Physical evidence for distinct mechanisms of translational control by upstream open reading frames

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The *Saccharomyces cerevisiae* GCN4 mRNA 5'-leader contains four upstream open reading frames (uORFs) and the CPA1 leader contains a single uORF. To determine how these uORFs control translation, we examined mRNAs containing these leaders in cell-free translation extracts to determine where ribosomes were loaded first and where they were loaded during steady-state translation. Ribosomes predominantly loaded first at GCN4 uORF1. Following its translation, but not the translation of uORF4, they efficiently reinitiated protein synthesis at Gcn4p. Adding purified eIF2 increased reinitiation at uORFs 3 or 4 and reduced reinitiation at Gcn4p. This indicates that eIF2 affects the site of reinitiation following translation of GCN4 uORF1 *in vitro*. In contrast, for mRNA containing the CPA1 uORF, ribosomes reached the downstream start codon by scanning past the uORF. Addition of arginine caused ribosomes that had synthesized the uORF polypeptide to stall at its termination codon, reducing loading at the downstream start codon, apparently by blocking scanning ribosomes, and not by affecting reinitiation. The GCN4 and CPA1 uORFs thus control translation in fundamentally different ways.

Keywords: *Neurospora*/ribosome reinitiation/ribosome scanning/*Saccharomyces*/uORF

Introduction

Upstream open reading frames (uORFs) in mRNAs regulate translation in prokaryotes and eukaryotes (Lovett and Rogers, 1996; Geballe and Sachs, 2000; Morris and Geballe, 2000; Gong and Yanofsky, 2001). The best-understood examples of regulatory uORFs in fungi are in the 5'-leaders of transcripts whose products are

involved in amino acid biosynthesis. These are the multiple uORFs in the transcript of *Saccharomyces cerevisiae* GCN4, and an evolutionarily conserved single uORF in the mRNAs specifying the small subunit of carbamoyl phosphate synthetase (*S.cerevisiae* CPA1 and *Neurospora crassa* arg-2).

The *S.cerevisiae* GCN4 gene encodes a transcriptional activator that positively controls the expression of genes in response to amino acid limitation or imbalance as well as to other stresses (Hinnebusch, 1996, 1997). The GCN4 mRNA contains four uORFs (uORFs 1–4) that are responsible for conferring translational regulation (derepression) in response to amino acid limitation (Hinnebusch, 1996, 1997). Data from many experiments *in vivo* support a model for uORF control in which ribosomes initiate efficiently at uORF1 and then reinitiate downstream. Under repressing conditions, the level of functional eIF2 is high and ribosomes reinitiate at the starts of downstream uORFs in preference to Gcn4p. Under derepressing conditions, ribosomes reinitiate at Gcn4p instead of downstream uORFs because the level of functional eIF2 is reduced by phosphorylation of the α -subunit (Hinnebusch, 2000). In the absence of uORF2 and uORF3, near wild-type regulation is observed, indicating that these uORFs are not central to this regulatory process.

While the GCN4 system has served as the paradigm for the control of reinitiation, the evidence for reinitiation has been entirely genetic in nature and is open to alternative explanations. Thus, there was a strong need to test the genetic model with biochemical measurements. Here, by mapping the positions of ribosomes translating mRNA *in vitro* using toeprinting, and by using cycloheximide to identify the first initiation events occurring on an mRNA and initiation events occurring during steady-state translation, we observe that uORF1 translation enables reinitiation, and that increasing the level of functional eIF2 increases reinitiation at the downstream uORFs and decreases reinitiation at Gcn4p. These are the first *in vitro* data supporting the mechanism of reinitiation deduced from genetic data.

Saccharomyces cerevisiae CPA1 contains a single uORF encoding the arginine attenuator peptide (AAP). The translation of this uORF reduces Cpa1p synthesis in response to Arg surplus (Werner *et al.*, 1987). The sequence of the uORF-encoded peptide is critical for regulation (Werner *et al.*, 1987; Delbecq *et al.*, 1994, 2000; Wang *et al.*, 1999), in contrast to the situation for GCN4, in which the uORF-encoded peptide sequences are not important for regulation (Hinnebusch, 1996). This is because translation of the uORF-encoded AAP causes ribosomes to stall when the level of Arg is high (Wang *et al.*, 1999). The AAP sequence is highly conserved and each of the known fungal AAPs causes ribosomes to stall

...TAATACGACTCACTATAGATCG

GATCCACAAAACAAACAAACAAACAAACAACTCACACACAGGTACTCTCCCCCTAAATCAAATTTTTTTTGGCCATCAGTTT

CACTAGCGAATTATACAACCTACCAGCCACACAGCTCACTCATCTACTTCGCAATCAAAACAAATATTTTATTTTAGTTTCAGTTTAT

TAAGTTATTATCAGTATCGTATTAAAAATTAAAGATCATTTGAAAAATGGCTTGCCTAAACCGATTATATTTTGTGTTTAAAGTAGATT

ATTATTAGAAAATTATTAAGAGAATTATGTGTTAAATTTATTGAAAGAGAAAATTTATTTCCCTTATTAATTAAAGTCCTTTACTTT

TTTGTAAACTGTCAGTTTTTTGAAGAGTTATTTGTTTTGTTACCAATTGCTATCATGTACCCGTAGAATTTTATTCAGATGTTTCC

GTAACGGTTACCTTTCTGTCAAATTATCCAGGTTTACTCGCCAATAAAAAATTTCCCTATACTATCATTAAATTAATCATTATTATTAC

TAAAGTTTGTGTTACCAATTGTCTGCTCAAGAAAATAAATTAAATACAAATAAATGTCGGAATATCAGCTTCGAGTCACCGACGCC

AAAAACATAAAGAAAGCGCCGCCATTCTATCCGCTGGAAGATGGAACCGCTGGAGAGCAACTGCATAAGGCTATGAAGAGATACG

CCCTGGTTCCTGGAACAATTGCTTTTACAGATGCACATATCGAGGTGGACATCACTTACGCTGAGTACTTCGAAATGTCCGTTTCG...

Z W 4

Z W 18

GCN4

LUC

uORF1

uORF2

uORF3

uORF4

Fig. 1. Sequences of the 5' leader regions of *GCN4*–*LUC* genes used in this study. The sequence shown begins with the T7 RNA polymerase-binding site and ends within the LUC coding region. The 5' and 3' boundaries of the wild-type *GCN4* sequence are boxed. *GCN4* uORFs and the *GCN4* ATG_{ORF} are shown in bold and mutations are indicated below the wild-type sequence. Horizontal arrows below the sequence indicate the sequences for which the reverse complements were synthesized and used as primers ZW4 and ZW18 for toeprint analysis.

in high Arg (Fang *et al.*, 2000). The proposed basis for this regulatory mechanism is that ribosomes stalled during translation of the uORF in high Arg block the access of scanning ribosomes to the downstream initiation codon. Analyses of the function of the *N.crassa arg-2* AAP in *N.crassa* cell-free extracts indicate that ribosomes do not reinitiate after translating this uORF, since increasing initiation at the *arg-2* uORF decreases translation initiation downstream (Wang and Sachs, 1997a). However, it has been pointed out that the situation could potentially be different in *S.cerevisiae* (McCarthy, 1998; Delbecq *et al.*, 2000). Here we show that, in *S.cerevisiae* extracts, while efficient reinitiation follows translation of *GCN4* uORF1, it does not efficiently follow the translation of the *CPA1* or *arg-2* uORFs. In contrast, much more leaky scanning is observed past the *CPA1* and *arg-2* uORFs than *GCN4* uORF1, and Arg-regulated ribosome stalling blocks leaky scanning past the *arg-2* and *CPA1* uORFs. Thus the *GCN4* and *CPA1* uORFs regulate translation in different ways.

Results

Ribosomes progress linearly through the *GCN4* 5'-leader and recognize each of the *GCN4* uORF initiation codons

The 5'-leader and the first five codons of *GCN4* were fused to firefly luciferase (LUC) in a vector designed for the production of capped and polyadenylated synthetic mRNA. T7 RNA polymerase was used to transcribe mRNA containing the wild-type 5'-leader with uORFs 1–4 and mRNAs containing mutations that eliminated the start codons of uORFs 2–4, uORFs 1 and 2, uORFs 1–3, uORFs 2 and 3, or uORFs 1–4 (Figure 1 and Table I). Equivalent

Table I. Constructs used in this study

Construct	5'-leader
pPG101	<i>GCN4</i> uORFs 1–4
pPG102	<i>GCN4</i> uORF1
pPG103	<i>GCN4</i> uORF4
pPG104	<i>GCN4</i> no uORFs
pPG105	<i>GCN4</i> uORFs 1 and 4
pAG112	<i>GCN4</i> uORFs 3 and 4
pAG101	<i>CPA1</i> uORF
pPR101	<i>arg-2</i> uORF
pPT101	↑ <i>arg-2</i> uORF ^a

^a↑*arg-2* uORF is in an improved initiation context (Wang and Sachs, 1997a).

molar amounts of each mRNA were used to program *S.cerevisiae* cell-free extracts and the synthesis of LUC was measured enzymatically (Table II). Relative to the translation of mRNA in which all four uORF start codons were eliminated, uORF1 by itself had only a small inhibitory effect, uORF4 by itself had a large inhibitory effect, and the presence of uORF1 reduced the inhibitory effect of uORF4. The effects of these uORFs on translation *in vitro* were similar to their effects *in vivo* in cells that were derepressed for amino acid biosynthesis as determined previously using *GCN4*–*lacZ* reporter genes (Table II). Similar results were obtained using *N.crassa* cell-free extracts, except that uORF4 was less repressive (data not shown). Elimination of uORF2 and uORF3 had only a modest effect when uORF1 and uORF4 were present either *in vitro* or *in vivo* (Table II). The effect of uORFs 2 and 3 was different *in vitro* and *in vivo*; mRNA containing uORFs 1–4 appeared more active *in vitro* than mRNA

Table II. Effects of *GCN4* uORFs *in vitro* and *in vivo*

uORFs ^a	LUC ^b	Ribosomes at <i>GCN4</i> AUG ^c	LacZ ^d
None	100	100	100
1–4	43 ± 3.2	34	28
1,4	35 ± 2.6	37	35
1	56 ± 4.1	51	63
4	12 ± 1	17	2

^aConstructs used for analyses of LUC synthesis *in vitro* are described in Table I.

^bThe relative amount of LUC produced in reaction mixtures relative to the amount produced by translation of the construct containing no uORFs, which was assigned a value of 100. The average values and the standard errors shown were derived from data representing nine independent experiments employing five different RNA preparations and seven different extract preparations. LUC activity was measured at time-points between 15 and 30 min of incubation, depending on the experiment. The relative order of LUC activities shown here (none > 1 > 1–4 > 1,4 > 4) held for all experiments except one, in which RNA containing uORFs 1–4 had similar activity to the construct containing uORF4.

^cThe measurements of ribosomes at the LUC AUG codon were obtained using ImageQuant to analyze the data in Figure 6 lanes 12, 14, 16, 18 and 20), with values normalized as for the LUC assays.

^dThe *GCN4*–*lacZ* expression data are from Hinnebusch (1996); expression values were normalized as for the LUC assays.

containing only 1 and 4, and the reverse was observed *in vivo*. The long *GCN4* 5'-leader was in itself not strongly inhibitory to translation initiation at the *Gcn4p* start codon. Direct comparison of LUC synthesis from the *GCN4*–LUC transcript lacking uORFs to synthesis from a control transcript containing the *S.cerevisiae* *CPA1* 5'-leader without a uORF initiation codon (183 nt) indicated that the *GCN4* leader reduced synthesis of LUC by a factor of two (data not shown).

These data from extracts were consistent with the model that translation of uORF1 enables subsequent reinitiation at *GCN4*, while translation of uORF4 does not (Hinnebusch, 1996, 1997). We therefore examined this directly using the toeprinting assay in which reverse transcriptase is used to extend a radiolabelled primer on an RNA template in translation extracts, enabling the mapping of the positions of ribosomes on RNA (Wang *et al.*, 1999 and references therein). Firstly, we determined that ribosomes could be mapped to each of the *GCN4* uORF initiation codons by toeprinting during 'steady-state' translation. In both *S.cerevisiae* (Figure 2) and *N.crassa* (Wang *et al.*, 1998; Fang *et al.*, 2000) extracts, the time-course of the appearance of completed sea pansy LUC and firefly LUC shows that completed LUC accumulates at a constant rate after an initial lag in which polypeptide elongation is occurring but full-length polypeptide has not been produced (determined by [³⁵S]Met-labelling, data not shown). There appear to be two steady-state phases of LUC accumulation in *S.cerevisiae* (10–20 min and 20–50 min) and a single steady-state phase in *N.crassa* extracts before the synthesis of LUC plateaus. We used reaction time-points between 5 and 15 min to represent steady-state conditions in which ribosomes were actively synthesizing polypeptides. We did this to minimize potential complications arising from mRNA degrading with time and to maximize similarities in the protocols used to analyze the

Fig. 2. Time-course of translation in *S.cerevisiae* programmed with RNAs encoding firefly LUC and sea pansy LUC. A translation reaction (150 µl) was initiated using a mixture of 90 ng of *GCN4*–LUC RNA lacking uORFs and 9 ng of sea pansy LUC RNA and incubated at 25°C for the indicated periods when samples were removed for LUC assays (circles, *GCN4*–LUC; triangles, sea pansy LUC). LUC activity in relative light units (RLU) is expressed as the percentage of the activity observed at the final time-point.

two different extracts. The effects of uORFs were similar in experiments using *S.cerevisiae* extracts incubated for 35 min (data not shown).

By adding the elongation inhibitor cycloheximide to *S.cerevisiae* extracts under steady-state conditions, toeprints corresponding to ribosomes at each of the *GCN4* uORF start codons were observed (Figure 3). We established that each of these signals corresponded to a uORF initiation codon by the high-resolution mapping of each toeprint in both *S.cerevisiae* and *N.crassa* extracts to which cycloheximide was added in the steady state or prior to addition of mRNA template (see below), and by their loss when the corresponding mutated transcripts lacking that initiation codon were used to program extracts (Figures 3 and 4, and data not shown). For example, toeprints corresponding to ribosomes at the start codon of uORF2 were observed in both *S.cerevisiae* and *N.crassa* extracts only when the mRNA contained uORF2 (Figure 4A). The uORF2 initiation-codon toeprint maps 16 nt downstream of the AUG codon, indicating that it corresponds to ribosomes with the initiation codon in their P-site (Anthony and Merrick, 1992; Pestova *et al.*, 1996; Wang and Sachs, 1997a). Additional common premature termination products occur in all of the lanes; many of these were also observed in primer extension analyses of RNA in the absence of extract (e.g. Figure 5, lane 18 and Figure 6, lane 23) but not in primer extension analyses of extract in the absence of exogenously added mRNA (e.g. Figure 5, lanes 6 and 12, and Figure 6, lanes 11 and 22).

In *S.cerevisiae* extracts, strong toeprints corresponding to ribosomes in the *GCN4* mRNA leader were not observed in the absence of cycloheximide (Figure 5A, lanes 1–5). In contrast, in *N.crassa* extracts, toeprints corresponding to ribosomes involved in initiation, elongation and termination are observed in the absence of cycloheximide or other translation inhibitors (Wang and Sachs, 1997a; Wang *et al.*, 1998, 1999; Fang *et al.*, 2000). Mapping of *GCN4* uORF1 in *N.crassa* extracts showed ribosomes at the initiation codon, the two sense

Fig. 3. Detection of *S.cerevisiae* ribosomes at *GCN4* uORF initiation codons. Equal amounts (120 ng) of synthetic RNA transcripts were used to program translation mixtures derived from *S.cerevisiae*. The *GCN4-LUC* transcripts encoded uORFs 1–4, uORF1, uORFs 3 and 4, uORF4, or no uORFs in the 5'-leader as indicated. Transcripts were translated in reaction mixtures at 25°C for 10 min, then supplemented with cycloheximide followed by an additional 10-min incubation at 25°C. Radiolabelled primer ZW4 was used for primer extension analysis and for sequencing of the wild-type *GCN4* template (the four left hand lanes). The nucleotide complementary to the dideoxynucleotide added to each sequencing reaction for the wild-type *GCN4* template is indicated below the corresponding lane so that the sequence of the template can be directly deduced; the 5'-to-3' sequence reads from top to bottom. Premature transcription termination products corresponding to ribosomes bound at *GCN4* AUG_{uORFs} 1–4 are indicated by asterisks and numbers corresponding to the uORF.

codons, and the termination codon (Figure 4B). The most 5'-proximal toeprint observed at uORF1 maps 16 nt downstream of the AUG codon, corresponding to ribosomes with the initiation codon in the P-site. The most 3'-distal toeprint observed at uORF1 indicates that the ribosomes moved slightly past the uORF1 stop codon, or that the termination complex is larger at this stop codon than at the *arg-2* uORF termination codon, since the toeprint corresponding to the *arg-2* uORF terminator in the A-site maps 13-nt downstream from the first nucleotide of that codon in *N.crassa* extracts (Wang and Sachs, 1997a) and *S.cerevisiae* extracts (Wang et al., 1999).

In all cases in which one or more *GCN4* uORFs were present, the initiation codon closest to the mRNA 5'-end showed the greatest extent of ribosomes loaded during steady-state translation. Thus, elimination of the AUG-codons for uORFs 1 and 2 (Figure 3) or uORFs 1, 2 and 3 (Figure 3) resulted in the appearance of a much stronger signal that corresponded to ribosomes located at the start codon for the 5'-most proximal uORF. When all the uORFs were eliminated, ribosomes were found predominantly at the Gcn4p initiation codon (Figure 5, lane 10). These data indicated that ribosomes scanned linearly from the 5'-end of mRNA to initiate translation at each of these start codons and were not loading internally. Additional experimental evidence consistent with this is described below.

The relative intensity of signals corresponding to ribosomes located at the Gcn4p initiation codon during

Fig. 4. Detection of *S.cerevisiae* and *N.crassa* ribosomes translating uORFs 1 and 2 in *S.cerevisiae* *GCN4* mRNA. Equal amounts (120 ng) of synthetic RNA transcripts were used to program translation mixtures derived from *S.cerevisiae* and *N.crassa*. The *GCN4-LUC* transcripts encoded all of the uORFs (wild type, wt), uORF1, uORFs 1 and 4, uORF4, or no uORFs as indicated. (A) The uORF2 initiation codon (asterisk) in *S.cerevisiae* extracts (top) and *N.crassa* extracts (bottom). Transcripts were incubated at 25°C for 20 min in reaction mixtures supplemented with cycloheximide prior to incubation. Toeprinting and analysis with controls were accomplished as described in the legend to Figure 3 except that primer ZW18 was used. (B) Transcripts were translated in *N.crassa* reaction mixtures at 25°C for 15 min and toeprints were obtained in the absence of translational inhibitors. The positions of the uORF1 start and stop triplets are marked by vertical bars.

the steady-state translation of each of these RNAs and the relative amount of LUC synthesized as determined by LUC enzyme assays were comparable (Table II). Stronger toeprint signals corresponding to ribosomes at the Gcn4p initiation codon during steady-state translation correlated with higher levels of LUC synthesis. Thus, the direct measurement of ribosomes loaded at initiation codons *in vitro* indicates that the *GCN4* uORFs control Gcn4p expression by modulating initiation at the Gcn4p start codon.

Toeprint data are consistent with the model that ribosomes which translate uORF1 reinitiate downstream

As discussed above, the model for *GCN4* translational control is that ribosomes reach the downstream *GCN4* start codon by reinitiation after translation of uORF1 but not

Fig. 5. Reinitiation following GCN4 uORF1 translation. Equal amounts (120 ng) of synthetic *GCN4-LUC* RNA transcripts encoding either uORFs 1–4, uORF1, uORF4, uORFs 1 and 4, or no uORFs in the 5′-leader were used to program translation mixtures derived from *S.cerevisiae*. (A) Transcripts were incubated at 25°C for 20 min in reaction mixtures not supplemented with cycloheximide (none) or supplemented with cycloheximide added either prior to 25°C incubation (T₀) or after 5 min of incubation at 25°C (T₅). Toeprinting and analysis with controls were accomplished as described in the legend to Figure 3. Premature transcription termination products corresponding to ribosomes bound at AUG_{uORF1}, AUG_{uORF4} and AUG_{GCN4} on the *GCN4-LUC* RNA are indicated with arrowheads; products corresponding to AUG_{uORF2} and AUG_{uORF3} are indicated with asterisks. (B) Close-up of the regions containing toeprints for uORF1, uORF4 and *GCN4* at T₀ and T₅.

Fig. 6. Adding eIF2 increases initiation at uORF3 and uORF4 and decreases it at *GCN4*. Equal amounts (60 ng) of synthetic *GCN4-LUC* RNA transcripts encoding uORFs 1–4, uORF1, uORF4, uORFs 1 and 4, or no uORFs in the 5′-leader were used to program translation mixtures derived from *S.cerevisiae*. Transcripts were incubated at 25°C for 20 min in reaction mixtures supplemented with cycloheximide prior to incubation (T₀) or after 10 min of incubation at 25°C (T₁₀). Reaction mixtures were either not supplemented with eIF2 (–) or supplemented with purified eIF2 to a final concentration of 50 ng/μl (+). Toeprinting and analysis with controls were accomplished as described in the legend to Figure 3. Premature transcription termination products corresponding to ribosomes bound at AUG_{uORF1}, AUG_{uORF4} and AUG_{GCN4} are indicated with arrowheads. Asterisks indicate premature transcription termination products corresponding to ribosomes bound at AUG_{uORF3}.

uORF4. To test this directly, we compared the results of toeprints when cycloheximide was added to extracts prior to adding RNA template (T_0) or added after translation of the RNA was underway (T_5) (Figure 5). Cycloheximide at T_0 should reveal where ribosomes first initiate translation, since the drug interferes with elongation, not initiation. Adding drug in the steady state should reveal where primary initiation events and reinitiation events occur (see Figure 8).

When cycloheximide was added at T_0 , toeprints corresponding predominantly to the start codon nearest the 5'-end of the mRNA were observed (Figure 5A, lanes 7–11). Events at the uORF1, uORF4 and Gcn4p start codons from Figure 5A are shown magnified in Figure 5B. When uORF1 was present, ribosomes loaded predominantly at its start codon at T_0 (Figure 5A, lanes 7, 8 and 11; Figure 5B). When uORFs 2 and 3 were present in addition to uORF1 and uORF4, small but detectable signals corresponding to their initiation sites were observed (Figure 5A, lane 7); when they were absent, a signal corresponding to the initiation site of uORF4 was detected (Figure 5A, lanes 9 and 11; Figure 5B). When uORFs 2, 3 or 4 were present, few ribosomes were observed at the downstream Gcn4p start codon (Figure 5A, lanes 7, 9 and 11; Figure 5B). When uORF1, but not the other uORFs, was present, some ribosomes were observed at the Gcn4p start codon (Figure 5A, lane 8; Figure 5B). When no uORFs were present, substantial loading of ribosomes occurred at the *GCN4* start codon (Figure 5A, lane 10; Figure 5B). These results indicate that most ribosomes initiated translation at uORF1; those that did not scanned downstream to initiate at one of the following start codons.

In contrast to the results obtained by adding cycloheximide at T_0 , adding the drug to extracts engaged in the steady-state translation of mRNAs containing uORF1 revealed substantial loading of ribosomes at the Gcn4p start codon in addition to the uORF1 start codon (Figure 5A, compare lanes 13, 14 and 17 to lanes 7, 8 and 11; Figure 5B). These data indicate that ribosomes that translated uORF1 reinitiated downstream at the Gcn4p start codon. A lower level of reinitiation was apparent at uORFs 2, 3 and 4 under these conditions (Figure 5A, compare lanes 7 and 13). When there were no uORFs in the transcript, little difference was seen in ribosome loading at the Gcn4p initiation codon when cycloheximide was added at T_0 versus during steady-state translation (Figure 5A, compare lanes 10 and 16; Figure 5B). These biochemical results are consistent with the model that the increased signal at Gcn4p arises from reinitiation when transcripts contained uORF1 [presently individual ribosomes cannot be followed on a single mRNA to determine the exact mechanism(s) of reinitiation]. When only uORF4 was present, ribosomes primarily initiated translation there but showed little capacity to reinitiate at the Gcn4p start codon (Figure 5, compare lane 9 with 15; Figure 5B).

Increasing eIF2 increases reinitiation at uORFs and decreases reinitiation at Gcn4p

The *S.cerevisiae* translation extracts appear derepressed for Gcn4p translation, with relatively efficient translation of Gcn4p occurring after translation of uORF1. Therefore, we tested whether purified eIF2 added to extracts could relieve derepression. We measured the amount of

eukaryotic initiation factor (eIF)2 γ in extracts by immunoblotting (data not shown) and added approximately an order of magnitude more eIF2. Parallel analyses of extracts to determine the initial loading of ribosomes and the steady-state loading of ribosomes revealed that addition of eIF2 had a slight effect on the sites where ribosomes loaded initially and a greater effect on where they were loaded in the steady state. Specifically, for transcripts containing all of the uORFs, eIF2 increased the loading of ribosomes at uORF3 and reduced it at Gcn4p (Figure 6, compare lanes 12 and 13). In transcripts containing uORFs 1 and 4 only, eIF2 increased ribosome loading at uORF4 and decreased it at Gcn4p (Figure 6, compare lanes 20 and 21). These effects were small (<1.5-fold) but were reproducible in three independent experiments. Similar loading at uORF1 was seen in all cases. These data indicate that the level of eIF2 affects the reinitiation site in the cell-free system as determined by toeprinting. This effect was not discernable by measurement of LUC activity; this may reflect the relatively small magnitude of the effect under these conditions.

The *CPA1* and *arg-2* mRNAs each contain a uORF that regulates ribosome scanning

Ribosomes stall after translating an evolutionarily conserved uORF in the *S.cerevisiae* *CPA1* and *N.crassa* *arg-2* mRNAs in the presence of high Arg (Wang *et al.*, 1999 and references therein). Studies on the *arg-2* uORF in *N.crassa* extracts are consistent with the model that stalling blocks scanning ribosomes from reaching the downstream start codon. By examining where ribosomes first loaded at initiation codons and where they were loaded during steady-state translation, we tested whether this model applied to *CPA1* regulation in *S.cerevisiae*.

When cycloheximide was added at T_0 to *S.cerevisiae* extracts programmed with *CPA1*-LUC mRNA and the positions of ribosomes analyzed by toeprinting, ribosomes were observed at both the uORF and LUC initiation codon (Figure 7A, lane 1). Thus, in striking contrast to the results obtained when cycloheximide was added early to translation extracts programmed with mRNA containing the *GCN4* uORFs (Figure 5), significant leaky scanning past the *CPA1* uORF initiation codon was observed. When cycloheximide was added early, Arg had no effect on leaky scanning, since a similar level of initiation at the LUC start codon was detected in low or high Arg (Figure 7A, compare lanes 1 and 2). These data support the regulatory model that Arg-specific translational control requires the synthesis of the uORF-encoded peptide. In contrast, when cycloheximide was added later (15 min in the experiment shown here), Arg caused ribosomes at the uORF termination codon to stall, and fewer ribosomes were observed at the downstream LUC start codon (Figure 7A, compare lanes 3 and 4). It appears that ribosomes reach the downstream start codon in the *CPA1* mRNA by leaky scanning, not by reinitiation, and Arg-regulated ribosome stalling at the uORF terminator blocks ribosomes from scanning. Previous data using *N.crassa* extracts to which no translation inhibitors were added indicate that these stalled ribosomes also block 80S ribosomes that had initiated subsequently at the uORF start codon (Wang and Sachs, 1997a; Wang *et al.*, 1998).

Fig. 7. Leaky scanning past the *CPA1* and *arg-2* uORFs. Equal amounts (120 ng) of synthetic RNA transcripts were used to program translation mixtures derived from *S. cerevisiae* or *N. crassa*. (A) *Saccharomyces cerevisiae* *CPA1*-LUC RNA containing the wild-type *CPA1* uORF and intergenic region in the 5'-leader was incubated at 25°C for 20 min in *S. cerevisiae* translation extracts. Reaction mixtures were supplemented with cycloheximide added either prior to 25°C incubation (T₀) or after 15 min of incubation at 25°C (T₁₅), and contained either 10 μM Arg (-) or 500 μM Arg (+) as indicated. (B) Predicted improvement of the *arg-2* uORF initiation context increases initiation at that site and decreases downstream initiation. *N. crassa arg-2*-LUC RNA containing the wild-type *arg-2* uORF in a poor wild-type (wt) initiation context or in an improved initiation context (↑) were incubated in *S. cerevisiae* or *N. crassa* translation extracts at 25°C for 20 min; reaction mixtures were as for panel A for 20 min. Reaction mixtures containing 10 μM arginine were supplemented with cycloheximide added either prior to incubation (T₀) or after 5 min of incubation (T₅). Toeprinting and analysis with controls were performed as described in Figure 3. The positions of cDNA extension products corresponding to the mRNA 5'-end, uORF initiation and termination codons, and the LUC initiation codon, are indicated diagrammatically in each panel.

Fig. 8. uORF control of reinitiation and leaky scanning. (A) Reinitiation. Scanning ribosomes load at the uORF start codon and initiate translation there; subsequently, they reinitiate translation downstream. (B) Leaky scanning. Ribosomes scan and load at either the upstream or downstream start codons. (C) Discriminating reinitiation from leaky scanning. Adding cycloheximide (CYH) at T₀ will trap ribosomes at the positions where they first load on the mRNA; adding it during steady-state translation (T₅-T₁₅) will trap ribosomes where they are loaded following the primary initiation event and subsequent reinitiation events.

The context in which a start codon is found affects the extent to which it captures scanning ribosomes in reticulocyte lysates (Kozak, 1998). Studies on initiation context in *S. cerevisiae* indicate that it can also influence the extent to which ribosomes initiate (e.g. Welch and Jacobson, 1999 and references therein). Using calculations that evaluate favorable or unfavorable initiation contexts

for *S. cerevisiae* (Miyasaka, 1999), the start codons of *GCN4* uORF1 and Gcn4p are predicted to be in relatively good contexts, and the *CPA1* uORF in a relatively poor context (Table III). The other *GCN4* uORF start codons are all in better contexts than the *CPA1* uORF start codon (data not shown). This is consistent with the results that recruitment of ribosomes by each of the *GCN4* initiation

Table III. Predicted strengths of initiation codons

Initiation codon	Sequence context	AUG CAI ^a
<i>CPA1</i> AAP	CATTATATGTTT	0.287
<i>arg-2</i> AAP	GCCCTTATGAAC	0.174
improved <i>arg-2</i> AAP	GCCACAATGAAC	0.424
<i>GCN4</i> uORF1	TGAAAAATGGCT	0.751
<i>GCN4</i> uORF4	TTCAAGATGTTT	0.493
<i>GCN4</i> Gcn4p	ATAAAAATGTCC	0.707

^aThe AUG codon adaptation index was calculated as described (Miyasaka, 1999).

codons tested was efficient when it was the initiation codon nearest the 5'-end, precluding recruitment at downstream start codons. In contrast, recruitment by the *CPA1* uORF was relatively inefficient, allowing considerable scanning to the *CPA1*p start.

Previous experiments with the *N.crassa arg-2* uORF in a predicted poor versus good initiation context showed that, in *N.crassa* extracts in which initiation events could be seen without adding cycloheximide, a poor context increased the extent of scanning past the uORF (Wang and Sachs, 1997a). Figure 7B shows an analysis of the effects of initiation context in both *N.crassa* and *S.cerevisiae* extracts, with cycloheximide added at T₀ or added during steady-state translation. When the uORF was in a poor initiation context, fewer ribosomes loaded there, and more were loaded downstream at the LUC initiation codon, either initially or in the steady state. Consistent with this, less LUC was produced when the uORF was in a good context as measured by enzyme assay in reaction mixtures incubated for longer periods (not shown). These data indicate that the translational machinery of these fungi is responsive to the initiation context of a start codon, and indicate that initiation at the *arg-2* and *CPA1* uORFs is normally not efficient. Experiments similar to these, but in which high Arg was present, yielded results similar to those in Figure 7A (data not shown). Arg caused ribosomes to stall at the uORF termination codon and reduced loading at the downstream start codon when cycloheximide was added during steady-state translation but not when cycloheximide was added at T₀.

Discussion

We have obtained direct physical evidence that translation of *GCN4* uORF1 results in efficient reinitiation at the downstream *GCN4* start codon. This evidence is not consistent with alternative mechanisms to explain how ribosomes reach the *GCN4* start codon, such as by leaky scanning or internal initiation. For ribosomes to reach a downstream start codon through reinitiation, they must first initiate translation at an upstream start codon (Figure 8A). Therefore, when cycloheximide is added to arrest translation elongation in cell-free translation extracts before ribosomes can initiate translation (T₀), ribosomes will collect at the upstream start codon but not at the downstream start codon, if ribosomes reach the downstream codon by reinitiation. When it is added to arrest steady-state translation (after 5–15 min of incubation), ribosomes will be observed at both upstream and

downstream start codons (Figure 8C). This behavior is what we observed for transcripts containing *GCN4* uORF1 and downstream coding regions (Figures 5 and 6), indicating that ribosomes efficiently initiate at uORF1 and then reinitiate downstream.

When ribosomes reach a downstream start codon by leaky scanning, they will load at that downstream start codon without prior translation of a uORF (Figure 8B). Therefore, whether cycloheximide is added to extracts at T₀ or under steady-state conditions, ribosomes will collect at both upstream and downstream start codons (Figure 8C). This pattern was observed for ribosomes loaded on transcripts containing the *CPA1* or *arg-2* uORFs (Figure 7). A similar pattern would be predicted if internal initiation occurred; but, for internal initiation, the loading of ribosomes at the downstream site should not be affected by the context of the upstream initiation codon, or by ribosomes stalled during translation of the uORF. When the initiation context of the *arg-2* uORF is improved, more ribosomes load at that codon and fewer load downstream, consistent with the leaky scanning, and not with reinitiation or internal initiation (Figure 7B). Arg-induced stalling of ribosomes that synthesized the uORF peptide reduced the loading of ribosomes at the downstream start codon, also consistent with their reaching the downstream codon by leaky scanning (Figure 7A). In addition to blocking scanning ribosomes from reaching the downstream start codon, as demonstrated through the experiments with cycloheximide shown here, ribosomes stalled at the uORF termination codon appear to block 80S ribosomes involved in subsequent rounds of translation of the uORF (Wang and Sachs, 1997a; Wang *et al.*, 1998).

While the *CPA1* and *arg-2* uORFs decrease loading at the downstream start codon by causing ribosomes that have translated the uORF to stall, we see no evidence that uORF4 of *GCN4* reduced translation by causing stalling, a possibility that is consistent with the genetic data. Toeprints from the uORF4 termination codon are not observed under conditions in which it is repressing translation, while such toeprints are observed under conditions in which the *CPA1* and *arg-2* uORFs reduce translation. This is consistent with data that the peptide encoded by uORF4 does not have a role in regulation, since, for *CPA1* and *arg-2*, as well as other uORFs that stall ribosomes, the encoded peptide sequence is important (Lovett and Rogers, 1996; Geballe and Sachs, 2000; Morris and Geballe, 2000; Gong and Yanofsky, 2001; Law *et al.*, 2001).

Considerable genetic and biochemical evidence indicate that the derepression of *GCN4* translation *in vivo* is triggered by a decrease in the concentration of the eIF2/GTP/Met-tRNA_i^{Met} ternary complex (TC). This results from phosphorylation of eIF2 and attendant inhibition of eIF2B, the guanine nucleotide exchange factor (GEF) for eIF2, when the eIF2α kinase Gcn2p is activated in amino acid-starved cells (Hinnebusch, 2000). According to the current model, all ribosomes scanning from the 5'-end of the mRNA translate uORF1, and ~60% resume scanning as 40S subunits. Under nonstarvation conditions, virtually all of these reinitiating 40S ribosomes rebind the TC and reinitiate at uORF4, after which they dissociate from the mRNA. Under starvation conditions, when phosphorylation of eIF2α lowers the TC level, ~50% of the ribosomes

scanning from uORF1 will reach uORF4 before rebinding the TC, and lacking Met-tRNA^{Met}, will bypass uORFs 2–4 (or just uORF4 in constructs containing only uORFs 1 and 4). Most of these ribosomes rebind the TC before reaching Gcn4p and reinitiate translation there instead. Thus, the reduction in TC levels elicited by Gcn2p activation allows a fraction of reinitiating ribosomes to bypass inhibitory uORF4 and reinitiate at Gcn4p.

The *S.cerevisiae* extracts used here appeared to be derepressed, with relatively high levels of uORF1-dependent synthesis of Gcn4p from constructs containing uORFs 1 and 4. This suggests that the concentration of TC in the extracts is lower than in yeast cells growing under non-starvation conditions where Gcn2p is quiescent. Accordingly, we predicted that supplementing the extracts with excess eIF2 would repress *GCN4* translation by elevating TC levels and increasing the probability of reinitiation at uORFs 3 and 4 at the expense of the *GCN4* start codon. Our findings confirmed this prediction, providing the first *in vitro* evidence that TC levels influence the site of reinitiation on *GCN4* mRNA. However, the addition of excess eIF2 did not fully repress *GCN4* translation to the level seen with uORF4 alone, whereas this degree of repression is nearly achieved in living cells under nonstarvation conditions (Hinnebusch, 1996). One possible explanation for this discrepancy is that the purified eIF2 added to the extracts was present in the GDP-bound form and the GEF activity of eIF2B, required to produce eIF2–GTP for TC formation, may have been low in the extracts. Perhaps the eIF2B was inactivated by phosphorylated endogenous eIF2 generated during preparation of the extracts, as Gcn2p is constitutively activated in yeast cell extracts (Zhu *et al.*, 1996).

Another discrepancy between the *in vivo* and *in vitro* results for *GCN4* translational control is that the 1–4 construct containing all four uORFs produced somewhat higher levels of Gcn4p in the translation extract than did the 1,4 construct containing uORFs 1 and 4, whereas the opposite was true in derepressed yeast cells (Table II). According to the regulatory model described above, the two constructs should yield identical amounts of Gcn4p synthesis under derepressing conditions since scanning ribosomes that bypass uORF4 because they failed to rebind the TC should necessarily bypass uORFs 2 and 3 for the same reason. However, recall that only a fraction of ribosomes scan past uORFs 2–4 when TC levels are reduced, and the remainder still translate these uORFs and subsequently dissociate from the mRNA. It is possible that the presence of ribosomes translating uORFs 2 or 3 will delay the progression of ribosomes scanning from uORF1 to uORF4 and increase the time available for TC re-binding. This would increase the frequency of reinitiation at uORF4 and thereby decrease *GCN4* translation as observed *in vivo*. How can we explain the results obtained in the extracts where the presence of uORFs 2 and 3 in the 1–4 construct led to greater *GCN4* translation than that given by the 1,4 construct? One possibility is that the frequency of reinitiation following translation of uORFs 2 or 3 may be greater in the extract than in cells. In this event, ribosomes which translate these uORFs, either by reinitiating after uORF1 translation or by leaky-scanning past uORF1, will scan past uORF4 at high frequencies because of their proximity to uORF4.

The model for reinitiation by the *GCN4* uORFs may be a conserved regulatory mechanism because other fungal homologs of *GCN4* contain multiple uORFs. Two uORFs are found in the *N.crassa cpc-1* (Davis, 2000; Paluh *et al.*, 1988; Sachs, 1996), *Aspergillus niger cpcA* (Wanke *et al.*, 1997) and *Candida albicans GCN4* (DDBJ/EMBL/GenBank accession No. AF205716) transcripts. Examination of the distribution of ribosomes on the *N.crassa cpc-1* transcript in cells in which *cpc-1* expression is repressed or derepressed show that this mRNA is also subject to translational regulation (Luo *et al.*, 1995). *N.crassa* also contains another key element of the regulatory pathway, *cpc-3*, which is the homolog of *S.cerevisiae GCN2* (Sattlegger *et al.*, 1998).

Emerging evidence indicates that the control of reinitiation is important in regulating the expression of mammalian genes. uORF-mediated reinitiation occurs in the synthesis of CAAT/EBP (Lincoln *et al.*, 1998; Calkhoven *et al.*, 2000). uORFs in the *Fli-1* mRNA are translated, and their translation precludes scanning while accentuating the production of a shorter Fli-1 polypeptide from a downstream AUG codon, indicating that their translation enables reinitiation (Sarrazin *et al.*, 2000). Interestingly, the action of the eIF2 kinases GCN2 and PERK negatively affects the translation of many mRNAs but selectively activates the translation of *ATF4* mRNA (Harding *et al.*, 2000), which contains two uORFs. Their elimination affects the translational regulation of *ATF4*, although the mechanism may be different than is observed for *GCN4*. The control of reinitiation following uORF translation is implicated in the glucose-mediated regulation of the synthesis of macrophage scavenger receptor CD36, with implications for the development of atherosclerosis in diabetics (Griffin *et al.*, 2001).

In contrast to the situation for *GCN4*, Arg-regulation through the evolutionarily conserved *S.cerevisiae CPA1* and *N.crassa arg-2* uORFs appears to be exerted by the control of the movement of scanning ribosomes and not by modulating reinitiation. Thus, control through these uORFs is more similar to the control exerted by cytomegalovirus *gpUL4* uORF2 (Cao and Geballe, 1995, 1996a,b) than the *GCN4* uORFs. *In vivo*, the *CPA1* and *arg-2* uORFs have relatively small effects on gene expression (Delbecq *et al.*, 1994; Luo and Sachs, 1996) in the absence of exogenous Arg, consistent with a high level of leaky scanning occurring *in vivo* in low Arg. This observation is also consistent with efficient reinitiation occurring (Delbecq *et al.*, 1994), but the *in vitro* data obtained do not support this.

These experiments, which examine where ribosomes first initiate the translation of mRNA and where they initiate translation in the steady state, demarcate two distinct uORF-mediated regulatory mechanisms. They demonstrate the possibilities and the advantages of the toeprinting technique for *in vitro* investigation of mechanisms of translational control.

Materials and methods

Templates for RNA synthesis

Linearized plasmid templates were designed to produce capped and polyadenylated synthetic RNA encoding firefly LUC with RNA 5'-leaders containing either all four *GCN4* uORFs (pPG101), uORF1

alone (pPG102), uORF4 alone (pPG103), no uORFs (pPG104), uORFs 1 and 4 (pPG105) or uORFs 3 and 4 (pAG112). These 5'-leaders followed by the first five codons of *GCN4* were fused directly in-frame to firefly LUC. *GCN4* fragments were PCR-amplified from plasmids p243, p248, p249, p250, pB196 and p204. *Bam*HI- and *Xho*I-digested PCR products were gel-purified and ligated to *Bam*HI- and *Xho*I-digested pV101 vector which is essentially pHLUC + NFS4 (Wang and Sachs, 1997b) with an introduced *Bam*HI site 3' of the *Xho*I site. Primers for PCRs were: ZW10 (5'-CGCGGATCCACAAAACAAAACAAAAC-3'), which includes a 5'-*Bam*HI site and ZW12 (5'-CGGCTCGAGGCTGATATTCGGACA-TTT-3'), which includes a 3'-*Xho*I site. Templates for the synthesis of RNAs containing the *CPA1* and *arg-2* 5'-leaders were described previously (Wang and Sachs, 1997a; Wang *et al.*, 1999), as was the template used to produce capped and adenylated synthetic mRNA encoding sea pansy LUC to serve as an internal control for translation reactions (Wang *et al.*, 1998).

Plasmid DNA templates were purified as described (Wang and Sachs, 1997a). Capped and polyadenylated RNA was synthesized with T7 RNA polymerase from *Nsi*I-linearized *GCN4* plasmid DNA templates and from *Eco*RI-linearized *CPA1* and *arg-2* plasmid DNA templates. RNA quantification was as described (Wang and Sachs, 1997b).

Cell-free translation and primer extension-inhibition (toeprint) analyses

The preparation of translation extracts and reaction conditions were as described (Wang *et al.*, 1998, 1999; Fang *et al.*, 2000). eIF2 was prepared as described (Pavitt *et al.*, 1998). Cycloheximide was added to a final concentration of 0.5 mg/ml from a 10-mg/ml stock solution. Addition of cycloheximide at T_0 was accomplished by supplementing reaction mixtures with cycloheximide prior to the addition of extract. Primer extension assays were accomplished as described (Wang and Sachs, 1997a) except that for *GCN4* primer extension incubation at 37°C was for 35 min instead of 30 min.

The preparation of 5'-³²P-labelled ZW4 and ZW18 primers was simplified from the previously described procedure (Wang and Sachs, 1997a). The reaction volume was reduced to 50 µl and the addition of EDTA after incubation at 37°C was omitted. The volume of phenol-chloroform for organic extraction was reduced to 60 µl and the volume of TE used to back-extract the organic phase was reduced to 40 µl. Chloroform extraction was omitted. The aqueous phases were combined and 45 µl applied to a spin column for chromatography (Princeton Separations, Cat.#CS-101). Spin columns were hydrated with 650 µl of either TE or DEPC-treated water prior to use. The concentration of eluted radiolabelled oligonucleotides was adjusted with either TE or DEPC-treated water to ~0.1 µM based on a presumed 70% recovery from the spin column. The eluted material was predominantly radiolabelled primer as determined by checking for inorganic phosphate and unreacted ATP by polyethylenimine thin-layer chromatography (Wang and Sachs, 1997a).

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