu4*G4*. The 10 nt downstream of the *YAP2* uORF1 stop codon were also inserted 3′ of the *YAP1* uORF in pu*Y1*, creating pu*Y1*du1*Y2*. The *YAP1* uORF–*LUC* overlap construct pu*Y1*::*LUC* (Fig. 5B) was derived from construct pu*Y1*, 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). pu*Y1*Δ::*LUC* is identical to pu*Y1*::*LUC* except for a T→A substitution which changes the ATG codon of the *YAP1* uORF to AAG.

Spot test assay

The *yap1* transformants expressing different levels of the *YAP* genes [pu*Y1*, pu*Y1*Δ, pAu*Y1*Δdu4*G4*, pu*Y2* and pu*Y2*Δ(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 Cd^{2+} 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 *YAP1* 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 *YAP1* 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 positioned –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

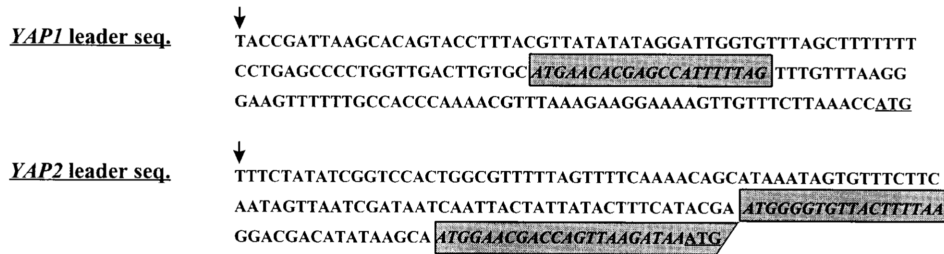


Figure 1. The 5′-UTRs of the *YAP1* and *YAP2* mRNAs contain uORFs. The 5′-UTRs of *YAP1* and *YAP2* contain one and two upstream open reading frames (solid boxes) respectively. The *YAP1* leader has one 7 codon uORF, whereas the *YAP2* leader has one 6 codon uORF (uORF1) and an overlapping reading frame (uORF2) of 23 codons. The transcription start sites are indicated by arrows and the initiator codons of the main reading frames are underlined.

blotting techniques (data not shown). Indeed, SAGE (serial analysis of gene expression) analysis (42) showed that the *YAP* mRNAs are present at very low levels (~0.5 mRNA molecules/cell; 43). For the purposes of the present work it was important to be able to accurately assess the quantitative influence of the *YAP* leaders on gene expression. We therefore combined the respective leader sequences with the *LUC* gene (Fig. 2), while the same 5′-UTRs were tested for their ability to influence stress responses mediated by the *YAP* genes (Fig. 3). Moreover, these leader–gene combinations were expressed using a modular form of the *TEF1* promoter (P_{TEF1}), which supports an increased level of transcription (41). We investigated the effects of mutating each of the start codons of the individual uORFs to AAG. The *YAP* uORFs mediated totally different effects on expression of the downstream genes: whereas in the case of *YAP1* the presence of the uORF has a very small inhibitory effect on expression of luciferase, *YAP2* uORF1 and *YAP2* uORF2 act as strong translational barriers. Moreover, although the first *YAP2* uORF mutant partly abolishes repression, only the double mutation also involving the second initiation codon allows efficient expression of *LUC* (Fig. 2). In polysomal gradient experiments removal of the two *YAP2* uORFs was also shown to result in a major shift in the localization of *YAP2* mRNA from monosomes to polysomes (data not shown). Measurements of the steady-state mRNA levels corresponding to the respective constructs revealed that these were also strongly affected by the *YAP2* uORFs. Correction of the luciferase activities for these mRNA levels allowed us to estimate the component of change in each case that was attributable only to translational modulation. For the sake of comparison the mRNA levels and corrected luciferase values were normalized to those of the *YAP1* leader construct (pu*Y1*). This resulted in increased relative luciferase values for the *YAP2* leader constructs after correction.

Examination of the results obtained with the equivalent *YAP* constructs reveals a consistent picture. Removal of the *YAP1*

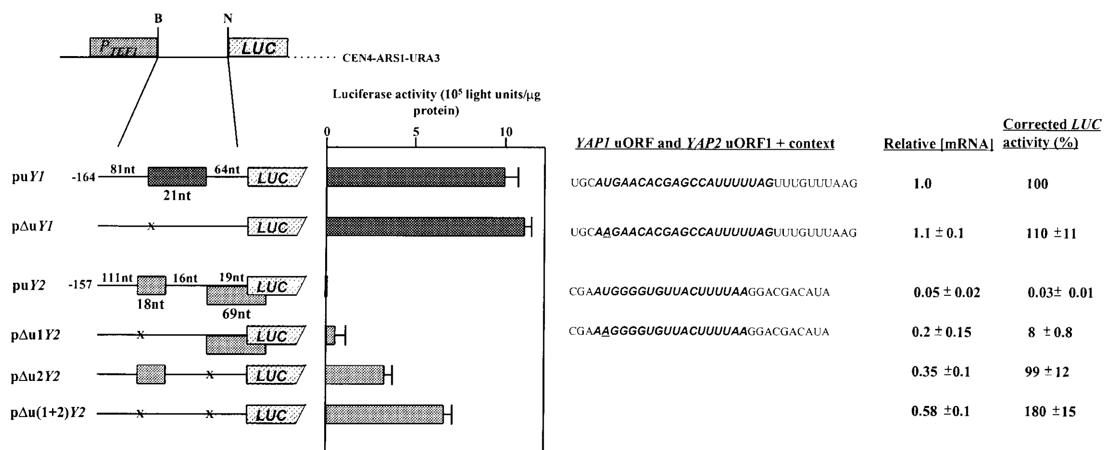


Figure 2. Differential control of gene expression by the *YAP* uORFs. Schematic representations indicate how the *YAP* leaders and their derivatives were combined with the *LUC* gene. The plasmid designations employed throughout this paper conform to a systematic terminology that reflects the identities and order of the components used in their construction (see Table 1). The restriction sites *Bam*HI (B) and *Nde*I (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. *YAP2* uORF2 overlapped –1 relative to the *LUC* reading frame. 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 pu*Y1*.

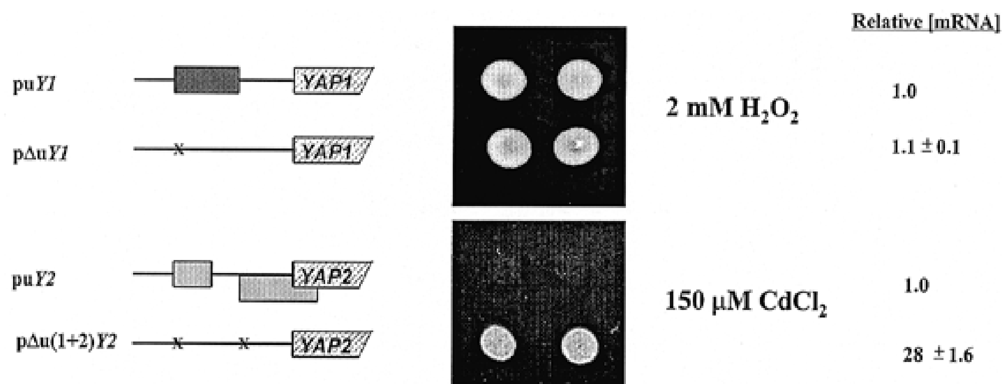


Figure 3. The roles of the respective *YAP* uORFs in controlling yeast stress responses. The figure shows the ability of a *yap1* strain expressing different levels of *YAP1* and *YAP2* [compare constructs pu*Y1* and pΔu*Y1* and pu*Y2* and pΔu(1+2)*Y2* in Fig. 2] to tolerate growth in the presence of Cd^{2+} and H_2O_2. Equal numbers of cells were spotted (in duplicate) onto minimal medium plates containing 150 μM Cd^{2+} and 2 mM H_2O_2 respectively and cultured at 30°C for 48 h. No detectable growth was observed in cells transformed only with the *URA3*-containing plasmid (data not shown). The relative mRNA abundance (average of three independent measurements) for each construct is also tabulated (together with standard deviations) on the right hand side.

uORF has little effect on the resistance of yeast to H_2O_2, 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 pu*Y2* and pΔu(1+2)*Y2*] 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.

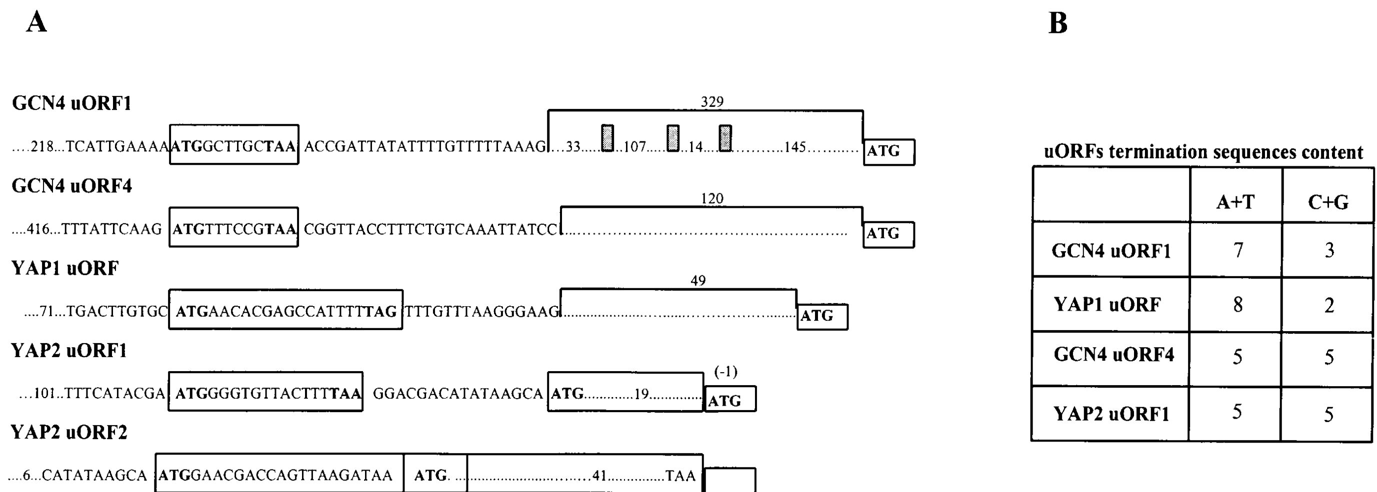
In the remainder of this paper we explore the causes of these differences in uORF function between the *YAP* genes. Given that both translational and mRNA decay effects can contribute to changes in post-transcriptional gene expression, we have analysed the influence of the *YAP* leaders on both translation 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 pu*Y1*du4*G4* 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 (pmu*Y1*du4*G4*). Furthermore, when the recognition context of the uORF start codon was improved in these constructs by changing the T at –3 to A (pAu*Y1*du4*G4* and pAmu*Y1*du4*G4*) 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 pAu*Y1*). In a further comparative experiment the *YAP1* uORF was replaced entirely by *GCN4* uORF4 (pu4*G4*), 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* uORF1 downstream sequence by the corresponding sequence from *YAP2* uORF1 (pu*Y1*du1*Y2*) resulted in an inhibitory effect equivalent to that obtained using the *GCN4* uORF4 downstream sequence (pu*Y1*du4*G4*).

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



	A+T	C+G
GCN4 uORF1	7	3
YAP1 uORF	8	2
GCN4 uORF4	5	5
YAP2 uORF1	5	5

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).

natural *YAP1* leader between the uORF and the main reading frame generated a –1 overlapping reading frame initiating at the uORF start codon and terminating at position +80 with respect to the start codon of the *LUC* coding region (Fig. 5B, pu*Y1*::*LUC*). There was strong inhibition of *LUC* expression from this construct. This inhibition was removed when the uORF start codon was mutated to AAG (pu*Y1*Δ::*LUC*). This indicates that the *YAP1* uORF is translated sufficiently well to be capable of diverting a considerable proportion of scanning ribosomes on the *YAP1* leader into translation of the –1 reading frame which, if extended, results in termination downstream of the main reading frame start codon, thus inhibiting the latter's translation. It follows that the *YAP1* uORF is not ignored by scanning ribosomes and that a large proportion of the 40S subunits initiating on the main reading frame have re-initiated subsequent to termination on this uORF.

Examination of the relative steady-state levels of mRNA again showed that the presence of an inhibitory uORF in the leader reduced the abundance of the mRNA. For example, the level of pAmu*Y1*du4G4 mRNA (Fig. 5A) was reduced by at least 30% relative to the corresponding value for pu*Y1* (data not shown), despite the fact that the uORF was only partially converted to a *YAP2*-type uORF. As with the results presented in Figure 2, this indicated that the YAP2-type uORF influences either transcription or mRNA decay and we therefore investigated this phenomenon further.

The *YAP2* type of uORF accelerates mRNA decay

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 *MFA2* (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 (pu*Y1*) and 1.7 min for the *YAP2* leader (pu*Y2*; data not shown). Overall, the fact that destabilization of the *YAP* and reporter mRNAs occurs primarily via a *upf*-independent pathway rules out that these natural uORFs are merely triggering the nonsense-dependent decay pathway (46).

The destabilizing effect can be achieved in two ways: either by using the naturally occurring *YAP2*-type uORF (Fig. 6A and B)

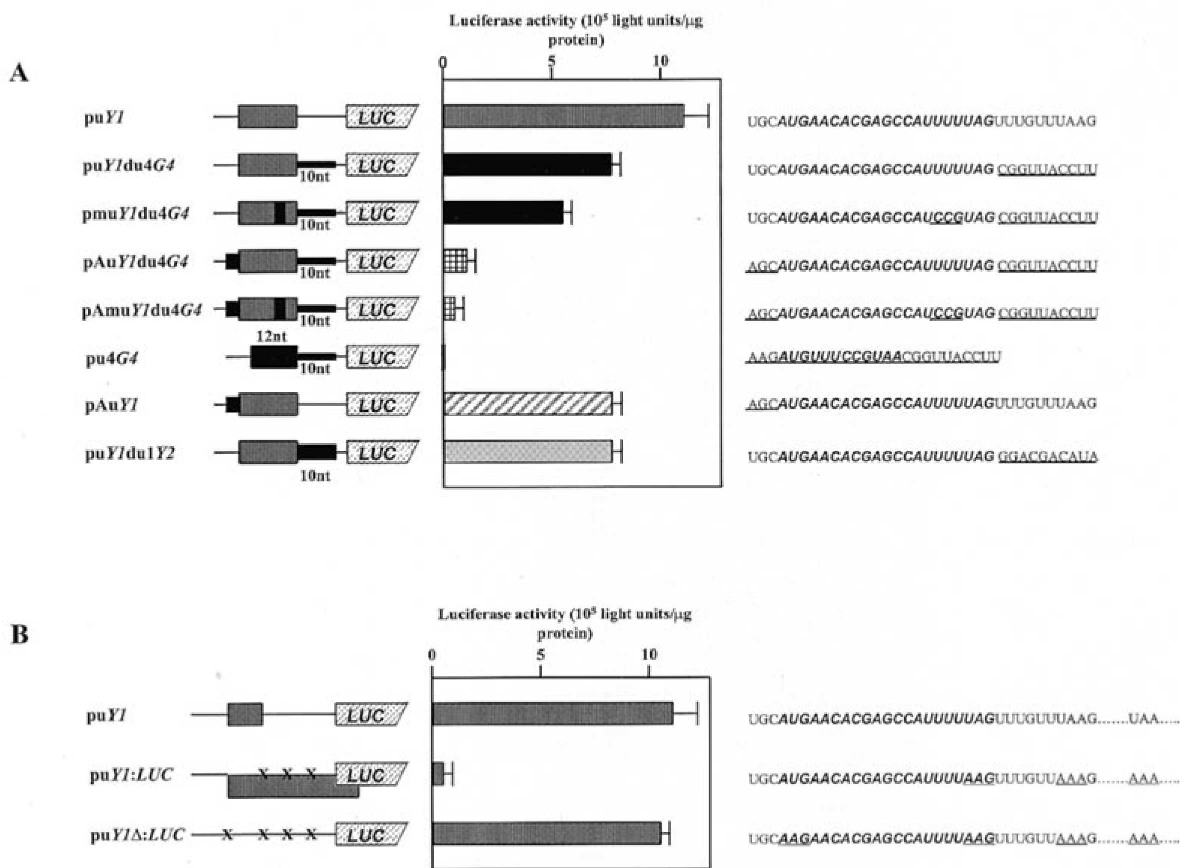


Figure 5. The sequence and nucleotide context of the *YAP* uORFs determine their effects on gene expression. (**A**) Replacement of the *YAP1* uORF downstream sequence and last codon with the corresponding G/C-rich sequences of *GCN4* uORF4 [solid (10 nt) black line and bar within the uORF respectively] or *YAP2* uORF1 (solid downstream black line) results in inhibition of *LUC* translation (see pu*Y1*du4*G4*, pmu*Y1*du4*G4* and pu*Y1*du1*Y2*). This effect is enhanced by improving the upstream context of the uORF start codon (solid upstream bar in pAu*Y1*du4*G4* and pAmu*Y1*du4*G4*). The constructs pAu*Y1*, in which the recognition efficiency of the wild-type *YAP1* uORF was improved, and pu4*G4*, 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. pu*Y1*::*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. pu*Y1*Δ::*LUC* is identical to pu*Y1*::*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

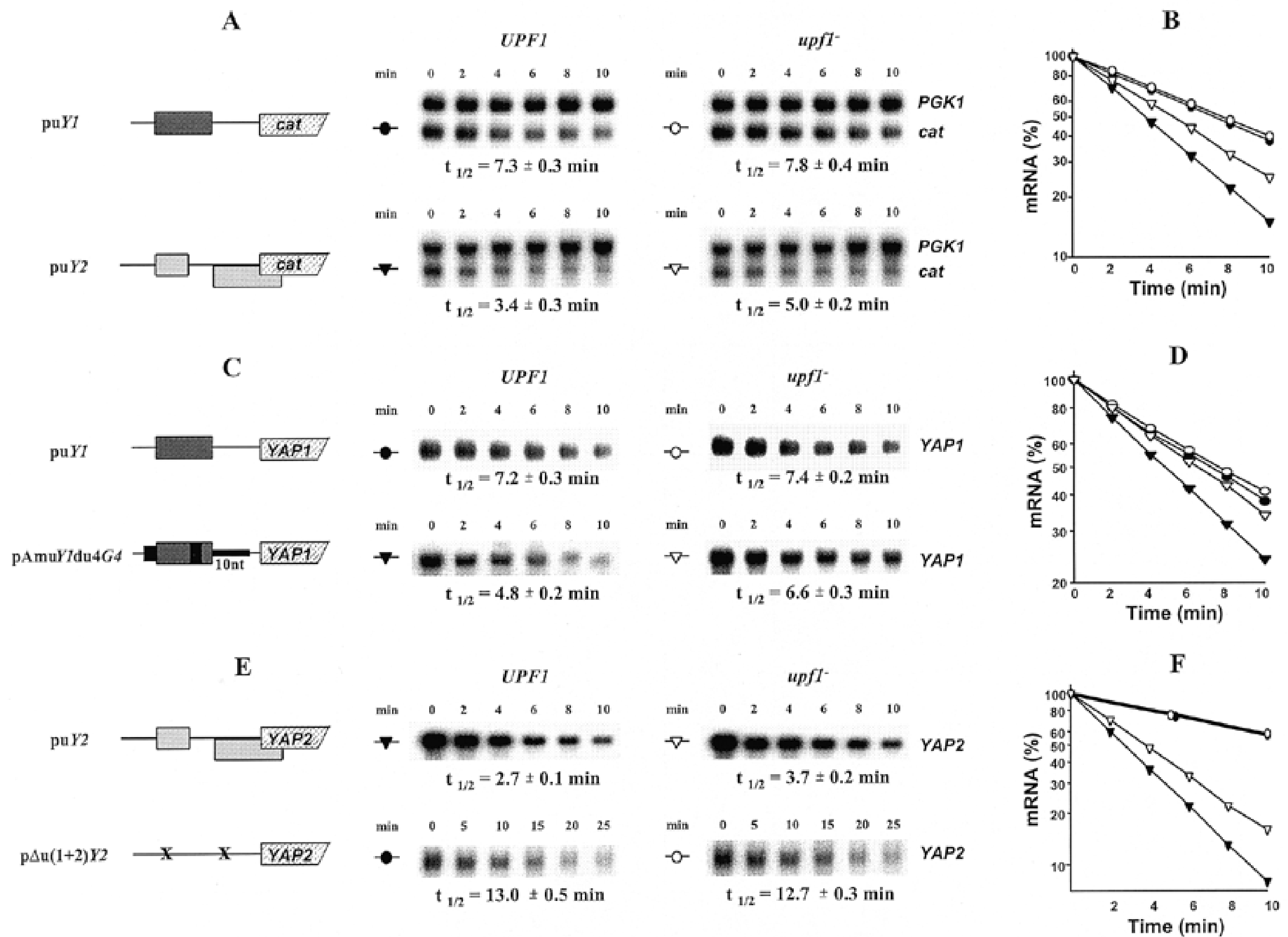


Figure 6. The uORFs are important determinants of mRNA stability. Northern blots (**A**, **C** and **E**) show the results of hybridization using RNA preparations from strains SWP154 (+) (*UPF1*$^+$) and SWP154 (–) (*upf1*$^-$) taken at various time points during half-life determination experiments. 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*

Gene	Length of major 5'UTR	No. and size of uORFs (codons)	Product
CBS1	101	uORF (4)	*PET* gene envolved in the 5'end processing of the cytochrome *b* (51)
CPA1	244	uORF (26)	Small subunit of cytosolic carbamoyl phosphate synthetase (52)
DCD1	33	uORF (4)	dCMP deaminase (53)
GCN4	591	uORF1 (4) uORF2 (3) uORF3 (4) uORF4 (4)	Transcriptional activator of amino acid biosynthetic pathway (18)
HAP4	~280	uORF1 (10) uORF2 (4)	Subunit of transcriptional activator complex binding CCAAT (54)
HOL1	~385	uORF (6)	Major facilitator family (drug resistance subfamily) of putative transport proteins (55)
LEU4	85	uORF1 (13)	a-isopropylmalate synthase (cytoplasmic) (56)
PET111	459	uORF1 (6) uORF2 (31) uORF3 (11) uORF4 (30)	Mitochondrial translational activator (57)
PPR1	50	uORF (6)	Regulatory protein controlling transcription of two genes in pyrimidine biosynthesis pathway (58)
SCHO9	~600	uORF (55)	Protein kinase that positively regulates the progression of yeast through G1 phase (59)
SCO1	~150	uORF (3)	*PET* gene involved in the accumulation of cytochrome c oxidase subunits I and II (60)
TIF 4631	295	uORF1 (12) uORF2 (20) uORF3 (16) uORF4 (8) uORF5 (12) uORF6 (22)	Translation initiator factor p150 (61)
YAP1	164	uORF (7)	Stress related transcription factor (17)
YAP2	157	uORF1 (6) uORF2 (23)	Stress related transcription factor (15)

upf-dependent and non-*upf*-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 (TGYYGATGYYYYY) 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. 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.

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

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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