THE *SACCHAROMYCES CEREVISIAE* ARGININE ATTENUATOR PEPTIDE REGULATES TRANSLATION AND mRNA DECAY

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A dissertation submitted to the faculty of the
OGI School of Science & Engineering
at Oregon Health & Science University
in partial fulfillment of the
requirements for the degree

Doctor of Philosophy
in
Biochemistry and Molecular Biology

July 2005

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ACKNOWLEDGEMENTS

I wish to express my gratitude towards my thesis advisor, Dr. Matthew S Sachs, for his inspiration, enthusiasm, and suggestions, which has made my Ph.D. studies full of excitement and reward. Matt's passion for data, his hands-on efforts in the lab, and his appreciation for a loud sound system in the lab have been essential for this thesis research.

Special thanks go to the dissertation committee members, Drs. Allan Jacobson, Gebre Woldegeorgis, and Peter Zuber. I am indebted to Allan for his commitment to my studies and for his guidance as I prepare for the next stage of my career. I thank Dr. Adam Geballe for critical discussions at the Northwest Translational Control Group meetings, and Dr. David Morris for his critical comments and generous assistance in the lab. Thanks to Jacobson lab members for all of their technical assistance, especially Robin Ganesin and Dr. Feng He, to Dr. Michiko Nakano for guidance and support, and to members of the Nakano, Zuber, Woldegeorgis, and Cregg labs for generously sharing equipment and reagents.

Many thanks to Christina Spevak, Peng Fang, members of the Sachs lab who have kindly assisted me with experiments, Dr. Zhong Wang, who provided guidance with experiments and encouraged me to pursue yeast studies, and Drs. Geoff and Joan Lin Cereghino for all their support and delicious dinners. A very special thanks to department moms Nancy Christie and Terrie Hadfield for all their help and kindness throughout my graduate studies and to my Abuelita and brothers for much needed inspiration.

I am forever indebted to my mother and father for their continuous encouragement, advice, and support, which without, this thesis would not have been possible. For all their efforts, patience, and understanding, I dedicate this thesis to my parents.

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ABSTRACT

The *Saccharomyces cerevisiae* Arginine Attenuator Peptide Regulates Translation and mRNA Decay

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July 2005

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The *Saccharomyces cerevisiae CPA1* mRNA 5′-leader contains an evolutionarily conserved 25-codon upstream open reading frame (uORF), which genetic studies had suggested to encode a peptide involved in translational regulation in response to arginine (Arg). Using a cell-free translation system, studies with the *CPA1* homolog in *Neurospora crassa*, *arg-2*, showed that the *arg-2* uORF-encoded peptide, the Arg attenuator peptide (AAP), functioned to stall ribosomes at the uORF termination codon in response to Arg. To investigate *CPA1* AAP function, this study utilized *S. cerevisiae* and *N. crassa* cell-free translation systems to demonstrate that the *CPA1* AAP functions as its *N. crassa* counterpart to stall ribosomes at its uORF termination codon in response to Arg, resulting in reduced levels of ribosomes at a downstream reporter start codon. The charge status of Arg-tRNA and the termination of AAP translation did not appear critical for *S. cerevisiae* AAP function to stall ribosomes. Analyses of ribosomes at the *CPA1* uORF and at the uORFs present in *GCN4* mRNA indicated that these uORFs control translation in fundamentally different ways. Whereas the *CPA1* uORF regulates

ribosomes that scan past the uORF, the *GCN4* uORFs regulate ribosomes that reinitiate translation. *CPA1* uORF analyses using an *in vivo* reporter system with wild-type and nonsense-mediated mRNA decay (NMD)-deficient strains indicated that uORF-mediated translational control regulated *CPA1* mRNA levels by affecting transcript susceptibility to NMD. These studies of the *CPA1* uORF provide a novel example of regulated eukaryotic gene expression in which the synthesis of a nascent peptide controls translation and mRNA stability.

CHAPTER 1
INTRODUCTION

The coordinate and timely control of eukaryotic gene expression involves regulation at multiple levels such as transcription, post-transcriptional processing, mRNA stability, translation, post-translational modifications, and protein degradation. Whereas regulation of transcription can entail a delay before a precursor RNA is processed and ready for translation, regulation of translation from pre-existing mRNA can occur essentially instantaneously, thus allowing a cell to rapidly alter its protein synthesis in response to extracellular and intracellular signals. The multitude of steps and factors involved in translation provides various means by which translational regulation can finely control gene expression. Regulation of mRNA turnover is also highly important for control of gene expression and studies continue to provide evidence indicating that the processes of translation and mRNA turnover are interconnected. This thesis describes the investigation of a novel regulatory system that affects the translation and degradation of the *Saccharomyces cerevisiae CPA1* mRNA.

The *CPA1* transcript specifies the small subunit of arginine-specific carbamoyl phosphate synthetase and its 5′-leader contains an upstream open reading frame (uORF) encoding a 25-residue evolutionarily conserved peptide, named the arginine attenuator peptide (AAP). Cpa1p is critical for arginine (Arg) biosynthesis and its expression is negatively regulated in response to Arg surplus, which in turn, reduces Arg biosynthesis. Experimental results described in this thesis indicate that the *S. cerevisiae* AAP attenuates translation in the presence of Arg by forcing ribosomes to stall at the uORF sequence, thereby reducing translation at the downstream cistron. Critical for this regulatory mechanism, the *CPA1* uORF initiation codon is shown to inefficiently initiate translation, resulting in downstream initiation via leaky scanning of ribosomes. Analyses of ribosomes at *GCN4* uORFs indicated that the *CPA1* and *GCN4* uORFs regulate

translation through distinct mechanisms. Whereas ribosome stalling at the *CPA1* uORF termination codon regulates the movement of ribosomes that scan past the uORF, *GCN4* uORFs regulate ribosomes that reinitiate at uORFs 3 and 4. Finally, *in vivo* studies with a reporter system indicated that AAP-mediated ribosome stalling at the *CPA1* uORF termination codon controlled translation and mRNA steady-state levels by regulating transcript susceptibility to nonsense-mediated mRNA decay (NMD) and general 5′ to 3′ mRNA decay pathways. Taken together this thesis provides evidence for a model in which uORF-encoded peptide-induced ribosome stalling modulates translation as well as transcript turnover in response to Arg. To introduce these studies, this chapter will focus on mechanisms for eukaryotic translation and mRNA turnover, then review some general features of these controls, and last discuss previous studies that investigated the *CPA1* uORF and the corresponding uORF in the *N. crassa arg-2* transcript.

1.1 EUKARYOTIC TRANSLATION

Eukaryotic translation is achieved by orchestrated interactions between various *trans*-acting factors, such as eukaryotic initiation factors (eIFs), and mRNA structural elements (Figure 1.1) (Mignone et al., 2002). These elements include the 5′-cap structure (m7G(5′)ppp(5′)N), the 5′-leader, the open reading frame (ORF) region(s), the 3′-untranslated region (UTR), and the 3′-polyadenylate [poly(A)] tail. Cap-dependent scanning appears to be the most common mechanism for translation initiation on capped and adenylated mRNAs (Hershey and Merrick, 2000; Kozak, 1999). As the vast majority of eukaryotic mRNAs contain both a cap structure and a poly(A) tail (approximately 55–90 in yeast), cap-dependent scanning is likely to be the predominant pathway for eukaryotic translation initiation. The most common cap-independent pathway for eukaryotic translation initiation, known as internal initiation, is utilized by some cellular and viral mRNAs and has been recently reviewed (Komar and Hatzoglou, 2005), however, this mode of translation initiation will not be discussed further in this thesis. The following sections of this chapter will describe the initiation, elongation, and

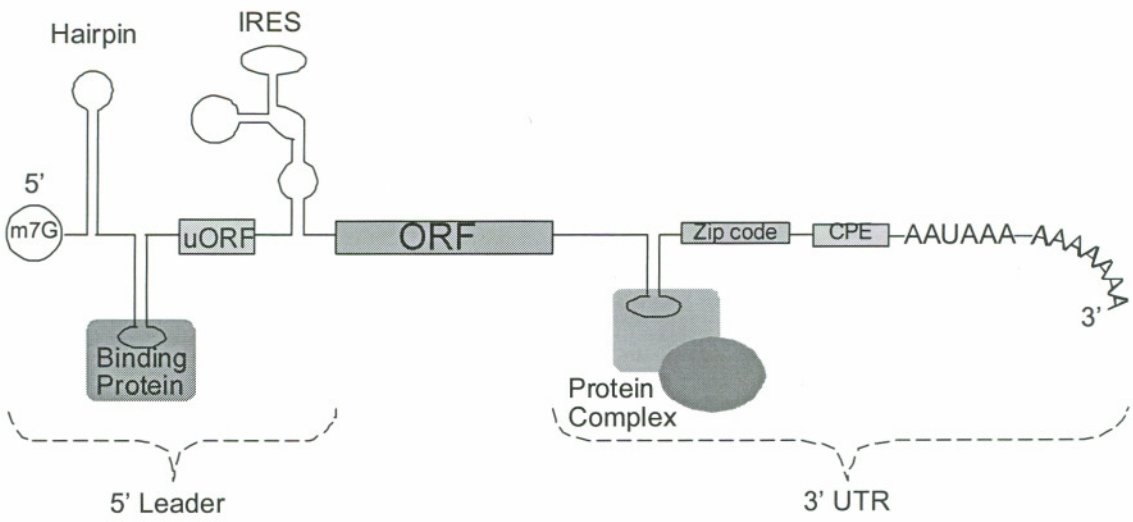

Figure 1.1. Cis-acting elements on mRNA that affect gene expression. Abbreviations (from 5' to 3'): UTR, untranslated region; m7G, 7-methyl-guanosine cap; hairpin,, hairpin-like secondary structures; uORF, upstream open reading frame; IRES, internal ribosome entry site; CPE, cytoplasmic polyadenylation element; AAUAAA, polyadenylation signal. Adapted from Mignone et al. (2002).

termination steps of the cap-dependent scanning pathway for eukaryotic translation initiation.

1.1.1 Initiation: Cap-Dependent Scanning

In both prokaryotes and eukaryotes, initiation of polypeptide synthesis is a multistep process that begins with recruitment of ribosomes to mRNAs. In prokaryotes, the small 30S ribosomal subunit is directed to mRNA primarily by base pairing between the 16S rRNA and the Shine-Delgarno sequence on mRNA (Calogero et al., 1988). In eukaryotes, the 40S ribosomal subunit is primarily directed to mRNA by protein-protein and protein-RNA interactions that place the small ribosomal subunit at the mRNA cap structure. The 40S subunit then scans the mRNA in a 5′-to-3′ direction until encountering a start codon, at which the 60S ribosomal subunit joins the 40S subunit to form the 80S initiation complex (Kozak, 1989). The cap-dependent scanning pathway described below is summarized as follows: Dissociation of 80S ribosomes into 40S and 60S subunits, 40S subunit binding to an active ternary complex to form the 43S preinitiation complex, binding of the 43S complex to mRNA, recognition of the initiator codon by the 43S complex, and joining of the 60S subunit to form an elongation-competent 80S ribosome.

1.1.1.1 ***Dissociation of Ribosomes into their Subunits.*** At physiological conditions, the free magnesium concentration (~1-2 mM) favors the association of 40S and 60S ribosomal subunits, giving rise to the first step for translation initiation, which is the dissociation of vacant 80S ribosomes into their subunits (Moldave, 1985; Russell and Spremulli, 1978; Russell and Spremulli, 1979). Although poorly understood, eIF3 and eIF1A (the homologue of bacterial initiation factor 1) are thought to promote this dissociation. One possibility is that eIF3 and eIF1A bind the 40S subunit thereby preventing its association with the 60S subunit by steric hindrance (Goumans et al., 1980). However, using cryo-EM at 48 Å resolution, eIF3 appears to be bound to 40S subunits at sites away from the subunit-subunit interface, arguing against a simple steric hindrance mechanism (Srivastava et al., 1992). It remains possible that eIF3 binding causes an allosteric effect by inducing a structural change in the 40S subunit. A third protein, eIF6,

has also been shown to affect ribosomal subunit association by binding to the 60S subunit and preventing its association with the 40S subunit (Raychaudhuri et al., 1984; Russell and Spremulli, 1979). More recently, phosphorylation of eIF6 has been reported to regulate the level of free 60S subunits (Ceci et al., 2003).

1.1.1.2 43S Preinitiation Complex Assembly. Subsequent to ribosomal subunit dissociation, a 43S preinitiation complex is assembled from a 40S ribosomal subunit, eIF1A, eIF3, and a ternary complex (TC) consisting of eIF2, GTP, and Met-tRNA$_i^{Met}$, with the aminoacylated initiator tRNA positioned at the P-site of the 40S subunit (Figure 1.2)(Benne et al., 1978). eIF1A and eIF3 stimulate the assembly of the 43S complex (Benne et al., 1978; Trachsel et al., 1977). eIF1A catalyzes TC binding to 40S ribosomal subunits, but is not required to stabilize the 43S complex (Chaudhuri et al., 1997; Thomas et al., 1980). eIF2C has been shown to stabilize the TC against disruption by RNA (Roy et al., 1988) and eIF3 appears to stabilize the 43S complex prior to mRNA binding, and is dispensable for this function subsequent to base pairing between Met-tRNA$_i^{Met}$ and an AUG start codon (Chaudhuri et al., 1999).

Start codon recognition triggers hydrolysis of GTP by eIF2 and releases from the ribosome a stable eIF2-GDP binary complex that is inactive for binding Met-tRNA$_i^{Met}$. Since eIF2-GTP is required for TC regeneration, activity of the guanine nucleotide exchange factor (GEF) eIF2B is required to exchange eIF2-bound GDP for GTP (Kimball, 1999). Yeast eIF2 is a heterotrimeric complex consisting of subunits α, β, and γ. Phosphorylation of a conserved serine residue at position 51 of the α subunit converts eIF2-GDP from a substrate to an inhibitor of eIF2B (Dever et al., 1992; Hershey, 1991), an event that can be utilized by the cell for general and mRNA-specific translational regulation that will be discussed in later sections (1.3.1 and *1.3.2.1*). The β subunit of eIF2 may be involved in binding Met-tRNA$_i^{Met}$ (Huang et al., 1997) and has been reported to have mRNA binding activity (Gonsky et al., 1992), which could aid in codon-anticodon interactions (Laurino et al., 1999). The eIF2 γ subunit appears to be directly involved in the binding of GTP/GDP and Met-tRNA$_i^{Met}$ (Dorris et al., 1995; Erickson and Hannig, 1996; Gaspar et al., 1994; Harashima and Hinnebusch, 1986).

1.1.1.3 Association of mRNA with the 43S Preinitiation Complex. In the cap-dependent scanning pathway, the 43S preinitiation complex binds the m^7G cap structure at the mRNA 5′-terminus in a reaction promoted by eIF3 (Benne et al., 1978; Benne and Hershey, 1976; Trachsel et al., 1977), eIF4E, eIF4G, eIF4A, eIF4B (Hershey and Merrick, 2000), and poly[A]-binding protein (Pab1p) (Sachs et al., 1997). eIF4A, eIF4E, and eIF4G form eIF4F, a heterotrimeric complex that binds the cap structure and Pab1p with its eIF4E and eIF4G subunits, respectively, thereby promoting circularization of mRNA (Wells et al., 1998). In mammals, recruitment of the 43S complex to mRNA involves direct interaction between eIF3 of the 43S complex and a central domain in eIF4G (Lamphear et al., 1995). In yeast, where this eIF4G domain is absent, it is unclear how eIF3 promotes 43S complex binding to mRNA. The eIF4A subunit of eIF4F is an ATP-dependent RNA helicase that can unwind secondary structure in the mRNA 5′-leader (Preiss and W., 2003). This helicase activity is aided by eIF4B (Rogers et al., 2001) and is most efficient when eIF4A is present in the eIF4F complex (Rozen et al., 1990).

The cap structure and poly(A) tail on mRNA function synergistically to promote ribosome loading at the mRNA 5′ terminus. Observations that eIF4E and eIF4G are required for stimulation of translation (Etchison et al., 1982), and that Pab1p is required for synergy (Tarun and Sachs, 1995) have led to models that involve simultaneous binding of eIF4F and Pab1p to the cap structure and poly(A) tail, respectively, while the eIF4G subunit of eIF4F interacts with Pab1p. Upon such interactions, mRNA circularization is expected and has been supported by the demonstration of circular polyribosomes in electron micrographs of serial sections through ER membranes (Christensen et al., 1987) and by the use of atomic force microscopy to detect circular RNA in response to assembly of recombinant eIF4E, eIF4G, and Pab1p on double-stranded RNA that contained a 5′-capped strand and a 3′ polyadenylated strand (Wells et al., 1998). Although it is currently unclear, cap and poly(A) tail synergy may reflect conformational differences in eIF4F, Pab1p,or other factors, that occur in response to eIF4F-Pab1p interactions, resulting in an increased affinity by any one or more of these factors for a translational apparatus component. Allosteric transitions in eIF4G upon its binding to eIF4E support this model (Kessler and Sachs, 1998; Otero et al., 1999).

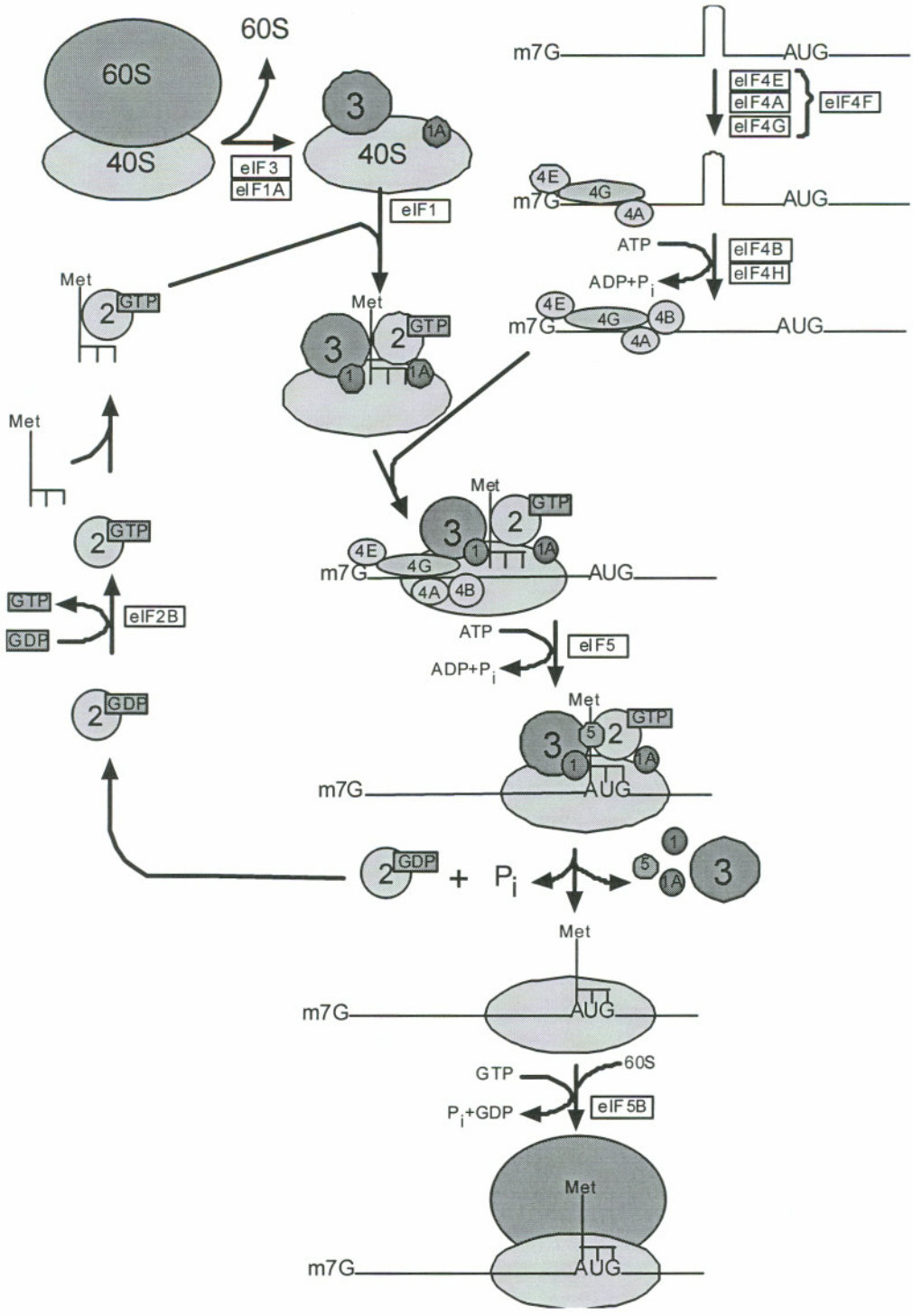

Figure 1.2. Initiation pathway in eukaryotes. Initiation factors are shown as gray circles. See text for detail. Adapted from Hershey and Merrick (2000).

1.1.1.4 Scanning of the 5′-leader and AUG start codon selection. Following recruitment to the mRNA 5′-terminus, the scanning model proposes that the 43S preinitiation complex scans the 5′-leader 5′ to 3′ and selects an AUG codon as the appropriate translation initiation site (Kozak, 1999; Kozak, 2002). If all AUG codons act equally efficient for initiating translation, this model would predict that AUG codons closest to mRNA 5′-ends serve as primary initiation sites, and this appears true for most known mRNAs (Kozak, 1989), however, some criteria are influential. The nucleotide sequence around an AUG codon, known as the initiation context, is highly influential for AUG selection, and explains why some 5′-proximal AUG codons do not serve as primary sites of initiation (Kozak, 1989; Kozak, 2002). An initiation context that supports efficient AUG recognition will closely match the sequences ACCAUGG and AAAAUGU in mammals and yeast, respectively (Kozak, 1999; Miyasaka, 1999). In these systems, most critical for efficient initiation is a highly conserved purine, usually A, at the –3 position of the initiation context (three nucleotides before the AUG codon). AUG codons with a pyrimidine at –3 are associated with inefficient initiation and promote leaky scanning – a process by which some scanning 40S ribosomal subunits bypass an upstream AUG and initiate translation at a downstream AUG codon (Kozak, 1989). The distance from an AUG codon to the mRNA 5′-terminus can also affect initiation efficiency such that AUG codons residing less than 12 nt from the mRNA 5′-terminus inefficiently initiate translation regardless of their initiation context (Kozak, 1991b; Slusher et al., 1991). In genetic screens, eIF1, eIF2, and eIF5 have each been found to affect the fidelity of initiation codon selection (Castilho-Valavicius et al., 1990; Cigan et al., 1989; Cui et al., 1998; Donahue et al., 1988; Hashimoto et al., 2002; Huang et al., 1997; Yoon and Donahue, 1992); however it remains unclear how these factors contribute to initiation codon recognition.

Much remains to be mechanistically understood about the scanning process. It has been reported to require ATP (Kozak, 1980); however it is not clear what this energy is used for. As eIF4F appears to contribute to the scanning process, it is possible that the ATP-dependent RNA helicase subunit of eIF4F, eIF4A, utilizes energy from ATP hydrolysis to unwind structures in mRNA, thereby coupling mRNA unwinding and the scanning movement (Pestova and Kolupaeva, 2002). Biochemical reconstitution

experiments showed that a minimal complex comprising a 40S subunit, eIF3, and the TC can bind to an mRNA 5′-end, the identification of a start codon however, required eIF1 (Pestova and Kolupaeva, 2002), which is an essential and conserved initiation factor (Yoon and Donahue, 1992) (Kyrpides and Woese, 1998). It has been suggested that eIF1 base pairing with mRNA sequences may influence initiation codon recognition by reducing the rate of scanning and facilitating recognition of AUG codons by Met-tRNA$_i$ (Kozak, 1999). Perhaps consistent with this role for eIF1, human eIF1 contains a unique domain consisting of two α-helices tightly packed on one side of a five-stranded β-sheet, which contains two parallel and three anti-parallel stands (Fletcher et al., 1999). It is unclear how this domain is involved in start codon recognition.

*1.1.1.5 **Assembly of the 80S Ribosome.*** Formation of an elongation-competent 80S ribosome involves two distinct GTP hydrolysis steps. The initial hydrolysis, by eIF2, is of GTP bound to the eIF2 γ subunit. This hydrolysis occurs in response to start codon selection, and is aided by the GTPase-activating protein (GAP) eIF5 (Chakravarti and Maitra, 1993; Huang et al., 1997; Merrick and Hershey, 1996). The carboxy-terminal two-thirds of the eIF2 β subunit and eIF5 show significant homology (Das et al., 1997), raising the possibility that the homologous domains in these factors interact or compete with each other, thereby influencing GTP hydrolysis by eIF2. Codon-anticodon interactions are likely to trigger a conformational change in eIF2 that leads to the hydrolysis of eIF2-bound GTP (Hinnebusch, 2000) and generation of a GDP-eIF2 binary complex believed to be released from the ribosome.

More recently, *in vitro* reconstitution experiments have shown that a second GTPase, eIF5B, the eukaryotic homolog of bacterial IF2, has GTPase activity that is coupled to, but not required for, 80S ribosome assembly (Lee et al., 2002b; Pestova et al., 2000). GTP-bound eIF5B stimulates 60S subunit joining and hydrolysis of this GTP occurs after 80S formation (Pestova et al., 2000). Yeast with GTPase-deficient eIF5B show reasonable health, however, translation initiation at uAUG codons is reduced (Shin et al., 2002), implicating eIF5B function in start codon recognition. Although more characterization is needed to understand eIF5B's role in translation initiation, one

possibility is that GTP hydrolysis by eIF5B serves predominantly as a final checkpoint for correct 80S assembly.

1.1.2 Elongation

Amino acids are sequentially added to the carboxy-terminal end of a nascent peptide during the elongation stage of protein synthesis, which is a process involving three tRNA binding sites in the large ribosomal subunit: The A-site, the initial aminoacyl-tRNA binding site; the P-site, where peptidyl-tRNA binds after a translocation reaction; and the E-site, where deacylated tRNA binds before it is released from the ribosome. Unlike initiation and termination stages of translation, the machinery used during translation elongation has been highly conserved across the three kingdoms of life. This conservation has led to the assumption that the mechanisms underlying elongation are the same in eukaryotes as they are in bacteria and Archaea (Ramakrishnan, 2002; Spahn et al., 2001). Elongation of protein synthesis in prokaryotes and eukaryotes consists of three major steps: (i) binding of aminoacyl-tRNA to the ribosomal A-site; (ii) transpeptidation, the peptide bond formation step, and (iii) translocation, the transfer of peptidyl-tRNA from the A- to P-site.

Ribosomes begin peptide chain elongation with a vacant A-site and peptidyl-tRNA in their P-site. Together with eukaryotic elongation factor (eEF) 1A (EF-Tu in bacteria) and GTP, aminoacyl-tRNA forms a ternary complex that binds to the ribosomal A-site (Moldave, 1985). Noncognate aminoacyl tRNAs can enter the A-site but are incapable of forming the proper codon-anticodon interactions to induce GTP hydrolysis. By contrast, codon-anticodon base paring between mRNA and cognate tRNA alters the conformation of rRNA in the small ribosomal subunit which then appears to activate eEF1A/EF-Tu's GTPase activity (Ogle et al., 2001; Rodnina and Wintermeyer, 2001). Following GTP hydrolysis, GDP-eEF1A is released from the ribosome, and recycled to GTP-eEF1A by the GEF eEF1B, leaving the cognate aminoacyl-tRNA in the A-site ready for the transpeptidation reaction.

The peptidyl transferase center in the large ribosomal subunit catalyzes peptide bond formation between a peptidyl-tRNA in the P-site and an aminoacyl-tRNA in the A-site to produce a carboxy terminus-extended nascent peptide (Moore and Steitz, 2003). A

resulting deacylated tRNA is then in a hybrid state with its acceptor end in the E-site of the large ribosomal subunit and its anticodon end in the P-site of the small ribosomal subunit (Green and Noller, 1997). In a similar hybrid state, the peptidyl-tRNA contains its acceptor end in the P-site of the large subunit and its anticodon end in the A-site of the small subunit. Next, translocation is promoted by hydrolysis of eEF2- (EF-G in bacteria) GTP to eEF2-GDP (Wintermeyer et al., 2001), positioning the deacylated tRNA completely in the E-site, the peptidyl tRNA completely in the P site, and moves the mRNA by three nucleotides thereby placing the next codon of the mRNA into the A site. As a prerequisite for the next elongation cycle, eEF2-GDP is released from the ribosome, allowing the next eEF1A·GTP·aminoacyl-tRNA ternary complex to enter the cycle, which continues until the termination process is initiated at a termination codon. GEFs for EF-G and eEF2 have not been identified.

A notable fungal factor that makes exception to the conservation in translation elongation machinery is eEF3, a factor for which homologs have not been found in bacteria, archeabacteria, or in higher eukaryotes, and therefore appears exclusive to fungal protein synthesis. eEF3 is found mostly in polysome fractions, possesses ribosome-dependent ATPase and GTPase activities (Dasmahapatra and Chakraburtty, 1981) and is essential for yeast viability (Qin et al., 1990). eEF3 interacts with eEF1A (Kovalchuke et al., 1998) and this interaction is important for protein synthesis *in vivo* (Anand et al., 2003). These observations have led to the thought that eEF3 functions to facilitate release of deacylated tRNA from the E-site and enables efficient binding of eEF1A·GTP·aa-tRNA ternary complex to the A site (Triana-Alonso et al., 1995). It remains unclear why fungal ribosomes should uniquely require a distinct elongation factor to facilitate E-site clearance and A-site loading. It may be possible that eEF3-like function is in fact evolutionarily conserved, not as a dissociating factor, but as an intrinsic function of the ribosome.

1.1.3 Termination

In prokaryotic and eukaryotic cells, termination codons (UAA, UGA, and UAG) lack cognate tRNAs and are recognized in the ribosomal A-site by class 1 release factors. In a reaction believed to be catalyzed by the peptidyl transferase center of the ribosome,

class 1 release factors promote hydrolysis of the ester bond linking the polypeptide to the peptidyl tRNA in the P-site (Arkov et al., 1998; Arkov et al., 2002; Caskey et al., 1971; Seit-Nebi et al., 2001; Zavialov et al., 2002). In prokaryotes, UAG is recognized by release factor RF1, UGA is recognized by release factor RF2, and UAA is recognized by both RF1 and RF2(Scolnick et al., 1968). Class 1 release factor activity is stimulated by the GTPase class 2 release factors (Frolova et al., 1996; Mikuni et al., 1994; Stansfield et al., 1995; Zhouravleva et al., 1995). One class 2 release factor, RF3, is present in bacteria and is required in a GTP-dependent manner to eject RF1 and RF2 from the ribosome following peptidyl tRNA hydrolysis (Freistroffer et al., 1997; Grentzmann et al., 1994; Mikuni et al., 1994). Thus in bacteria, two codon-specific class 1 release factors, RF1 and RF2, posses overlapping codon specificity for the UAA stop codon and their activities are stimulated by a single class 2 release factor, RF3.

The only class 1 release factor present in eukaryotes is eRF1, which can recognize any of the three stop codons and promote hydrolysis of peptidyl-tRNA (Dontsova et al., 2000; Frolova et al., 1994; Konecki et al., 1977). Similar to bacteria, eukaryotes contain only one class 2 release factor, eRF3, which, unlike its bacterial counterpart, has not been experimentally verified to promote release of eRF3 from the ribosome following peptidyl tRNA hydrolysis. There is significant difference in the reported crystal structures for human eRF1 and *E. coli* RF2 (Song et al., 2000; Vestergaard et al., 2001) and whereas eRF3 is essential for eukaryotic viability, bacteria are viable in the absence of RF3 (Grentzmann et al., 1994; Mikuni et al., 1994; Wilson and Culbertson, 1988). These observations and the fact that, aside from the GTPase domain in class 2 release factors, there is no significant sequence similarity between eukaryotic and prokaryotic release factor sequences, open the possibility that prokaryotic and eukaryotic release factors belong to two distinct protein families (Frolova et al., 1994).

Functional and structural characterization of eRF1 supports a model in which the structure of eRF1 mimics that of tRNA, thus allowing eRF1 to act at the peptidyl transferase center of the large ribosomal subunit and to recognize codons in mRNA within the small ribosomal subunit (Song et al., 2000). Studies with human eRF1 suggest that a GGQ motif, which is conserved in eukaryotic and prokaryotic class 1 release factors, is analogous to the aminoacyl group attached to the CCA-3′ sequence present in

the aminoacyl stem of tRNA (Song et al., 2000). As a mechanism for the ester bond hydrolysis step of translation termination, it has been proposed that the terminal glutamine of the GGQ motif coordinates a water molecule and mediates a nucleophilic attack on the ester bond of the peptidyl-tRNA in the P-site. Although eRF1 and eRF3 are known to interact and form a complex mediated by their C termini (Eurwilaichitr et al., 1999; Merkulova et al., 1999; Zhouravleva et al., 1995), stimulation of eRF1 by eRF3 is a poorly understood process.

The efficiency of a termination event signaled by a termination codon could be affected by nucleotide sequences. In *E. coli*, the most efficient and inefficient termination sequences are UAAU and UGAC, respectively (Pavlov et al., 1998; Poole et al., 1995). In eukaryotes, UAA(A/G) and UGA(A/G) are the preferred termination sequences (Brown et al., 1990). Genetic studies in yeast indicate that the termination sequence can be critical for ribosomes engaged in translation termination to avoid dissociating completely from mRNA, allowing scanning of 40S ribosomal subunits following termination (Grant and Hinnebusch, 1994; Miller and Hinnebusch, 1989). In addition to these *cis*-acting sequence effects, translation termination efficiency can also be affected by *trans*-acting factors. In yeast, the activities of Upf1p, Upf2p/Nmd2p, and Upf3p appear to affect the efficiency of translation termination since mutations in these *UPF1/NMD* genes results in a nonsense suppression phenotype (Leeds et al., 1992; Weng et al., 1996a; Weng et al., 1996b). The UPF/NMD factors are involved in the nonsense-mediated mRNA decay pathway (see section 1.2.2) and appear to either interact sequentially or form a complex (He et al., 1997; Weng et al., 1996a; Weng et al., 1996b). eRF1 and eRF3 have been shown to interact with Upf1p, and eRF3 prevents formation of a Upf1p:RNA complex, suggesting that eRF3 and mRNA may compete for binding to Upf1p (Czaplinski et al., 1998), which in turn could affect eRF1 activity. The poly(A) tail of mRNA might also affect translation termination as shown by an interaction between eRF3 and PABP that appears to affect cooperative binding of PABP to the poly(A) tail (Hoshino et al., 1999), suggesting that the proposed circularization of mRNA (see section 1.1.1.3) may be affected by terminating ribosomes.

1.2 EUKARYOTIC mRNA STABILITY

The cellular levels of mRNA can be controlled by posttranscriptional mechanisms that involve interactions between mRNA's structural elements (Figure 1.1) and specific *trans*-acting factors. These interactions are emerging as regulated events that control mRNA susceptibility to specific mRNA decay activities. Studies primarily with yeast and mammalian cells have identified two general mRNA decay pathways and two specialized decay pathways. The majority of cellular transcripts that are thought to degrade by the general decay pathways while the specialized decay pathways are more discriminatory with their transcript substrates. Transcripts containing premature termination codons are selectively degraded by the nonsense-mediated mRNA decay (NMD) pathway and transcripts lacking termination codons are targeted by the nonstop decay pathway (Frischmeyer et al., 2002; van Hoof et al., 2002). The identification and studies of these pathways has aided in understanding the relationship between translation and mRNA degradation.

1.2.1 General mRNA Decay Pathways

The general mRNA decay pathways initiate with deadenylation, a process in which a variety of mRNA deadenylases shorten the mRNA poly(A) tail to an oligo(A) length of 10–12 nucleotides (Decker and Parker, 1993; Muhlrad et al., 1995; Parker and Song, 2004). Subsequently, deadenylated mRNA could follow the deadenylation-dependent 5′ to 3′ decay pathway, in which the 5′-cap structure is removed by the Dcp1p/Dcp2p decapping enzyme complex, thereby exposing the transcript to digestion by the 5′ to 3′ exonuclease, Xrn1p (Beelman et al., 1996; Dunckley and Parker, 1999; Hsu and Stevens, 1993; Muhlrad et al., 1995; Muhlrad and Parker, 1994). Alternatively, deadenylated transcripts could follow the 3′ to 5′ exosome-mediated pathway, in which the exosome, a ten-subunit 3′ to 5′ exonuclease complex, degrades transcripts in a 3′ to 5′ direction (Chen et al., 2001)(Mukherjee D et., EMBO 2002)(Anderson and Parker, 1998; Wang and Kiledjian, 2001). Studies have indicated that the 5′ to 3′ decay pathway is the major mRNA decay activity in yeast (Anderson and Parker, 1998; Cao and Parker, 2001; Muhlrad et al., 1995). However, microarray analyses demonstrated that inactivation of

Dcp1p or Xrn1p up regulated, by 2-fold or more, fewer than 20% of yeast genes (He et al., 2003), suggesting that the 3′ to 5′ decay pathway, and not the 5′ to 3′ decay pathway, provides most of the mRNA decay activity in yeast.

1.2.2 Nonsense-Mediated mRNA Decay

Mutations in genomes and errors in gene expression can give rise to mRNAs containing premature in-frame termination codons and translation of these transcripts could result in expression of polypeptide fragments that are harmful to the cell. To safeguard against this threat, eukaryotic cells utilize the nonsense-mediated mRNA decay (NMD) pathway, which rapidly degrades a subset of nonsense-containing transcripts, including aberrant and wild-type mRNAs (He et al., 2003; Jacobson and Peltz, 2000) (Lee and Schedl, 2004; Mendell et al., 2004). In yeast, microarray analyses demonstrated that in the absence of cellular factors required for NMD, a diverse range of transcripts were up regulated at least 2-fold, and 324 of the 545 genes represented by these transcripts have assigned functions such as metabolism, energy generation, cell fate, cell cycle and DNA processing, cell rescue and defense, cellular transport and transport mechanisms, control of cellular organization, regulation of interaction with cellular environment, transcription, protein synthesis, protein fate, and transport facilitation (He et al., 2003). Similarly, microarray analyses have shown that in NMD-deficient human cells, specific classes of natural transcripts are NMD substrates (Mendell et al., 2004). These and other studies demonstrating NMD control of normal mRNAs (Dahlseid et al., 1998; Lew et al., 1998; Welch and Jacobson, 1999) indicate that, in addition to quality control, NMD may play a significant regulatory role for several specific processes in yeast.

NMD can degrade transcripts by a deadenylation-independent decapping mechanism, in which transcripts are decapped by the Dcp1p/Dcp2p complex, without prior deadenylation, and degraded 5′ to 3′ by Xrn1p, (Beelman et al., 1996; Coller and Parker, 2004; Hagan et al., 1995; Hatfield et al., 1996; Muhlrad and Parker, 1994). Nonsense-containing mRNAs that undergo deadenylation-independent decapping can also demonstrate accelerated deadenylation and 3′ to 5′ decay (Cao and Parker, 2003; Lejeune et al., 2003; Mitchell and Tollervey, 2003; Takahashi et al., 2003). In support of

5′ to 3′ decay activity by NMD, microarray studies in yeast showed that inactivation of the NMD pathway up regulated 792 probe sets, 608 (77%) of which were also up regulated by inactivation of Xrn1p (He et al., 2003), indicating that when NMD is active, NMD substrates mostly degrade by the 5′ to 3′ pathway and this degradation includes the activity of Xrn1p. Further analysis demonstrated that in the absence of a functional NMD pathway, transcripts that were NMD substrates continue to degrade by the 5′ to 3′ pathway (He et al., 2003). It is unclear if these transcripts could also degrade by the 3′ to 5′ pathway when NMD is active.

In yeast, the nonessential *UPF1*, *UPF2/NMD2*, and *UPF3* genes encode conserved central regulators of the NMD pathway. Mutations in the *UPF/NMD* genes selectively stabilize nonsense-containing mRNAs without affecting the decay rates of most wild-type mRNAs (Cui et al., 1998; Cui et al., 1996; Cui et al., 1999; Cui et al., 1995; He and Jacobson, 1995; Leeds et al., 1991; Leeds et al., 1992). These results and the observation that single, double, and triple mutations in these genes results in comparable stabilization of the nonsense-containing *CYH2* pre-mRNA (Cui et al., 1995; He et al., 1997; He et al., 1993), indicates that the *UPF/NMD* gene products are functionally related and act in a common pathway. In addition to mRNA stabilization, cells that lack a functional NMD pathway also show nonsense suppression, i.e., nonsense codon read-through (Keeling et al., 2004; Maderazo et al., 2000; Weng et al., 1996a; Weng et al., 1996b). Upf1p, which contains motifs of the superfamily group I helicases (Altamura et al., 1994; Koonin, 1992; Leeds et al., 1992), has been purified and shown to posses RNA binding activity in addition to RNA-dependent ATPase and RNA helicase activities (Czaplinski et al., 1995; Weng et al., 1996a; Weng et al., 1996b; Weng et al., 1998). Nmd2p contains MIF4G (middle portion of eIF4G) domains, one of which maps to a binding region for Upf3p (He et al., 1997; Serin et al., 2001). N- and C-terminal regions of Nmd2p have been mapped as Upf1p binding sites (He et al., 1996; Serin et al., 2001). Upf3p contains a ribonucleoprotein (RNP) domain and NLS- and NES-like segments, suggesting that it can shuttle between the nucleus and the cytoplasm (Lee and Culbertson, 1995; Shirley et al., 1998). A crystal structure of the complex between human Nmd2 and Upf3b proteins indicates that the RNP domain of Upf3b is involved in Nmd2 binding and that residues of its putative NES are critical for the interactions

(Kadlec et al., 2004). These analyses suggest that Upf3p NES mutations that abolish NMD (Serin et al., 2001; Shirley et al., 2002) affect Upf3 nuclear export by disrupting the Nmd2-Upf3 interaction. Consistent with a requirement for an Nmd2-Upf3b complex, Upf3b alone does not bind RNA, but the Nmd2-Upf3b complex does (Kadlec et al., 2004).

Rapid mRNA decay rates caused by NMD are lost by elimination of AUG start codons (Ruiz-Echevarria et al., 1998) and by the expression of nonsense-suppressing tRNA (Gozalbo and Hohmann, 1990; Losson and Lacroute, 1979), indicating that the translational apparatus must recognize NMD-inducing termination codons for the NMD pathway to promote rapid degradation. For some transcripts, the distinction of normal nonsense codons from those that induce NMD has been attributed to sequences 3′ to nonsense codons, referred to as downstream elements (DSEs)(Peltz et al., 1993; Zhang et al., 1995). Based on a degenerate DSE consensus sequence, DSE-like sequences are present in at least one copy in the coding regions of most yeast mRNAs (Hagan et al., 1995; Peltz et al., 1993; Yun and Sherman, 1995; Zhang et al., 1997). Another *cis*-acting sequence element, the stabilizing element (STE), appears to counteract the NMD-inducing effects of nonsense codons present in yeast *GCN4* and *YAP1* mRNAs (Ruiz-Echevarria et al., 1998; Vilela et al., 1998). The *YAP1* STE appears to interact with the RNA binding protein Pub1p, a factor that when inactivated results in destabilization of STE-containing transcripts (Ruiz-Echevarria and Peltz, 2000). In contrast to the uORF-containing *GCN4* and *YAP1* transcripts, the *CPA1* transcript, which contains a uORF and lacks an STE, is susceptible to NMD (He et al., 2003; Ruiz-Echevarria and Peltz, 2000), and was the first example of a naturally occurring wild-type uORF-containing transcript targeted by NMD. It is unclear if NMD of *CPA1* mRNA involves a DSE.

Pre-mRNA splicing results in the deposition of an exon junction complex (EJC) of proteins positioned 20-24 nucleotides 5′ of each exon-exon junction on newly spliced mRNAs (Le Hir et al., 2000). Studies indicates that eIF4AIII, a member of the eukaryotic translation initiation factor 4A family of RNA helicases (DExH/D box proteins), associates with the EJC (Chan et al., 2004), and is essential for NMD in humans (Shibuya et al., 2004) and for mRNA localization in *Drosophila* (Palacios et al., 2004). In mammals, translation termination at nonsense codons that reside more than 50-

55 nucleotides upstream of the 3'-most exon-exon junction typically induce NMD (Cheng et al., 1994; Thermann et al., 1998; Zhang et al., 1998a; Zhang et al., 1998b), which is thought to involve recruitment of Upf1p to EJC-associated Nmd2p (Lykke-Andersen et al., 2000; Maquat, 2004; Mendell et al., 2000). A predominant model for NMD suggests that the first ribosome to translate an mRNA during a "pioneer round" of translation removes EJCs and consequently renders the mRNA immune to NMD (Ishigaki et al., 2001; Lejeune et al., 2002). If an mRNA retains an EJC as a result of premature termination during the pioneer round of translation, then Upf1p recruitment to the EJC is thought to activate NMD. In yeast, the Hrp1/Nab4 protein, which can interact with Upf1p and bind to a DSE (Gonzalez et al., 2000), serves a similar role in promoting NMD as the mammalian EJC. Evidence that NMD is restricted to a pioneer round of translation includes the observation that NMD reduces the abundance of nonsense-containing mRNAs bound to the nuclear cap-binding protein (CBP) 80 to a similar extent as corresponding mRNAs bound to eIF4E, which replaces the CBP80-CBP20 heterodimer at the mRNA cap and is the major cytoplasmic cap binding protein (Ishigaki et al., 2001; Lejeune et al., 2002). These studies did not exclude the possibility that NMD involves eIF4E-bound mRNA, as shown recently in a yeast strain that lacks the primarily nuclear cap-binding complex (Cbc) 1p (Gao et al., 2005).

Unlike with the mammalian NMD model, NMD in yeast does not appear to be limited to an early round of translation. Yeast studies that utilized a galactose inducible promoter to repress or induce the *UPF/NMD* proteins demonstrated that the steady-state levels of NMD substrate transcripts were elevated when the NMD pathway was inactive, and activation of NMD caused a rapid reduction in steady-state levels of the transcripts (Maderazo et al., 2003), indicating that NMD could target steady-state mRNA. Consistent with these results, inactivation of transcription and addition of cycloheximide caused an accumulation of nonsense-containing transcripts on polysomes, and when the drug was washed away, the nonsense-containing transcripts on polysomes degraded (Zhang et al., 1997), as predicted if translation of steady-state mRNA could induce NMD. In addition to these studies, the mammalian NMD model is challenged by experiments indicating that (i) NMD of a nonsense-containing mammalian *HEXA* transcript does not require downstream introns (Rajavel and Neufeld, 2001), (ii) NMD in *Drosophila* occurs

independent of EJC components and exon boundaries (Gatfield et al., 2003), and (iii) NMD can target nonsense-containing unspliced viral RNA (LeBlanc and Beemon, 2004).

A second NMD model suggests that interactions between a terminating ribosome and a specific mRNP structure or set of factors located 3′ of stop codons leads to proper termination and normal mRNA decay rates (Hilleren and Parker, 1999; Jacobson and Peltz, 2000; Maderazo et al., 2003). In the absence of these interactions, improper termination results and NMD is triggered. This model predicts that a DSE acts a "faux" 3′-UTR that lacks regulatory factor(s) required for proper termination, and therefore causes impaired termination, which is thought to allow *UPF/NMD* factor binding and subsequent activation of NMD. Consistent with the *faux* UTR model prediction that premature and normal termination are biochemically distinct events, toeprint analyses with yeast extracts demonstrated that prematurely terminating ribosomes fail to release efficiently, indicating that premature termination is aberrant (Amrani et al., 2004). This effect was not observed with extracts prepared from cells lacking Upf1p or when the nonsense codon was flanked with a normal 3′-UTR. Furthermore, tethering Pab1p downstream of a nonsense codon, which mimics a normal 3′-UTR, resulted in efficient termination and stabilization of nonsense-containing mRNAs. These analyses support the faux 3′-UTR model for NMD and provide strong evidence that a properly configured 3′-UTR is required for proper termination and mRNA stability.

1.2.3 Upstream Open Reading Frames and mRNA Stability

The *S. cerevisiae YAP2* transcript is one of few wild-type uORF-containing mRNAs investigated for uORF-mediated effects on transcript stability. The transcript contains two uORFs and is relatively unstable with a half-life of approximately 2.5 min. when containing both uORFs (Vilela et al., 1999). Elimination of uORF1 or uORF2 results in mRNA half-lives of approximately 4.5 and 7.5 min., respectively, and elimination of both uORFs increases the transcript half-life to 13 min., indicating that in an additive manner, each of the uORFs destabilizes the *YAP2* transcript. These same degrees of transcript destabilization induced by the *YAP2* uORFs were also observed with *YAP2-LUC* chimeric reporter transcripts which were used to discriminate between translational and mRNA stability effects mediated by the *YAP2* uORFs (Vilela et al.,

1998). Luciferase activity measurements showed that, relative to when the *YAP2* mRNA 5′-leader lacked uORFs, uORFs 1 and 2 were approximately 1.8- and 23-fold inhibitory to downstream luciferase translation (Vilela et al., 1998), consistent with predicted poor and good initiation contexts for *YAP2* uORFs 1 and 2, respectively. These studies demonstrate that although the *YAP2* uORFs mediate translational and mRNA destabilizing effects, the magnitudes of these effects are different for the two uORFs. Whereas uORF1 acquires low levels of ribosomes and moderately destabilizes the *YAP2* transcript, uORF2 acquires relatively more ribosomes and is more destabilizing to the *YAP2* transcript.

Although it is unclear exactly how uORFs alter mRNA stability, one possibility is that translation events associated with uORFs do not affect mRNA decay directly; rather they simply reduce downstream translation, which in turn, reduces the ability of mRNA to enter a decay pathway. Alternatively, the utilization of a uORF may directly induce mRNA decay by either rendering the mRNA susceptible to mRNA decay pathway(s) or for endonucleolytic cleavage. It should be noted that in *Drosophila*, decay of nonsense-containing mRNA has been shown to initiate by endonucleolytic cleavage in the viscinity of the nonsense codon, followed by degradation of the 5′- and 3′-fragments by the exosome and Xrn1p, respectively (Gatfield and Izaurralde, 2004). Endonucleolytic cleavage at uORF termination codons may serve a similar role as nonsense codons in *Drosophila* to initiate mRNA decay. It is also conceivable that the translation of a uORF induces one mRNA decay pathway, while the translation of downstream cistrons induces another decay pathway, thereby allowing multiple decay pathways to target an mRNA and control its decay rate. For example, translation of a uORF could trigger the NMD pathway and cause rapid mRNA decay, whereas translation of a downstream cistron could trigger a general mRNA decay pathway that more slowly degrades the mRNA.

1.2.3.1 Upstream Open Reading Frames and Nonsense-mediated mRNA Decay. Many studies have shown that yeast transcripts rapidly degrade by NMD when uORFs are introduced into their 5′-leaders (Jacobson and Peltz, 2000; Linz et al., 1997; Ruiz-Echevarria et al., 1996; Welch and Jacobson, 1999). These analyses however, focused on uORF-containing transcripts that are not normal constituents of the yeast

transcriptome, as are the uORF-containing YAP1, YAP2, GCN4, and CPA1 transcripts. Directed studies of these transcripts indicated that the transcript-destabilizing effects mediated by the YAP2 uORFs occur independent of the NMD pathway (Vilela et al., 1999), and the uORFs in YAP1 and GCN4 mRNAs neither mediate NMD nor destabilize their respective mRNAs (Ruiz-Echevarria et al., 1998; Vilela et al., 1998). By contrast, the uORF-containing CPA1 transcript is rapidly degraded by NMD (He et al., 2003; Ruiz-Echevarria and Peltz, 2000). Although the CPA1 transcript has been shown to be a NMD substrate, the uORF has not been investigated for its role in NMD. These studies indicate that not all uORF-containing transcripts are NMD substrates, consistent with microarray analyses that showed accumulation of only a portion of cellular uORF-containing mRNAs in upf1Δ, nmd2Δ, or upf3Δ strains (He et al., 2003; Ruiz-Echevarria and Peltz, 2000), indicating that additional criteria to the uORF itself are required for NMD of uORF-containing transcripts.

A recent study in *Caenorhabditis elegans* demonstrated that the *gna-2* mRNA, which contains two uORFs, is targeted by NMD (Lee and Schedl, 2004). Elimination of these uORFs resulted in *gna-2* mRNA insensitivity to the NMD pathway. These studies further showed that the *trans*-acting factor, GLD-1, binds the *gna-2* mRNA 5′-leader thereby repressing uORF translation and protecting the *gna-2* transcript from NMD. The *gna-2* transcript appears to be the target of a global NMD regulatory circuit because GLD-1 reduces NMD of *gna-2* and other nonsense-containing mRNAs (Lee and Schedl, 2004). Thus, *cis*-acting elements such as uORFs can modulate transcript stability by acting in concert with *trans*-acting factors that regulate translation.

1.3 TRANSLATIONAL CONTROL OF EUKARYOTIC GENE EXPRESSION

Regulation of eukaryotic translation can occur on a global scale or can target translation of specific mRNAs. The phosphorylation status of specific translation factors is highly important for global translational control (Matthews et al., 2000) and has been shown to be critical for the specific induction of *GCN4* mRNA translation in a mechanism dependent on the presence of upstream open reading frames (uORFs)(Geballe and Sachs, 2000; Hinnebusch, 1996; Morris and Geballe, 2000). Translational control

mediated by uORFs can also be transcript specific, as with the uORFs present in the 5′-leaders of the *S. cerevisiae CPA1* and *N. crassa arg-2* transcripts (see section 1.3.2.3).

1.3.1 Global Control of Eukaryotic Translation

Global control of translation is primarily governed by the phosphorylation state of specific initiation factors, elongation factors, ribosomal proteins, aminoacyl tRNA synthetases (Matthews et al., 2000), and eIF4E-binding proteins (4E-BPs); proteins that when bound to eIF4E, prevent eIF4F assembly (Haghighat et al., 1995) and reduce cap-dependent translation (Pause et al., 1994). Hormones, cytokines, mitogens, G-protein-coupled receptor ligands, and adenovirus infection can induce the phosphorylation of 4E-BP1, which in turn, appears to reduce 4E-BP1 interaction with eIF4E and increase global translation rates (Gingras et al., 1999). Decreased phosphorylation of 4E-BP1 resulting from serum starvation (von Manteuffel et al., 1996), picornavirus infection (Gingras et al., 1996), heat shock (Vries et al., 1997), osmotic shock (Parrott and Templeton, 1999), and amino acid deprivation (Kimball and L.S., 2000), leads to a higher affinity by 4E-BP1 for eIF4E, and reduces global cap-dependent translation. The PI3K-Akt/PkB and FRAP/mTOR signaling pathways are involved in the phosphorylation of 4E-BPs, eIF4G, and eIF4B, whereas the ERK and p38 MAPK pathways modulate eIF4E phosphorylation (Kimball and L.S., 2000). Although it is not completely understood how the activities of these factors are affected by signaling pathways and phosphorylation, it is clear that cells utilize signaling pathways to regulate eIF4F assembly as a means to globally regulate translation in response to various extracellular signals.

1.3.2 Upstream Open Reading Frames and Translational Control

According to the scanning model for translation initiation (see section *1.1.1.4*), AUG start codon selection involves 5′ to 3′ directed ribosome scanning of mRNA (Kozak, 1999; Kozak, 2002) and predicts that AUG codons located in mRNA 5′-leaders can serve as translation initiation sites. The identification of transcripts containing such upstream AUG (uAUG) codons has been problematic due to incorrect or unavailable mRNA end-mapping data (Kozak, 1996). Further issues arise from the use of alternative transcription start sites and alternative splicing activity, events which can lead to multiple

transcript isoforms with different uAUG-containing 5′-leader regions. Despite these complications, surveys estimate that 11 to 42% of vertebrate mRNAs and 20 to 48% of human mRNAs contain at least one uAUG codon (Davuluri et al., 2000; Kozak, 1987; Pesole et al., 2000; Pesole et al., 1997; Suzuki et al., 2000). Studies in yeast suggest that uAUG codons are present in at least 200 (3%) of the approximate 6000 mRNAs that comprise the *S. cerevisiae* transcriptome (Vilela et al., 1998), however these studies, similar to the aforementioned vertebrate and human studies, are limited by incomplete mRNA 5′-end mapping data. Approximately two-thirds of oncogenes, and many genes involved in cell growth control and differentiation, contain uAUG codons (Kozak, 1987; Kozak, 1991a; Morris, 1995), indicating that uAUG codons are not uncommon in genes with critical cellular roles.

Studies investigating uAUG codons associated with upstream open reading frames (uORFs) support the scanning model for translation initiation (Geballe and Sachs, 2000; Kozak, 1999; Kozak, 2002; Morris and Geballe, 2000). Translation initiation at uORFs, as with initiation at the principle ORFs in mRNAs, is subject to global control of translation initiation (see section 1.3.1) (Matthews et al., 2000) and can be influenced in *cis* by the distance from the uORF AUG start codon to the mRNA 5′-terminus (Kozak, 1991b; Slusher et al., 1991), secondary structure in the mRNA 5′-leader (Fletcher and Jackson, 2002; Garlapati and Wang, 2002; Wang et al., 1995), and by the uORF initiation context (Kozak, 1999; Miyasaka, 1999). A number of uORFs have been identified and investigated in mammals, DNA viruses, RNA viruses, plants, and fungi (see Table 1.1). Examples of mammalian uORFs will be discussed followed by a detailed discussion of the best-understood examples of regulatory uORFs in fungi, which are those present in the *S. cerevisiae GCN4* transcript, and an evolutionarily conserved single uORF in mRNAs encoding the small subunit of carbamoyl phosphate synthetase (*S. cerevisiae CPA1* and *Neurospora crassa arg-2*). Characterization of the *GCN4* and *CPA1* uORFs has identified two distinct uORF-mediated regulatory mechanisms that control downstream translation initiation.

Table 1.1

Genes with uORFs That Are Involved in Translational Control[a]

Organism	Gene	References
Viruses	baculovirus gp64	(Chang and Blissard, 1997)
	cauliflower mosaic virus 35S RNA	(Fütterer and Hohn, 1992; Pooggin et al., 1998)
	cytomegalovirus UL4*	(Alderete et al., 1999; Degnin et al., 1993; Schleiss et al., 1991)
	influenza NB/NA	(Williams and Lamb, 1989)
	reovirus S1	(Belli and Samuel, 1993; Fajardo and Shatkin, 1990)
	Rous sarcoma virus	(Donzé and Spahr, 1992; Moustakas et al., 1993)
	SV40 16S and 19S RNAs	(Grass and Manley, 1987; Perez et al., 1987; Sedman et al., 1989; Sedman and Mertz, 1988)
Fungi	arg-2*	(Freitag et al., 1996; Luo and Sachs, 1996)
	brlA	(Han et al., 1993)
	CLN3	(Polymenis and Schmidt, 1997)
	CPA1*	(Werner et al., 1987)
	cyc1-362	(Pinto et al., 1992)
	GCN4	(Hinnebusch, 1997)
	HOL1	(Wright et al., 1996)
	INO2	(Eiznhamer et al., 2001)
	stuA	(Wu and Miller, 1997)
	YAP2	(Vilela et al., 1998; Vilela et al., 1999)
Plants	S-adenosylmethionine decarboxylase*	(Franceschetti et al., 2001; Hanfrey et al., 2002)
	arginine decarboxylase	(Chang et al., 2000)
	Lc	(Wang and Wessler, 1998)
	Opaque-2	(Lohmer et al., 1993)
	ornithine decarboxylase	(Kwak and Lee, 2001)
	pma1	(Michelet et al., 1994)
	pma3	(Lukaszewicz et al., 1998)
Amphibia	Cx41	(Meijer et al., 2000)
Mammals	S-adenosylmethionine decarboxylase*	(Hill and Morris, 1992; Hill and Morris, 1993; Mize et al., 1998)
	ADH5	(Kwon et al., 2001)
	ß2 adrenergic receptor*	(Parola and Kobilka, 1994)
	ATF-4	(Harding et al., 2000)
	bcl-2	(Harigai et al., 1996)
	BTEB	(Imataka et al., 1994)
	CD36	(Griffin et al., 2001)

	C/EBPα	(Calkhoven et al., 2000; Lincoln et al., 1998)
	C/EBPß	(Calkhoven et al., 2000; Lincoln et al., 1998)
	CHOP	(Jousse et al., 2001)
	erythrocyte carbonic anhydrase inhibitor	(Bergenhem et al., 1992)
	estrogen receptor alpha uORF	(Kos et al., 2002)
	fibroblast growth factor 5	(Bates et al., 1991)
	fli-1	(Sarrazin et al., 2000)
	glucocorticoid receptor 1a	(Diba et al., 2001)
	her-2/neu	(Child et al., 1999a; Child et al., 1999b)
	huntingtin	(Lee et al., 2002a)
	lck	(Marth et al., 1988)
	major vault protein	(Holzmann et al., 2001)
	mdm-2	(Brown et al., 1999a)
	c-mos	(Steel et al., 1996)
	muscle acylphosphatase	(Fiaschi et al., 1997)
	ornithine decarboxylase	(Manzella and Blackshear, 1990; Shantz and Pegg, 1999)
	placental growth factor	(Maglione et al., 1993)
	PR65 (PP2A regulatory subunit)	(Wera et al., 1995)
	retinoic acid receptor ß2*	(Reynolds et al., 1996; Zimmer et al., 1994)
	serine hydroxymethyltransferase	(Byrne et al., 1995)
	suppressor of cytokine signaling 1 (socs-1)	(Schluter et al., 2000)
	transforming growth factor ß3	(Arrick et al., 1991)
	V(1b) vasopressin receptor	(Nomura et al., 2001)

[a]Deletion of uORF initiator codons alters gene expression; updated and expanded from (Geballe and Sachs, 2000).

*The uORF peptide sequence is established to be important for controlling gene expression.

1.3.2.1 Mammalian uORFs. A role for uORF function has been suspected for the translational control of mdm2 mRNA, which contains two uORFs and encodes for the oncoprotein MDM2. Some human tumors contain elevated MDM2 levels due to increased mdm2 mRNA translation (Capoulade et al., 1998; Landers et al., 1997; Landers et al., 1994), however it is unclear if the mdm2 uORFs contribute to increased mdm2 mRNA translation in tumors. Using a human growth hormone reporter system in HeLa cells, reporter translation efficiency was reduced approximately 40 and 25% by uORFs 1 and 2, respectively, and the uORFs together reduced translation efficiency by approximately 97% (Brown et al., 1999b). In JEG-3 cells, reporter translation efficiency was reduced by uORFs 1 and 2 by approximately 80 and 50%, respectively, and the uORFs together reduced translation efficiency by approximately 96%. These results indicate that the uORFs reduce downstream translation; however, it is unclear how this inhibitory effect is regulated. Some insight has been gained by the observation that uORF1 in human mdm2 (huMDM2) and mouse mdm2 (muMDM2) mRNAs encodes a 14 amino acid peptide that contains 4 conserved amino acids (excluding the initiator methionine), suggesting that the huMDM2 and muMDM2 uORF1-encoded peptides posses regulatory function (Jin et al., 2003). Consistent with this possibility, missense mutations that affected some of the conserved amino acids of the uORF1-encoded peptide were shown to reduce uORF1-mediated repression of translation efficiency (Jin et al., 2003). A regulatory mechanism utilized by mdm2 uORFs to regulate translation has not been identified.

The mRNA encoding for the polyamine biosynthetic enzyme S-Adenosylmethionine decarboxylase (AdoMetDC) contains a uORF that encodes a six amino acid peptide with the sequence MAGDIS (Hill and Morris, 1992). In T cells, translation of the MAGDIS peptide appears required for negative translational regulation of AdoMetDC in response to polyamine levels because missense mutations that alter the MAGDIS sequence relieve translational repression (Hill and Morris, 1993; Mize et al., 1998; Shantz and Pegg, 1994; Shantz et al., 1994). In addition to translational control mediated by the MAGDIS peptide, the efficiency of uORF translation is also regulated in a cell-specific manner and can occur independent of the uORF-encoded peptide. In

nonlymphoid cells the uORF is largely ignored by ribosomes and AdoMetDC translation is relatively uninhibited, whereas in T cells ribosomes efficiently initiate translation at the uORF and AdoMetDC translation is repressed (Ruan et al., 1994). Using a cell-free wheat germ translation system, polyamine translational regulation was mediated by the wild-type uORF-encoded peptide, which was shown to stall ribosomes in the vicinity of the uORF termination codon in response to polyamine levels (Law et al., 2001). *Cis*-acting mutations that were shown to eliminate uORF-mediated regulation of AdoMetDC translation *in vivo*, were also shown to eliminate polyamine-induced ribosome stalling in the cell-free system (Law et al., 2001). Although it is unknown how the uORF-encoded peptide stabilizes ribosomes to mRNA, a consequence of this effect is an accumulation of a MAGDIS-tRNA ^{Ser}, which suggests that the regulated step occurs after the formation of this peptidyl-tRNA molecule and before hydrolysis of the peptidyl-tRNA bond (Raney et al., 2002).

The CD36 mRNA contains three upstream open reading frames and encodes for a macrophage scavenger receptor that mediates the uptake of oxidized low-density lipoprotein (OxLDL) (Endemann et al., 1993; Nozaki et al., 1995; Podrez et al., 2000). Using a luciferase reporter system with human monocyte-macrophages, the CD36 uORFs were shown to increase luciferase translation efficiency in response to glucose concentrations in the growth medium (Griffin et al., 2001). These studies suggested that translational regulation mediated by the CD36 uORFs involves a reinitiation mechanism that is modulated by glucose. CD36 uORF-mediated translational control in response to glucose has been proposed to be physiologically relevant to diabetes because macrophage uptake of lipid is thought to lead to the development of atherosclerosis, a major vascular complication of diabetes (Boring et al., 1998; Griffin et al., 2001; Semenkovich and Heinecke, 1997).

The mRNA encoding the mammalian activating transcription factor 4 (ATF4) contains two uORFs that induce translation of ATF4 in response to eIF2α phosphorylation, which can occur in response to various cellular stresses such as amino acid starvation (Dever et al., 1992), viral infection (Kostura and Mathews, 1989), iron deficiency (Chen et al., 1991), and accumulation of malfolded proteins in the ER (Harding et al., 1999). Using a GFP reporter in mouse fibroblasts, ATF4 uORF2 was

shown to inhibit reporter translation. The uORF2-mediated effect was reduced when both uORFs were present resulting in derepression of reporter translation, which was responsive to eIF2α phosphorylation (Lu et al., 2004). These analyses are consistent with a mechanism by which translation of uORF1 allows ribosomes to bypass the inhibitory effect of uORF2 and reinitiate translation at the downstream ATF4 initiation codon. The ATF4 uORFs are presumed to function analogously to the *S. cerevisiae GCN4* uORFs to increase downstream translation in response to stresses that cause eIF2α phosphorylation (see section 1.3.2.2).

The mRNA encoding the oncoprotein HER-2, a transmembrane receptor tyrosine kinase, contains a uORF that has been shown to repress downstream HER-2 and reporter translation (Child et al., 1999a; Child et al., 1999b). Despite conservation of the HER-2 uORF in mammalian species, multiple missense mutations do not affect uORF-mediated translational repression (Child et al., 1999b), suggesting that the peptide may serve an alternative function to translational control. The HER-2 uORF has been shown to mediate downstream reinitiation of translation (Child et al., 1999b), which presumably can occur at any of three in-frame HER-2 initiation codons. It is unknown if reinitiation is regulated in response to specific signals.

The retinoic acid receptor-β2 (RARβ2) mRNA contains five uORFs that are conserved in humans and mice (de The et al., 1987). Effects of these uORFs on LacZ reporter gene expression have been investigated at different developmental stages in transgenic mice that express RARβ2-LacZ reporter transcripts (Zimmer et al., 1994). In most tissues of transgenic mice, the RARβ2 uORFs did not reduce LacZ translation. In heart and brain regions however, the uORFs were shown to inhibit LacZ translation, suggesting that the uORFs function to modulate RARβ2 expression in a tissue specific manner. Tissue-specific differences in translation initiation factor activities may explain why RARβ2 uORF-mediated repression of translation is specific to mouse heart and brain tissues.

β_2 adrenergic receptor mRNA from several mammalian species contain a single uORF that encodes a conserved 19 amino acid peptide with positively charged residues near the carboxy terminus (Parola and Kobilka, 1994). In COS-7 cells, translation of the uORF-encoded peptide was shown to inhibit translation of β_2 adrenergic receptor mRNA

and mutation of positively charged conserved residues reduced uORF-mediated inhibition of translational (Parola and Kobilka, 1994). Thus, the β_2 adrenergic receptor uORF-encode peptide appears to regulate translation; however, it is unclear how the uORF-encoded peptide functions in translational control. Similarly, disease related genes such as Huntington disease (HD) (Lee et al., 2002a), branched-chain α-ketoacid dehydrogenase (BCKD) kinase (Muller and Danner, 2004), and beta amyloid cleaving enzyme 1(BACE-1) (De Pietri Tonelli et al., 2004) contain uORFs that have been shown to reduce translation, however further analyses are required for an understanding of the regulatory mechanisms mediated by these uORFs.

*1.3.2.2 **GCN4 uORFs: Regulation of Reinitiation.*** The transcriptional activator Gcn4p positively controls the expression of genes in response to stresses such as amino acid limitation or imbalance (Hinnebusch, 1996; Hinnebusch, 1997). When cells are amino acid-starved, regulation of GCN4 mRNA translation is the principle means for induction of Gcn4p expression and involves four short uORFs (uORFs 1 – 4) in the GCN4 mRNA 5′-leader (Hinnebusch, 1996). Studies indicate that uORF-coding sequence is not important for this regulation and that uORFs 1 and 4 are sufficient for nearly wild-type translational control (Hinnebusch, 1996; Hinnebusch, 1997). A predicted efficient initiation context for uORF 4 is consistent with in vivo studies that showed this uORF alone to be approximately 99% inhibitory to downstream GCN4 translation (Hinnebusch, 1996). Further analyses indicated that, although uORF1 supports efficient initiation as predicted by its initiation context, its presence alone reduced downstream translation by only approximately 50%. Replacing the last sense codon and ten nucleotides immediately 3′ of the uORF1 termination codon with those sequences from uORF4 resulted in an approximate 15-fold reduction in downstream translation, thus identifying a sequence region that affects GCN4 uORF1-mediated repression of downstream translation (Miller and Hinnebusch, 1989). Genetic evidence indicates that this region of uORF1 acts as a critical cis-acting element that allows ribosomes to remain associated with GCN4 mRNA after translation of uORF1, thereby allowing ribosomes to reinitiate translation at the downstream start codon of GCN4. By contrast, ribosomes that have translated uORF4 do not reinitiate and are assumed to

dissociate from the mRNA, as is generally thought to occur after translation termination. Although the molecular basis for reinitiation is unknown, mechanistic insight may be gained from the observation that uORFs 1 and 4 contain AU- and GC- rich termination regions (i.e., the last codon and ten nucleotides immediately 3′ of the uORF) respectively, which are suspected to reflect differences in interactions between nucleotides in these regions and components of the translational machinery (Grant et al., 1994).

Many *in vivo* studies support a model for *GCN4* uORF control in which ribosomes scan the *GCN4* mRNA 5′-leader, initiate efficiently at uORF1, and approximately 50% of these ribosomes subsequently reinitiate downstream. Under nonstarvation conditions, the level of functional eIF2 (see section *1.1.1.2*) is high and reinitiation occurs soon after uORF1 translation, resulting in reinitiation at uORFs 2 – 4 in preference to the *GCN4* ORF. Under starvation conditions, phosphorylation of the eIF2 α-subunit by the protein kinase Gcn2p converts eIF2 from a substrate to an inhibitor of the guanine nucleotide exchange factor eIF2B (see section *1.1.1.2*) and thereby lowers the levels of eIF2•GTP•Met-tRNA$_i^{Met}$ ternary complex (TC) available for initiation (Hinnebusch, 1996). Consequently, starvation causes reinitiating ribosomes (i.e., ribosomes that have translated uORF1 and remain associated with the mRNA) to rebind TC at a slower rate, which provides sufficient time for reinitiating ribosomes to scan past uORFs 2 – 4 before rebinding TC. Reinitiating ribosomes that rebind TC downstream of uORF4 can then reinitiate at the *GCN4* ORF start codon. Thus, moderate reduction in TC levels allows a fraction of reinitiating ribosomes to bypass the highly inhibitory uORFs and initiate translation at the downstream *GCN4* start codon.

1.3.2.3 CPA1 and arg-2 uORFs: Regulation of Leaky Scanning. The *S. cerevisiae CPA1* mRNA encodes the small glutaminase subunit of arginine-specific carbamoyl phosphate synthetase (CPS-A) and its 5′-leader contains an evolutionarily conserved uORF that was identified almost 20 years ago as a negative element involved in translational repression of *CPA1* expression (Delbecq et al., 1994; Werner et al., 1987). These studies utilized a reporter system that coupled CPSase A activity with ornithine carbamoyltransferase activity (EC 2.1.3.3) to determine levels of *CPA1* gene expression in yeast grown in minimal media that lacked or contained arginine (Arg). Results from

these analyses indicated that Arg caused an approximate 4.5-fold reduction in Cpa1p while *CPA1* mRNA levels were relatively unaffected. Interpretation of the minimal effects of Arg on *CPA1* mRNA levels are challenging because *CPA1* mRNA is transcriptionally regulated by Gcn4p. Given that Gcn4p responds to amino acid limitation and imbalance, quantitation of *CPA1* mRNA requires appropriate controls to correct for possible Arg-induced transcriptional effects. The Arg-induced effect was absent in strains that contained either an AUG to UUG mutated uORF start codon (ΔAUG) or a single nucleotide Asp to Asn missense mutation at codon 13 (D13N), suggesting that translation of the uORF and the amino acid sequence of the uORF-encoded peptide are critical for reduction of Cpa1p in response to Arg. Further support for this notion was provided by genetic studies that demonstrated *CPA1* uORF-dependent Arg-induced repression of Cpa1p when sequences 5′ of the uORF were deleted, when the uORF was fused to *LacZ*, and when the uORF contained silent mutations that altered the mRNA sequence but left unchanged the uORF-encoded amino acid sequence (Delbecq et al., 1994). Taken together, these analyses suggested that the *CPA1* uORF-encoded peptide is critical for regulation of Cpa1p translation in response to Arg.

Genetic studies also identified an unlinked recessive *trans*-acting mutation, *cpaR*, which reduced regulation by Arg (Thuriaux et al., 1972; Werner et al., 1987). In a *cpaR* strain grown in Arg-containing medium, *CPA1* mRNA levels were reported to be elevated 3-fold relative to an isogenic wild-type strain (Messenguy et al., 1983), consistent with subsequent identification of the *cpaR* mutation as an allele of *UPF1*, an essential gene for NMD (see section 1.2.2) (Messenguy et al., 2002). A possible role for NMD in the *CPA1* regulatory system was initially suspected from studies indicating destabilization of *CPA1* mRNA in wild-type cells grown in Arg-containing minimal medium, as shown by ^{3}H-uridine pulse-chase analyses (Crabeel et al., 1990). In addition to reducing the *CPA1* mRNA half-life, Arg was also reported to reduce the rate of *CPA1* mRNA synthesis (Crabeel et al., 1990). Direct evidence that the *CPA1* transcript is a substrate for NMD was provided by transcription inhibition experiments that demonstrated the *CPA1* mRNA half-life to be 3 min in a wild-type strain and approximately 18 min in an isogenic *upf1Δ* strain (Ruiz-Echevarria and Peltz, 2000).

A mechanistic understanding of the translational control mediated by the *CPA1* uORF was gained by applying the scanning model for translation initiation (see *1.1.1.4*) to results that indicated similar Cpa1p levels in *ΔAUG* and isogenic wild-type strains when the growth medium lacked Arg, but not in Arg-containing medium, in which Cpa1p levels appeared 4.5-fold lower in the wild-type strain (Werner et al., 1987). From these studies a model was formulated proposing that during growth in Arg-devoid medium, ribosomes scan from the 5′-end of the *CPA1* transcript, inefficiently initiate translation at the uORF initiation codon, and predominantly initiate translation at the *CPA1* ORF start codon, consistent with studies suggesting inefficient and efficient initiation contexts for the *CPA1* uORF and ORF, respectively (Kozak, 1999; Miyasaka, 1999). During conditions of repression by Arg, the uORF-encoded peptide was proposed recognize the *CPAR* gene product (Upf1p) and induce the formation of a complex that inhibits ribosomes from scanning downstream of the uORF, thereby reducing translation of Cpa1p. Although a precise role for Upf1p in the *CPA1* uORF regulatory system remains to be defined, the apparent involvement of Upf1p in translation termination (Czaplinski et al., 1998) suggests that *CPA1* uORF translation termination may be critical for modulation of *CPA1* mRNA stability (Crabeel et al., 1990; Messenguy et al., 2002; Ruiz-Echevarria and Peltz, 2000).

The *CPA1* homolog in *Neurospora crassa*, *arg-2*, contains a 24-codon uORF that is predicted to encode a 23-amino acid peptide. Studies with a wild-type *N. crassa* strain grown in medium that lacked or contained Arg showed that Arg-supplementation reduced the average size of polysomes associated with *arg-2* transcripts, indicating reduced *arg-2* translation in response to Arg (Luo et al., 1995). Consistent with these results, pulse-labeling analyses indicated that Arg2p synthesis, when compared to the level of *arg-2* transcript, was reduced 2-fold within 10 min of exposure to Arg (Luo et al., 1995). These analyses provided direct biochemical evidence for a translational component to the control of *arg-2* expression in response to Arg availability.

Expression of *arg-2-lacZ* fusion genes containing either the wild-type *arg-2* uORF or a mutated sequence lacking the uORF start codon showed that strains expressing the wild-type fusion gene negatively regulated β-galactosidase activity in response to Arg but strains expressing the mutant fusion gene did not(Luo et al., 1995). This effect was

posttranscriptional because the levels of *arg-2-lacZ* transcripts were unaffected by Arg-supplementation. Studies with *arg-2-hph* fusion genes encoding *E. coli* hygromycin phosphotransferase showed that a single nucleotide mutation resulting in an Asp to Asn change at codon 12 of the *arg-2* uORF (D12N) eliminated regulation of hygromycin phosphotransferase activity by Arg (Freitag et al., 1996), indicating that the mutated uORF was no longer acting as a regulatory element, as was observed with the corresponding mutation, D13N, in the *S. cerevisiae CPA1* uORF (Werner et al., 1987). In addition, Arg was shown to reduce the average size of polysomes associated with *arg-2-hph* transcripts when the wild-type *arg-2* uORF was present but not when the uORF contained the D12N mutation (Freitag et al., 1996; Luo et al., 1995), indicating that the missense mutation eliminated translational regulation. These results are consistent with the notion that translation of the uORF-encoded peptide is required for negative-translational regulation in response to Arg.

In contrast to *arg-2-lacZ* transcript levels, which were unaffected by Arg, endogenous *arg-2* transcript levels were reduced approximately 3- and 6-fold in response to Arg-supplementation in two studies, (Freitag et al., 1996; Luo et al., 1995), respectively. Inability to detect differences in *arg-2-lacZ* transcript levels in response to Arg may reflect the absence of 5′ and 3′sequences in the *arg-2-lacZ* fusion genes, such as the intercistronic sequence and the 3′-UTR. Consistent with such sequence requirements, *N. crassa* cells expressing *arg-2-hph* reporter transcripts, which contained the complete wild-type *arg-2* mRNA 5′-leader, showed that Arg-supplementation reduced the steady-state levels of *arg-2-hph* and endogenous *arg-2* transcripts approximately 2.5 and 6-fold, respectively (Freitag et al., 1996). Steady-state levels of reporter transcripts that contained the D12N mutation however, were unaffected by Arg-supplementation, indicating that the uORF-encoded sequence is critical for Arg-regulation of *arg-2* transcript levels. That Arg reduced steady-state levels of *arg-2-hph* transcripts to a lesser degree than endogenous *arg-2* transcripts may reflect the absence of *arg-2* 5′-leader introns in *arg-2-hph* reporter genes because studies with higher eukaryotes indicate that RNA splicing can affect mRNA nuclear export and NMD (Kim et al., 2001; Le Hir et al., 2001; Lykke-Andersen et al., 2001). Collectively, these *in vivo* analyses are consistent

with a requirement for translation of the uORF-encoded peptide for negative regulation of Arg2p translation and *arg-2* mRNA steady-state levels in response to Arg.

Translational control mediated by the *arg-2* uORF was reconstituted in a homologous, cell-free *in vitro* translation system, with which synthetic *arg-2-luc* transcripts that contained the wild-type intron-less *arg-2* leader fused to firefly luciferase (*LUC*) showed negative regulation of *LUC* activity in response to Arg, while reporter transcripts that lacked the uORF initiation codon or contained the D12N mutation did not (Wang and Sachs, 1997b). This *in vitro* system allowed for primer-extension inhibition (toeprinting) analyses that enabled the detection and mapping of ribosomes on *arg-2-luc* transcripts during conditions of Arg-regulated *LUC* activity (Wang and Sachs, 1997b). These toeprinting studies showed that, ribosomes stall at the wild-type *arg-2* uORF termination codon in response to Arg but not at the corresponding positions on *arg-2-luc* transcripts that lacked the uORF initiation codon or contained the D12N mutation, consistent with *arg-2* and *CPA1* uORF sequence-dependent regulation observed *in vivo* (Freitag et al., 1996; Werner et al., 1987). Concomitantly with ribosome stalling at the uORF termination codon, a reduction in ribosomes associated with the downstream *LUC* initiation codon was detected, indicating decreased translation initiation at the *LUC* start codon in response to Arg (Wang and Sachs, 1997a). These results are consistent with a mechanism in which translation of the uORF-encoded peptide is required for Arg-induced ribosome stalling, which in turn, reduces the access of ribosomes to downstream initiation codons. Due to this *cis*-acting ability to negatively regulate translation, the *arg-2* uORF-encoded peptide has been called the arginine attenuator peptide (AAP)(Wang et al., 1998). Synthetic transcripts that encoded the wild-type AAP fused to the N terminus of LUC showed that Arg negatively regulated *LUC* activity and stalled ribosomes, effects that were absent when fusion transcripts contained the D12N mutation (Wang et al., 1998). These results demonstrated that the *arg-2* intercistronic sequence and uORF termination codon are dispensable for Arg-induced AAP-mediated ribosome stalling, and that Arg could induce AAP-mediated stalling of ribosomes engaged in translation elongation.

The *arg-2 in vitro* system has thus allowed for key insights into the mechanism of *arg-2* uORF-mediated translational control. A similar *in vitro* system for *CPA1* would be

beneficial because physical evidence for *CPA1* uORF-mediated translational control is lacking. The research for this thesis begins with a description of studies that used a *S. cerevisiae* cell-free translation system to investigate the regulatory role of the *CPA1* uORF at the level of translation, followed by a description of studies that utilized the *in vitro* system to distinguish between the translational control mechanisms mediated by the *CPA1* and *GCN4* uORFs, and lastly a description is given of *in vivo* studies in *S. cerevisiae* that investigated the translational and mRNA stability components of *CPA1* uORF-mediated control of gene expression.

CHAPTER 2

A HIGHLY CONSERVED MECHANISM OF REGULATED RIBOSOME STALLING MEDIATED BY FUNGAL ARGININE ATTENUATOR PEPTIDES THAT APPEARS INDEPENDENT OF THE CHARGING STATUS OF ARGINYL-tRNAs[1]

2.1 INTRODUCTION

Upstream open reading frames (uORFs) in the 5-leader regions of eukaryotic and prokaryotic transcripts can serve critical regulatory functions (Geballe, 1996; Jackson and Wickens, 1997; Konan and Yanofsky, 1999; Lovett and Rogers, 1996). The fungal mRNAs specifying the small subunit of carbamoyl phosphate synthetase contain a uORF encoding an evolutionarily conserved peptide (Figure 1A). In the cases of *Saccharomyces cerevisiae CPA1* and *Neurospora crassa arg-2*, the capacity to translate this uORF peptide is essential for establishing Arg-specific negative regulation of gene expression *in vivo* (Delbecq et al., 1994; Freitag et al., 1996; Luo et al., 1995; Luo and Sachs, 1996; Werner et al., 1987). Evolutionarily conserved uORFs are also found in mammalian mRNAs including those specifying HER-2/NEU (Child et al., 1999b and references therein), BCL-2 (Harigai et al., 1996; Salomons et al., 1998) and CCAAT/enhancer binding protein (Lincoln et al., 1998). While these mammalian uORFs affect translation, their regulatory roles remain unknown. Understanding how the *CPA1* and *arg-2* uORFs exert regulatory effects should provide insight into such uORF-mediated control mechanisms.

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Wang, Z., Gaba, A., and Sachs, M. S. (1999). A highly conserved mechanism of regulated ribosome stalling by fungal attenuator peptides that appears independent of the charging status of arginyl tRNAs. *J. Biol. Chem.* **274**, 37565-37574.

The evidence for a role of *CPA1* uORF translation in regulation is based on mutational studies (McCarthy, 1998 and references therein) and a variety of regulatory models are consistent with the existing data concerning the *CPA1* uORF (McCarthy, 1998). For *arg-2*, mutational studies have been combined with direct biochemical studies. Addition of Arg to growing cells causes a rapid decrease in the rate of ARG-2 polypeptide synthesis and a decrease in the association of the *arg-2* mRNA with ribosomes (Luo et al., 1995). Experiments with reporter genes show that translation of the wild-type *arg-2* uORF is critical for Arg-specific translational control. mRNAs in which uORF translation is eliminated or in which the evolutionarily conserved peptide sequence is altered no longer show decreased association with ribosomes when Arg is added to cells (Freitag et al., 1996; Luo and Sachs, 1996).

Further insight into the mechanism of Arg-specific translational attenuation mediated by the *arg-2* uORF-encoded peptide was gained using cap-, poly(A)- and amino acid-dependent translation extracts derived from *N. crassa* in which regulation is reconstituted (Wang and Sachs, 1997a). Using reaction mixtures containing low or high Arg concentrations and a primer extension inhibition (toeprint) assay to map the positions of ribosomes on capped and polyadenylated synthetic RNA templates, high Arg concentrations are observed to cause ribosome stalling with the wild-type uORF termination codon at the ribosomal A site (Wang and Sachs, 1997b). To ensure that equal amounts of synthetic RNA was added to reactions, trace amounts of [α-^{32}P] UTP were added to *in vitro* transcription reactions and the incorporated ^{32}P-UTP was quantiated to determine the concentrations of synthetic RNAs. Linearized DNA templates contained a poly(T) tract for *in vitro* transcription of poly(A) tails. Since reinitiation following uORF translation does not appear to be efficient in this case, these data provide the basis for a regulatory model in which the Arg-stalled ribosomes prevent trailing scanning ribosomes from reaching the downstream start codon (Kozak, 1999; Wang and Sachs, 1997b).

Based on its *cis*-acting ability to repress the translation of downstream RNA sequences, the *arg-2* uORF-encoded peptide was named the Arg attenuator peptide (AAP). Dissection of sequences outside of the 24-amino acid coding region established that little else of the original *arg-2* mRNA is required for the AAP's regulatory function

(Wang et al., 1998). Neither deletion of the intercistronic sequences nor changing the UAA termination codon to UGA or UAG alters the AAP's regulatory capacity. Furthermore, direct fusion of the AAP at the N-terminus of a polypeptide results in Arg-specific stalling of ribosomes involved in elongation in the region immediately downstream of the AAP, indicating that the termination codon is dispensable for stalling.

Here we examined the generality of AAP-mediated regulation by studying the function of the *CPA1* and *arg-2* AAPs in translation extracts derived from *S. cerevisiae*, *N. crassa* and wheat germ. Both *CPA1* and *arg-2* AAPs mediated translational attenuation in each of these systems as determined by LUC assays. Thus, factors that permit AAP-mediated translational attenuation can be found in plant as well as fungal systems. In the *S. cerevisiae* and *N. crassa* translation extracts, primer extension inhibition (toeprint) assays indicated that Arg-specific translational attenuation was associated with the stalling of ribosomes after AAP translation. The level of charged Arg-tRNA did not appear to be responsible for effecting Arg-specific control because the tRNA was fully charged even at low Arg concentrations. In contrast, all other well-understood examples of translational regulation of amino acid biosynthetic genes in eukaryotes and prokaryotes that are mediated by uORFs respond to the level of tRNA charging (Hinnebusch, 1996; Landick et al., 1996). Thus, AAP-mediated ribosome stalling appears to be an evolutionarily conserved *cis*-acting control mechanism that regulates the expression of a fungal Arg-biosynthetic gene in response to Arg independent of the level of charged tRNA.

2.2 RESULTS

2.2.1 AAP-Mediated Arg-specific Translational Attenuation in Three Cell-Free Translation Systems

We examined Arg-specific regulation mediated by the *S. cerevisiae* and *N. crassa* AAPs in translation extracts derived from *S. cerevisiae*, *N. crassa* and wheat germ. Capped and polyadenylated synthetic RNAs were synthesized from templates in which the *S. cerevisiae CPA1* AAP or the *N. crassa arg-2* AAP were placed upstream of firefly LUC, either as uORFs or as in-frame N-terminal extensions (Figure 2.1B and 2.1C, Table

2.1). Equal amounts of each RNA were used to program translation extracts. As an internal control, a second capped and polyadenylated synthetic RNA that encoded sea pansy LUC (which lacked fungal regulatory sequences) was also added to the extracts (Wang et al., 1998). For each of the extracts used, the addition of 10 μM of each of the 20 amino acids to reaction mixtures was sufficient for near maximal translation of LUC (data not shown). Additional Arg could be added to translation extracts with relatively slight effects on protein synthesis from RNA templates lacking *arg-2* regulatory sequences (e.g., sea pansy LUC, Figure 2.5B).

Comparisons of the translation of firefly LUC in reaction mixtures supplemented with low (10 μM) or high (2 mM) Arg showed that the wild-type *CPA1* AAP, when present as a uORF (Figure 2.1B), reduced the translation of LUC when the concentration of Arg was high (Table 2.1). Translation of LUC from RNA containing the wild-type *N. crassa arg-2* AAP was also subject to Arg-specific negative regulation in each extract (Table 2.1). Introduction of the D13N mutation in the *S. cerevisiae* AAP coding region (Figure 2.1B) or the corresponding D12N mutation into the *N. crassa* coding region eliminated this regulatory effect in all cases (Table 2.1).

The wild-type *CPA1* and *arg-2* AAPs, when fused directly to LUC as N-terminal extensions (Figure 2.1C), also functioned to regulate translation in all three extracts (Table 2.1). The LUC polypeptide produced appeared to initiate at the AAP start codon because changing this codon from AUG to UUG resulted in unregulated and substantially reduced (50-fold in *N. crassa*, 30-fold in *S. cerevisiae*) LUC synthesis (data not shown). The *CPA1* AAP D13N mutation and the *arg-2* AAP D12N mutation eliminated the regulatory effect of Arg in all three systems, showing the strong dependence of Arg-regulation on the sequence of the AAP peptide and the lack of necessity of a uORF termination codon for regulation.

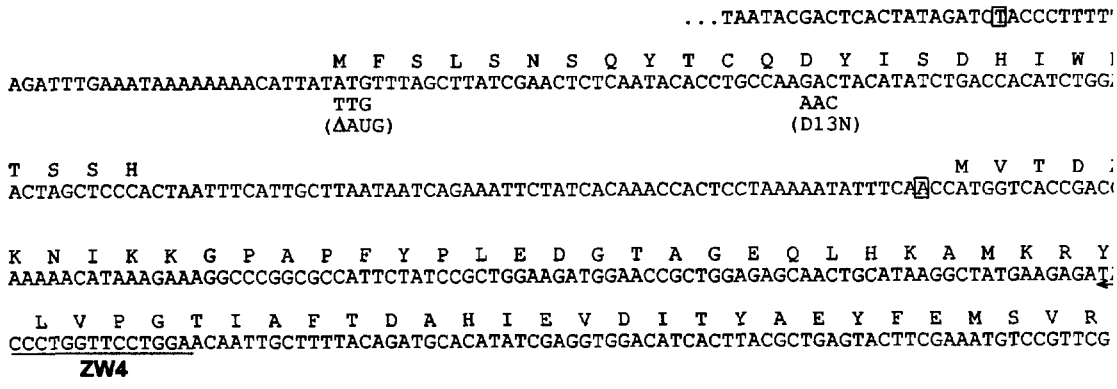
2.2.2 Ribosomal Stalling in High Arg is Mediated by the Wild-type *CPA1* and *arg-2* AAPs

Toeprint assays, in which reverse transcriptase is used for primer extension in translation extracts, enables the mapping of the positions of *N. crassa* ribosomes on RNA(Wang et al., 1998; Wang and Sachs, 1997b). Here we applied this technique to *S.*

A


Sc
Nc
Mg,Tv
An

B


...TAATACGACTCACTATAGATCTACCCTTTT
M F S L S N S Q Y T C Q D Y I S D H I W
AGATTTGAAATAAAAAAAACATTATATGTTTAGCTTATCGAACTCTCAATACACCTGCCAAGACTACATATCTGACCACATCTGG
TTG
(ΔAUG)
AAC
(D13N)
T S S H
M V T D
ACTAGCTCCCACTAATTTCATTGCTTAATAATCAGAAATTCTATCACAAACCACTCCTAAAAATATTTCAACCATGGTCACCGAC
K N I K K G P A P F Y P L E D G T A G E Q L H K A M K R Y
AAAAACATAAAGAAAGGCCCGGCGCCATTCTATCCGCTGGAAGATGGAACCGCTGGAGAGCAACTGCATAAGGCTATGAAGAGAT
L V P G T I A F T D A H I E V D I T Y A E Y F E M S V R
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ZW4

C

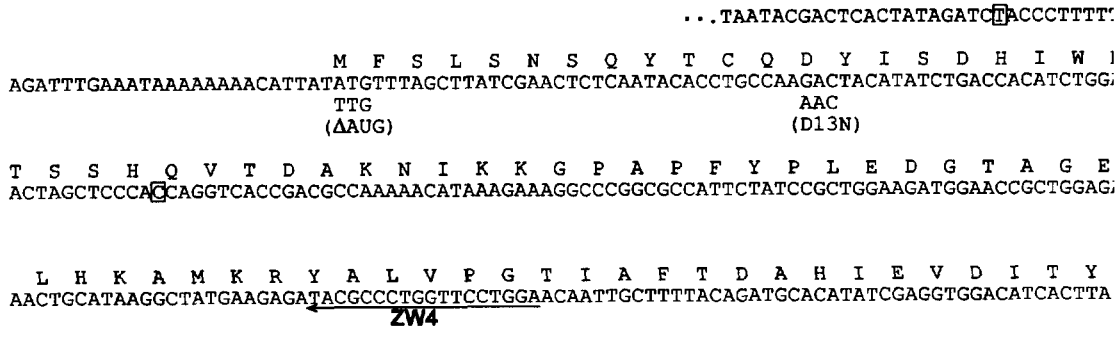

...TAATACGACTCACTATAGATCTACCCTTTT
M F S L S N S Q Y T C Q D Y I S D H I W
AGATTTGAAATAAAAAAAACATTATATGTTTAGCTTATCGAACTCTCAATACACCTGCCAAGACTACATATCTGACCACATCTGG
TTG
(ΔAUG)
AAC
(D13N)
T S S H Q V T D A K N I K K G P A P F Y P L E D G T A G E
ACTAGCTCCCACCAGGTCACCGACGCCAAAAACATAAAGAAAGGCCCGGCGCCATTCTATCCGCTGGAAGATGGAACCGCTGGAG
L H K A M K R Y A L V P G T I A F T D A H I E V D I T Y
AACTGCATAAGGCTATGAAGAGATACGCCCTGGTTCCTGGAACAATTGCTTTTACAGATGCACATATCGAGGTGGACATCACTTA
ZW4

Figure 2.1 Sequences of the fungal AAPs and the 5' leader regions of *CPA1–LUC* genes used in this study (also see Table 2.1). **(A)** Comparisons of the AAPs from *S. cerevisiae* (Sc`; Werner et al., 1987), *N. crassa* (Nc`; Orbach et al., 1990), *Magnaporthe grisea* (Mg`; Shen and Ebbole, 1997), *Trichoderma virens* (Tv`; Baek and Kenerley, 1998) and *Aspergillus nidulans* (An; Genbank AJ224085). **(B)** Sequences of wild-type and mutant templates in which the *CPA1* AAP is encoded by a uORF. The sequence shown begins with the T7 RNA polymerase-binding site and ends within the LUC coding region (Wang and Sachs, 1997b). The 5' and 3' boundaries of the *CPA1* region that was amplified by PCR are boxed. The amino acid sequences of the *CPA1* AAP and the N-terminus of LUC are indicated. Point mutations are shown below the wild-type sequence. The sequence for which the reverse complement was synthesized and used as primer ZW4 for toeprint analysis is indicated by a horizontal arrow below the sequence. **(C)** Sequences of wild-type and mutant templates containing *CPA1* AAP-LUC fusion genes. 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 *CPA1* region that was amplified by PCR are boxed. The amino acid sequence of the N terminus of the AAP_{sc}-LUC fusion polypeptide is indicated. Point mutations are shown below the wild-type sequence. The sequence for which the reverse complement was synthesized and used as primer ZW4 for toeprint analysis is indicated by a horizontal arrow below the sequence.

Table 2.1

Firefly LUC Constructs Used in This study and Their Regulation by Arg in Different Extracts

AAP	Construct	5′ leader structure[a]	LUC Activity[b] (Arg-/Arg+)		
			S.c.	*N.c.*	*Wh*
CPA1	pAG101	Wild-type	4.0 ± 0.5	2.8 ± <0.1	2.3 ± 0.1
	pAG103	D13N	0.9 ± 0.1	1.0 ± 0.2	0.9 ± 0.1
	pAG102	AAP-LUC	3.1 ± 0.1	4.3 ± 0.7	1.4 ± <0.1
	pAG104	D13N AAP-LUC	1.0 ± <0.1	1.2 ± 0.1	0.9 ± 0.1
	pAG105	ΔAUG	0.9 ± 0.1	1.0 ± <0.1	0.9 ± <0.1
arg-2	pPR101	Wild-type	1.6 ± 0.1	1.7 ± 0.1	2.5 ± 0.4
	pPS101	D12N	0.9 ± 0.2	0.9 ± 0.1	1.0 ± <0.1
	pRF107	↑AAP-LUC	2.9 ± 0.5	8.0 ± 0.2	1.4 ± <0.1
	pSF104	D12N ↑AAP-LUC	1.1 ± 0.3	0.9 ± <0.1	1.0 ± 0.1

[a] For details of how the *CPA1* constructs were made, see Figure 2.1 legend and materials and methods; for *arg-2* constructs, see (Wang et al., 1998; Wang and Sachs, 1997a). Wild-type represents the wild-type *CPA1* AAP or *arg-2* AAP as independent uORFs with wild-type initiation contexts. AAP-LUC represents the *CPA1* or *arg-2* AAPs as in-frame N-terminal fusions of the AAP to LUC. D13N and D12N represent amino acid substitutions in the AAPs that abolish regulation. ΔAUG represents an AUG to UUG mutation that eliminates the initiation codon for the uORF encoding the *CPA1* AAP. ↑AAP-LUC and D12N↑AAP-LUC represent wild-type and D12N *arg-2* AAP-LUC fusions in initiation contexts that yield greater translation in *N. crassa* extracts (Wang and Sachs, 1997a).

[b] The ratio of LUC enzyme activity produced after 30 min in translation extracts supplemented with 10 μM Arg to those supplemented with 2000 μM Arg. Extracts were from *S. cerevisiae* (S.c.), *N. crassa* (N.c.) or wheat germ (Wh) as indicated. Values were normalized to the sea pansy LUC internal control and are the mean values ± standard deviations of two independent translation reactions incubated in parallel (Wang et al., 1998).

cerevisiae ribosomes. To our knowledge, this is the first use of toeprinting to examine the positions of *S. cerevisiae* ribosomes on RNA. Primer extension from RNA templates containing the *CPA1* uORF in the absence of extract yielded cDNA products predominantly corresponding to full-length extension of the primer as well as other shorter transcription products (Figure 2.2 lanes 8 and 15). The shorter products are produced in relatively much lower quantities when the RNA is reverse-transcribed in buffer formulated for reverse transcription (data not shown) rather than the buffer formulated for *in vitro* translation necessary for these experiments. Extracts that were not programmed with RNA did not yield any of these signals (Figure 2.2 lanes 7 and 16), as predicted if these represented the products obtained from priming on the synthetic RNA template. When RNA containing the *CPA1* uORF in its 5'-leader was used to program *S. cerevisiae* extracts containing high Arg (500 or 2000 μM, Figure 2.2, lanes 2 and 3) but not low Arg (10 μM, Figure 2.2, lanes 1), new signals were observed that corresponded to ribosomes stalled with the uORF termination codon in the ribosome A site (confirmed by high resolution mapping on other gels, data not shown). The effect of Arg to stall ribosomes at the termination codon increased when the concentration of Arg was raised from 500 μM to 2000 μM. Arg also caused ribosome stalling at the *CPA1* uORF termination codon in *N. crassa* extracts (Figure 2.2, compare lanes 10 and 11 to lane 9). The *CPA1* AAP D13N mutation, which eliminates regulation *in vivo*, (Werner et al., 1987), eliminated Arg-specific effects on toeprints in both extracts (Figure 2.2, lanes 4-6; lanes 12-14), consistent with the loss of regulation observed by LUC assay (Table 2.1).

Puromycin, an inhibitor of translation that releases 80S ribosomes from RNA, would be expected to release Arg-specific signals if they arose from the stalling of ribosomes. Therefore, extracts were programmed with RNA and incubated for 15 min in low or high Arg; then puromycin was added to a final concentration of 1.3 mM (or water was added as a negative control) and incubation continued for 5 min. Extracts were then subjected to toeprint analysis. Puromycin released the Arg-specific toeprints observed in both *S. cerevisiae* and *N. crassa* extracts (data not shown). Thus, the Arg-specific signals appear to be a reversible consequence of the association of ribosomes with the RNA.

In *N. crassa* extracts, signals corresponding to ribosomes with the uORF and LUC initiation codons in their P-sites were observed in extracts programmed with RNA. For

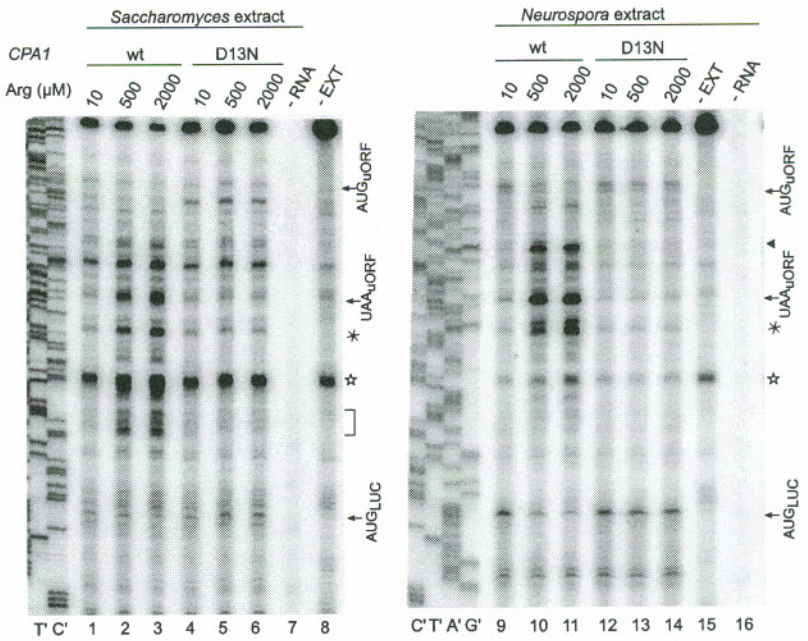


Figure 2.2 Effects of the *CPA1* AAP encoded as a uORF on Arg-specific regulation in translation extracts derived from *S. cerevisiae* and *N. crassa*. Equal amounts (120 ng) of synthetic RNA transcripts were used to program translation mixtures derived from *S. cerevisiae* or *N. crassa*. The transcripts encoded either the wild-type (wt) or D13N mutant *CPA1* AAP as a uORF in the 5' leader as indicated. The 20-μl reaction mixtures were supplemented with different concentrations of Arg (10, 500, or 2000 μM as indicated) and with 10 μM of each of the other 19 amino acids. After 20 min of incubation at 25°C, the reaction mixtures were toeprinted with primer ZW4 as described (Wang and Sachs, 1997b). The products obtained from primer extension of pure RNA (18 ng) in the absence of translation reaction mixture (-EXT) and from a translation reaction mixture not programmed with RNA (-RNA) are shown for comparison. The arrows indicate the positions of premature transcription termination products corresponding to ribosomes bound at AUG_{uORF}, UAA_{uORF}, or AUG_{LUC}. The arrowhead indicates the position of an additional toeprint site upstream of UAA_{uORF} observed in *N. crassa* extracts containing high Arg concentrations; asterisks indicate an additional toeprint site downstream of UAA_{uORF} observed in *S. cerevisiae* and *N. crassa* extracts containing high Arg concentrations. The bracket indicates additional toeprints observed downstream of UAA_{uORF} in *S. cerevisiae* extracts containing high Arg concentrations. The star indicates a strong signal observed from primer extension of RNA in the absence of extract (-EXT). Dideoxynucleotide sequencing reactions for the wild-type *CPA1* template are shown on the left; the nucleotide complementary to the dideoxynucleotide added to each sequencing reaction 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.

constructs containing the wild-type but not the D13N uORF, these signals were reduced when Arg was added. Similar results are observed for the wild-type *arg-2* uORF in *N. crassa* extracts and are interpreted to arise as a consequence of ribosome stalling at the uORF termination codon, which decreases ribosome loading at the LUC AUG, and decreases the capacity to detect signal at the uORF AUG (Wang et al., 1998; Wang and Sachs, 1997b).

The wild-type *CPA1* AAP caused additional Arg-specific effects in each extract, some common and some system-specific (Figure 2.2 and data not shown). In *N. crassa* extracts, an additional signal (arrowhead) approximately 30 nt upstream of the stop codon appeared in high Arg. These possibly represent ribosomes queued behind ribosomes that have stalled at the uORF termination codon (Wang et al., 1998; Wang and Sachs, 1997b). In both systems, Arg caused a substantial increase in (puromycin-releasable) toeprints in the intercistronic region (asterisk, approximately 12 and 16 nt downstream of the stop codon in *N. crassa*, and 16 nt downstream of the stop codon in *S. cerevisiae*). In *S. cerevisiae*, additional Arg-regulated toeprints were observed further downstream in the intercistronic region (star and bracket). One of these signals corresponds in position to a strong signal in the intercistronic region of the RNA which was also present in primer extension products obtained from the RNA in the absence of translation extract (Figure 2.2, lanes 8 and 15, star). The physical basis for these additional bands, which may arise for reasons similar or different than those responsible for the "echo band" phenomenon, in which a ribosome located at an initiation codon can cause a primary toeprint and an additional toeprint (Kozak, 1998), remain to be elucidated. Possibly, they could represent ribosomes or additional machinery recruited by ribosomes; alternatively, they could reflect increased secondary structure in the RNA arising as a consequence of ribosome binding.

In a manner highly similar to the wild-type *CPA1* AAP, the wild-type *arg-2* AAP (in these experiments placed in an improved initiation context (Wang and Sachs, 1997b)) caused ribosomes to stall at the uORF termination codon in response to Arg in both *S. cerevisiae* and *N. crassa* systems; the D12N mutation eliminated regulation (Figure 2.3). Thus, the two fungal AAPs acted similarly when present as uORFs to stall ribosomes in *S. cerevisiae* and *N. crassa* systems.

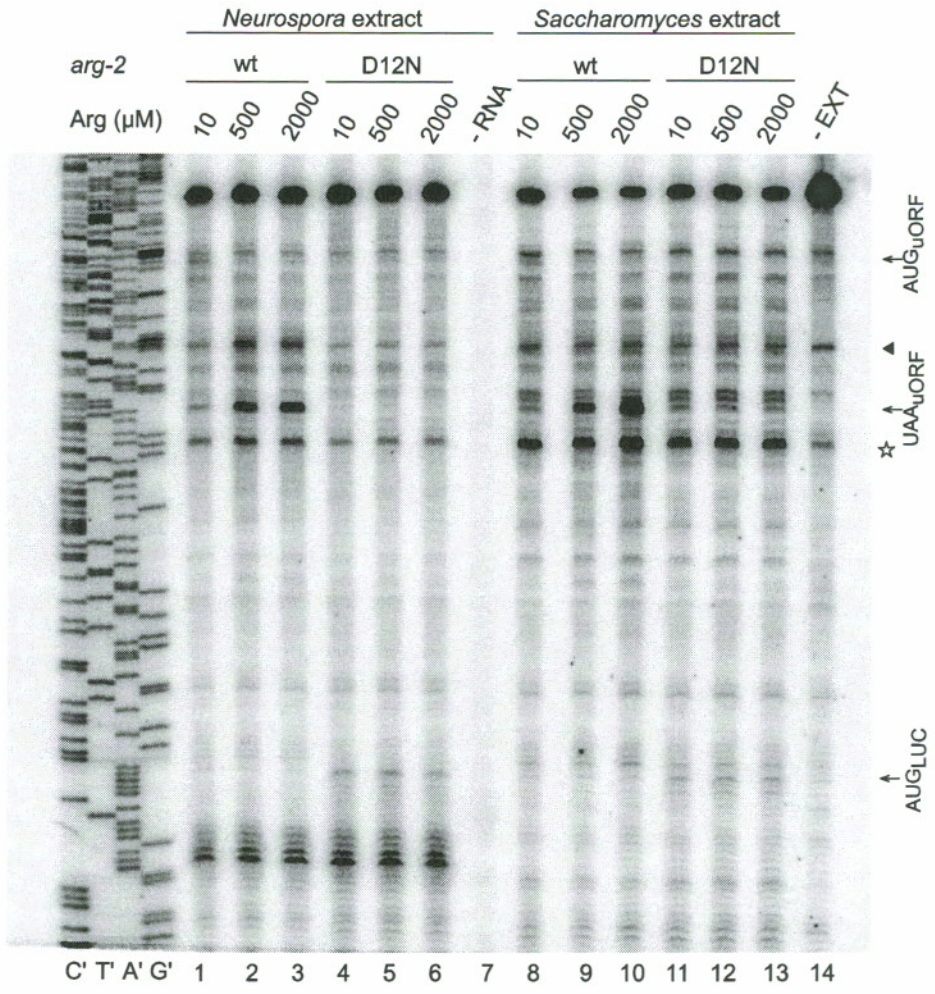


Figure 2.3 Effects of the *arg-2* AAP encoded as a uORF on Arg-specific regulation in translation extracts derived from *N. crassa* and *S. cerevisiae*. Equal amounts of synthetic RNA transcripts (120 ng) were translated in reaction mixtures and analyzed by toeprinting as described in the legend to Figure 2.2. The transcripts encoded either the wild-type (wt) AAP in an improved initiation context or the D12N AAP (Wang and Sachs, 1997b). Arrows indicate the positions of premature transcription termination products corresponding to ribosomes bound at AUG_{uORF}, UAA_{uORF}, or AUG_{LUC}. The arrowhead indicates the position of an additional toeprint site upstream of UAA_{uORF} observed in *N. crassa* extracts containing high Arg concentrations. The star indicates a signal observed from primer extension of RNA in the absence of extract (-EXT) that coincides with a toeprint signal that increases with high Arg (Wang and Sachs, 1997b). Dideoxynucleotide sequencing reactions for the wild-type *arg-2* template are shown on the left; the nucleotide complementary to the dideoxynucleotide added to each sequencing reaction 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.

While AAP-dependent regulation was observed using wheat germ extracts (Table 2.1), in primer extension experiments, no signals indicating Arg-specific stalling were apparent (data not shown). All signals, including full length cDNA products, were weaker in primer extension analyses using wheat germ. This failure to achieve results in the wheat germ system that were comparable to those obtained with the fungal systems is possibly attributable to the presence of an RNase H activity in wheat germ extracts (Haeuptle et al., 1986).

The effect of Arg-specific, AAP-mediated regulation on ribosomes involved in elongation was tested using *CPA1* AAP-LUC and *arg-2* AAP-LUC fusions in *S. cerevisiae* and *N. crassa* extracts. A high concentration of Arg substantially increased the intensity of a series of toeprints on the *CPA1* AAP-LUC RNA in both extracts (Figure 2.4, compare lanes 4 and 3, lanes 12 and 11). The D13N mutation eliminated these Arg-specific effects on toeprints (Figure 2.4, compare lanes 1 and 2, 9 and 10), as did treatment with puromycin after 15 min, as described above (Figure 2.4, lanes 5, 6, 13 and 14), indicating that they resulted from an interaction of ribosomes with the RNA. In both extracts, the most 5'-proximal of the Arg-specific toeprints corresponded to ribosomes translating the first codon following the AAP coding sequence (Figure 2.4, arrowheads). This stall site in the fusion polypeptide corresponds to the position, relative to the *CPA1* AAP coding sequence, of the uORF termination codon. This toeprint site was followed by additional Arg-induced toeprints corresponding to ribosomes stalled in the downstream LUC coding region (indicated by brackets). These signals extend further downstream in *S. cerevisiae* extracts.The length of the region in which ribosomes involved in elongation were stalled in response to Arg appeared determined by the source of the extract and not the source of the AAP. Both *CPA1* and *arg-2* AAPs yielded a more extended series of toeprint sites in *S. cerevisiae* than *N. crassa* (Figure 2.4; data not shown). The reasons for these extract-dependent differences in toeprinting are not known but might reflect faster translation elongation rates in *S. cerevisiae*-derived extracts (data not shown).

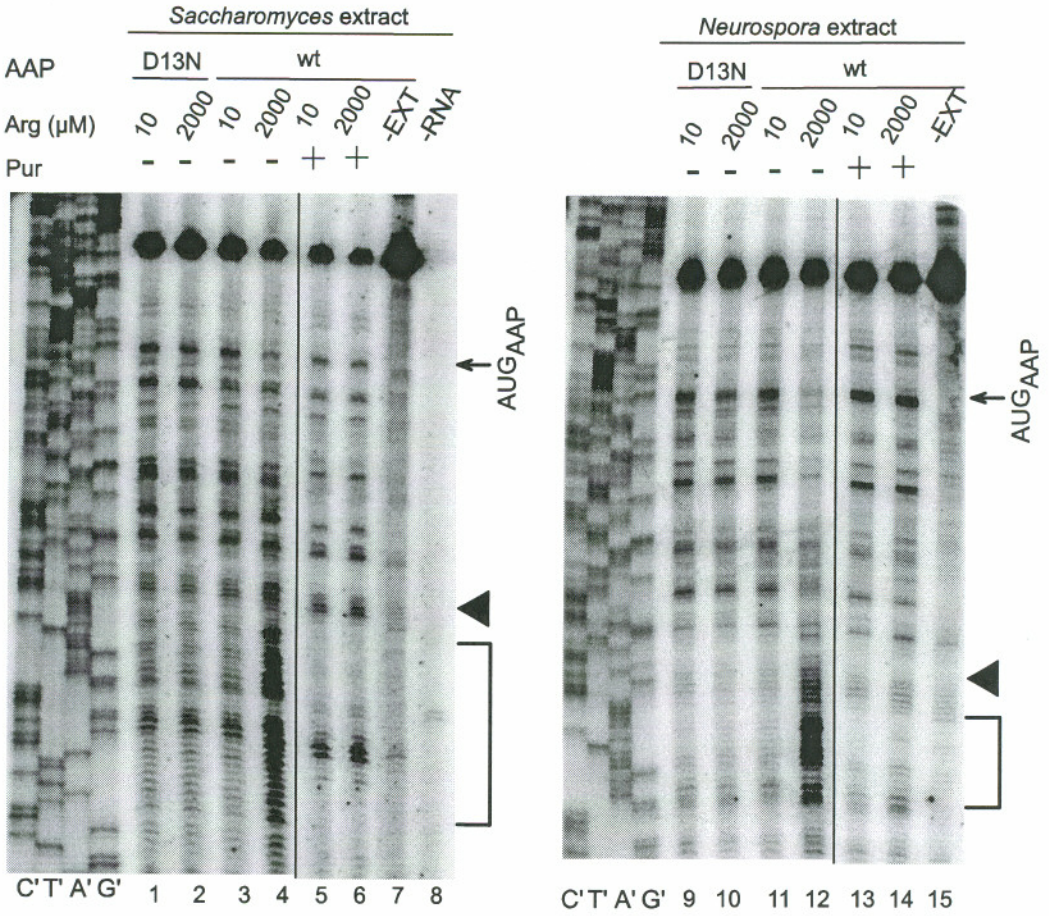
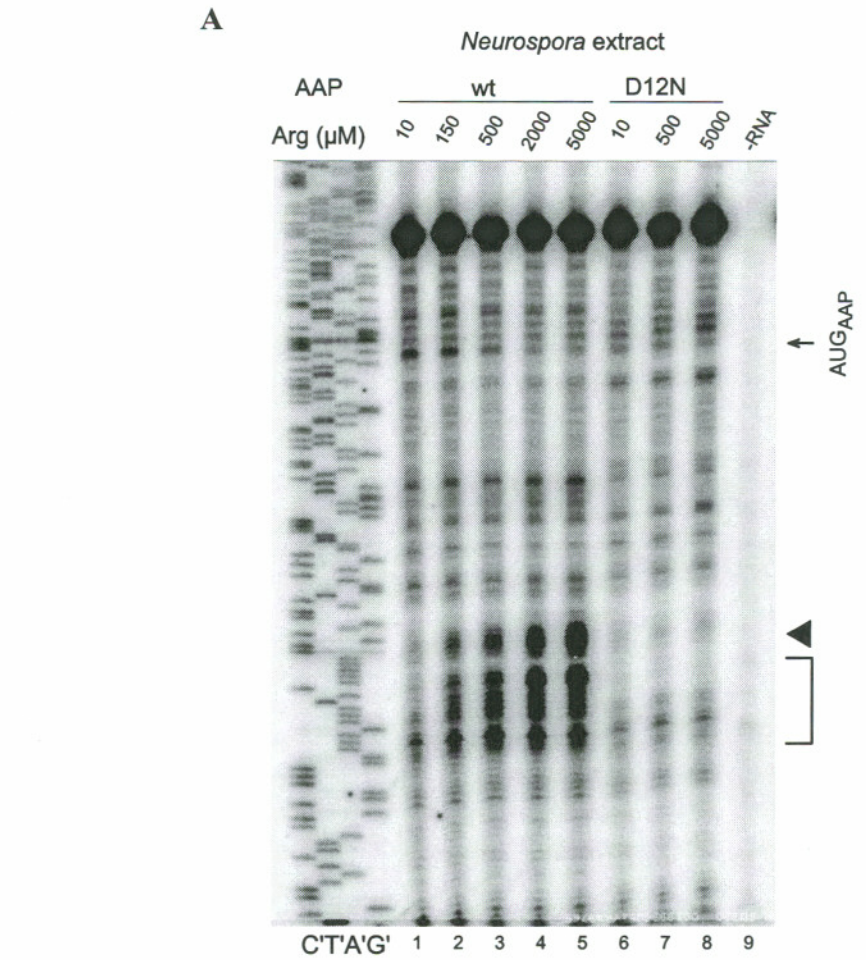


Figure 2.4 Effects of the *CPA1* AAP as an N-terminal fusion to LUC on Arg-specific regulation in translation extracts derived from *S. cerevisiae* and *N. crassa*. Equal amounts of synthetic RNA transcripts (120 ng) were translated in reaction mixtures and analyzed by toeprinting as described in Figure 2.2. The transcripts encoded either the wild-type (wt) AAP-LUC fusion or the D13N mutant AAP-LUC fusion as indicated. Puromycin (Pur) was added where indicated (+), as described in the text. The arrow indicates the position of premature transcription termination products corresponding to ribosomes bound at the AAP initiation codon (AUG_{AAP}). The arrowhead indicates the positions of premature termination products corresponding to ribosomes stalled at the codon immediately following the last codon of the AAP in *S. cerevisiae* and *N. crassa* extracts containing a high Arg concentration. The bracket indicates the position of premature termination products corresponding to ribosomes stalled in the LUC coding region in *S. cerevisiae* and *N. crassa* extracts containing a high Arg concentration. Dideoxynucleotide sequencing reactions for the wild-type *CPA1* AAP-LUC fusion template are shown on the left; the nucleotide complementary to the dideoxynucleotide added to each sequencing reaction 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.

2.2.3 Arg-specific Regulation Appears Independent of the Charging Status of Arginyl-tRNAs

When the effect of Arg on ribosome stalling on transcripts encoding the wild-type *arg-2* AAP-LUC fusion was examined in *N. crassa* translation extracts, the amount of stalling increased as the concentration of Arg increased (Figure 2.5A, lanes 1-5). Similar effects were observed with the *CPA1* AAP-Luc fusion in *N. crassa* extracts and with both fusions in *S. cerevisiae* extracts (data not shown). The *S. cerevisiae* D13N and *N. crassa* D12N mutants did not show stalling at any Arg concentration in either extract (Figure 2.4; Figure 2.5A, lanes 6-8; data not shown). Consistent with the observed increase in stalling of ribosomes on RNA containing the wild-type AAP-LUC fusion, the magnitude of Arg-specific regulation increased in *S. cerevisiae*, *N. crassa* and wheat germ extracts as the concentration of added Arg was increased from 10 μM to 5 mM (Figure 2.5B) as determined by LUC assay. It should be noted that, while the precision of measurements in a given experiment is high, the absolute magnitude of regulation by Arg differs between extract preparations and experiments (e.g., the extracts used in the experiment shown in Table 2.1 showed a lower-magnitude effect than those used in Figure 2.5B). Nonetheless, in multiple experiments using any of the amino acid-dependent fungal extracts prepared in our laboratory to date (28 independently prepared *N. crassa* extracts and 12 independently prepared *S. cerevisiae* extracts), Arg-specific regulation is always observed.

The level of charged arginyl-tRNA might be a signal for Arg-specific regulation mediated by the AAP. Transcriptional attenuation of the amino acid biosynthetic operons in bacteria is modulated by the level of charged tRNA (Landick et al., 1996). The charging status of tRNA controls the translation of Gcn4p in yeast (Hinnebusch, 1996). To determine whether the levels of aminoacylation of arginyl-tRNAs change when different Arg concentrations are present in translation extracts, we adapted a method that has been successful in determining the levels of aminoacylation of tRNAs *in vivo* (Varshney et al., 1991). Reaction mixtures were supplemented with increasing concentrations of Arg and incubated for 10 min. Then total nucleic acid was obtained under conditions in which tRNA charging is maintained and the tRNAs separated by polyacrylamide gel electrophoresis using conditions that resolve charged and uncharged


A
Neurospora extract
AAP
wt
D12N
Arg (μM)
10
150
500
2000
5000
10
500
5000
-RNA
AUG AAP
C'T'A'G'
1
2
3
4
5
6
7
8
9

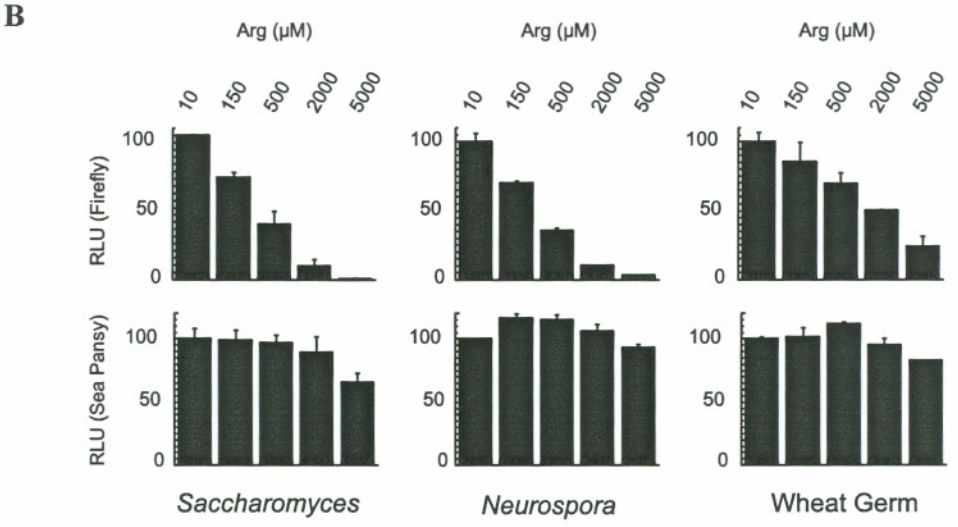

B
Arg (μM)
Arg (μM)
Arg (μM)
10
150
500
2000
5000
RLU (Firefly)
RLU (Sea Pansy)
100
50
0
Saccharomyces
Neurospora
Wheat Germ

Figure 2.5 Effects of Arg concentration on Arg-specific regulation. (**A**) Effects of *arg-2* AAP-LUC fusion in *N. crassa* translation extracts containing varying concentrations of Arg assayed by toeprinting. Equal amounts of synthetic RNA transcripts (120 ng) were translated in reaction mixtures that contained either 10, 150, 500, 2000, or 5000 μM Arg and a 10 μM concentration of each of the other 19 amino acids. Transcripts were analyzed by toeprinting as described in the legend to Figure 2.2. The transcripts encoded either the wild-type (wt) or the D12N mutant *arg-2* AAP as a uORF in the 5' leader. The arrow indicates the position of toeprint products corresponding to ribosomes bound at the AAP initiation codon (AUG_{AAP}). The arrowhead indicates the position of toeprint products corresponding to ribosomes stalled at the codon immediately following the last codon of the AAP in *N. crassa* translation extracts containing high Arg concentrations. The bracket indicates the positions of toeprint products corresponding to ribosomes stalled in the luciferase coding region in *N. crassa* translation extracts containing high Arg concentrations. Dideoxynucleotide sequencing reactions for the wild-type *arg-2* AAP-LUC fusion template are shown on the left; the nucleotide complementary to the dideoxynucleotide added to each sequencing reaction 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. (**B**) Effects of *arg-2* AAP-LUC fusion on Arg-specific regulation in translation extracts derived from *S. cerevisiae, N. crassa,* and wheat germ assayed by measuring luciferase enzyme activity. Equal amounts (12 ng) of *arg-2* AAP-LUC fusion RNA was translated in *S. cerevisiae, N. crassa,* and wheat germ extracts containing either 10, 150, 500, 2000, or 5000 μM Arg and a 10 μM concentration of each of the other 19 amino acids. Mean values and standard deviations from measuring the firefly luciferase enzyme activity in two independent translation reactions are given. Activities of sea pansy luciferase translated from a second RNA encoding this enzyme (an RNA lacking AAP regulatory sequences that was included as an internal control in each reaction mixture) are indicated below the corresponding firefly luciferase activities.

tRNAs. The charging status of different tRNA species were detected by northern blot hybridization using ^{32}P-labeled oligonucleotide probes complementary to the specific tRNAs of interest.

We first checked the charging status of *S. cerevisiae* tRNAs in yeast extracts with a positive control $tRNA_i^{Met}$ probe. Predominantly uncharged tRNA is observed after alkali treatment of the tRNAs isolated from extracts (Figure 2.6A, lane 1). tRNA is mostly uncharged in T_0 *S. cerevisiae* extracts which have not been incubated and which have not been supplied with an energy regeneration system or additional amino acids (Figure 2.6A, lane 2). In contrast, in complete translation extracts containing 10, 150, 500, or 2,000 μM of Arg and 10 μM of each of the other 19 amino acids that have been incubated for 10 min, the $tRNA_i^{Met}$ is predominantly fully charged (Figure 2.6A, lanes 3-6).

The arginyl-tRNAs detected with probes that should recognize three different $tRNA^{Arg}$ species showed the same pattern of charging as the $tRNA_i^{Met}$ control (Figure 2.6B and C). The arginyl-tRNAs were mainly uncharged in T_0 extracts but maximally charged at even the lowest concentration of Arg added to extracts (10 μM). Thus, in *S. cerevisiae* extracts, the charging status of arginyl-tRNAs did not appear to change in response to levels of Arg supplement that result in Arg-specific translational regulation.

Similar studies were attempted using *N. crassa* extracts. Both $tRNA_i^{Met}$ and $tRNA_{GAA}^{Phe}$ were maximally charged under normal translation conditions (data not shown). However, we were unable to detect *N. crassa* $tRNA^{Arg}$ with *S. cerevisiae* probes, and lacking *N. crassa* $tRNA^{Arg}$ sequences to design specific probes, we were unable to determine the charging status of these tRNAs.

Since AAP-mediated Arg-specific regulation was observed in wheat germ extracts (Figure 2.5B), we analyzed the charging status of methionyl- and arginyl-tRNAs in these extracts (Figure 2.6D-F). The results were similar to those obtained with yeast extracts except that wheat germ tRNAs were already charged in T_0 extracts. The reason for this difference between wheat germ and yeast extracts was not determined but it might reflect the presence of a high level of ATP in T_0 wheat germ extracts, since ATP and an ATP regenerating system are present in T_0 wheat germ extracts but not T_0 yeast extracts.

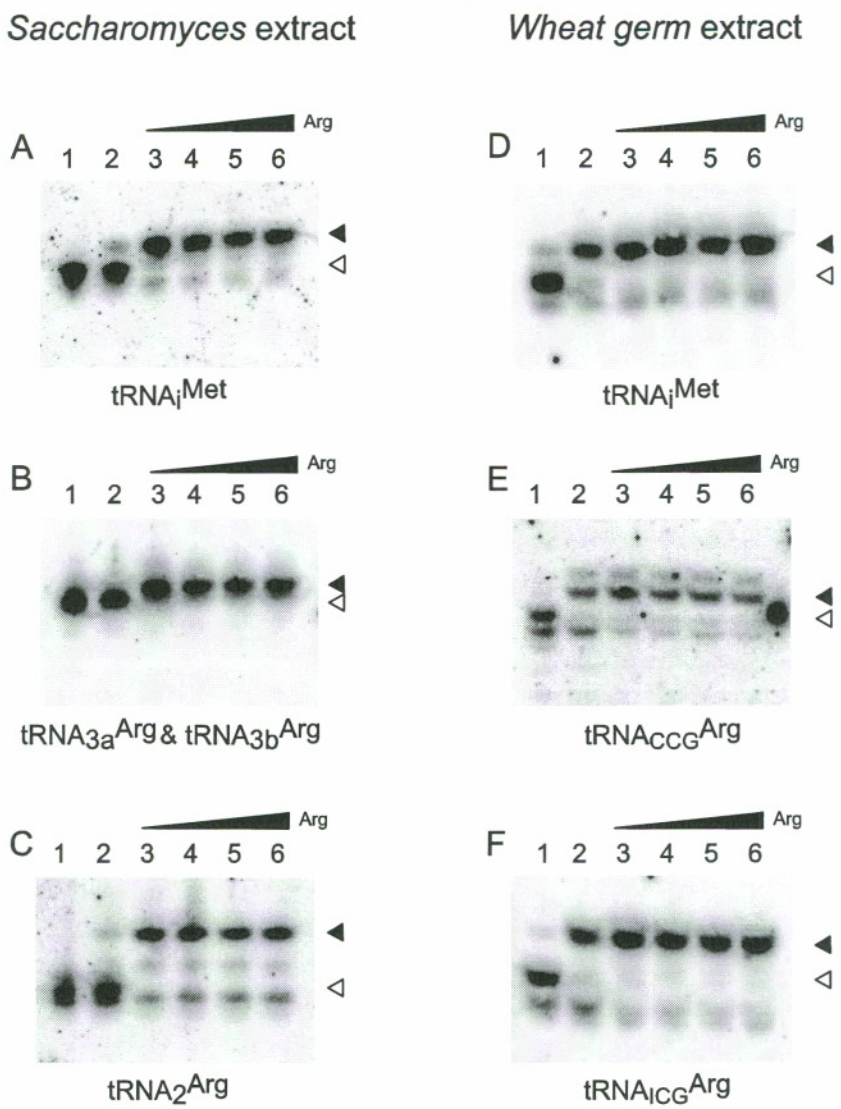


Figure 2.6 Charging status of tRNAs in *S. cerevisiae* and wheat germ extracts. Charged (aminoacylated) tRNAs (filled arrowheads) and uncharged (deacylated) tRNAs (open arrowheads) were separated on acid-urea-polyacrylamide gels followed by electrophoretic transfer to Nytran Plus membranes. Specific tRNA species were detected by northern blot hybridization (Varshney et al., 1991) using 5'-^{32}P-labeled oligonucleotides complementary to specific regions of the tRNA species that are indicated at the bottom of each panel. For each panel, lane 1: alkali-treated tRNAs (deacylated tRNAs) from the extracts indicated; lane 2: tRNAs in extracts at time 0 (T_0 extract); lanes 3-6: tRNAs in translation reaction mixtures after 10 min of incubation; reaction mixtures contained 10, 150, 500 and 2000 μM of Arg, respectively, and 10 μM of each of the other 19 amino acids.

2.3 DISCUSSION

The *S. cerevisiae* Arg biosynthetic gene *CPA1* contains a *cis*-acting control region functionally analogous to a bacterial operator in repressing gene expression in response to Arg (Thuriaux et al., 1972). Regulation is indicated to act at the level of translation because mutations causing constitutive expression affect the translation of a peptide encoded in the 5'-leader of the *CPA1* mRNA (Werner et al., 1987) that is evolutionarily conserved (Figure 2.1A). We examined the role of this AAP (arginine attenuator peptide) in Arg-specific regulation by programming translation extracts from *S. cerevisiae*, *N. crassa* and wheat germ with mRNA containing the AAP and firefly LUC reporter sequences. Using fungal extracts, in which the movement of ribosomes could be examined by primer extension inhibition, the wild-type but not mutant *CPA1* and *arg-2* AAPs acted similarly to stall the movement of ribosomes immediately after AAP translation. Regulation did not appear to be a response to the level of charged Arg-tRNA. The observation that tRNAs were maximally charged in extracts provided with 10 μM of exogenously supplied amino acids is consistent with the observation that this amount of supplement is sufficient for near maximal translational activity and consistent with the observed K_m of 1.5 μM for Arg of the purified *S. cerevisiae* arginyl-tRNA synthetase (Gangloff et al., 1976).

The absence of an apparent role for the level of charged tRNA in a case of translational regulation of an amino acid biosynthetic gene is unprecedented. tRNA charging is important for the transcriptional attenuation of amino acid biosynthetic genes in prokaryotes, in which lack of specific charged tRNAs cause critical stalls in the translation of upstream leader peptides (Landick et al., 1996), and in the translational regulation of *S. cerevisiae GCN4* through the *GCN2*-encoded kinase, which is activated by binding to uncharged tRNA (Hinnebusch, 1997). Yet, these latter control mechanisms that respond to tRNA charging are designed to respond to amino acid limitation. *CPA1* in fact responds to Arg limitation through a *GCN4*-mediated process (Kinney and Lusty, 1989) and *N. crassa arg-2* responds to amino acid limitation through a *cpc-1* mediated process (Ebbole et al., 1991; Freitag et al., 1996; Sachs and Yanofsky, 1991). *cpc-1* is

the homolog of *GCN4* (Paluh et al., 1988; Sachs, 1996), and the translation of its mRNA is also regulated by amino acid limitation (Luo et al., 1995). The available evidence concerning the signal for the CPC-1-mediated response to amino acid limitation in *N. crassa* indicates that it is uncharged tRNA (Sachs, 1996) as it is for the Gcn4p-mediated response in yeast. Strikingly, *N. crassa* contains a close homolog of *GCN2* known as *cpc-3*, and *cpc-3* mutants have phenotypes similar to *gcn2* mutants (Sattlegger et al., 1998). Thus it appears that *CPA1* and *arg-2* share both a conserved mechanism to respond to amino acid limitation (through *GCN4/GCN2* and *cpc-1/cpc-3* , respectively) and a conserved mechanism to respond to Arg surplus (through translation of the *cis*-acting AAP). The response to limitation appears mediated by the level of tRNA; the response to surplus appears to be mediated differently. Because fungi store large amounts of Arg in the vacuole (Davis, 1986) – the concentration of Arg in the vacuole of *S. cerevisiae* grown in Arg-containing medium is 430 mM (Kitamoto et al., 1988) and a high concentration of Arg is also stored in the vacuole of *N. crassa* (Keenan and Weiss, 1997) – it would seem logical that they possess a regulatory mechanism to modulate Arg biosynthesis in response to cytosolic concentrations of Arg far exceeding those necessary for the charging of tRNA.

The *S. cerevisiae* and *N. crassa* AAPs exerted regulatory effects on translation in plant as well as fungal systems. These data provide constraints for models of how Arg exerts its regulatory effect. Presuming that Arg – or a close metabolite – is directly responsible for regulation, then there are at least three ways that it could function to control the movement of ribosomes. High concentrations of Arg could result in modification of the translational machinery (in analogy with uncharged tRNA resulting in eIF2α phosphorylation). This modified machinery would then be sensitive to stalling by the wild-type AAP. Wheat germ and fungal systems might share regulatory pathways (or have independently derived regulatory pathways) that enable AAP-mediated Arg-specific control to be observed *in vitro*.

Second, Arg might not cause modification of a translational component, but instead might interact directly with the translational machinery, causing the machinery to become sensitive to AAP-mediated stalling. In addition to the well-established interaction of Arg with regulatory proteins such as the *E. coli* Arg repressor (e.g., Ni et al.,

1999), Arg can also interact with RNA. In the case of the Tetrahymena rRNA self splicing intron, Arg competes for GTP binding (Yarus, 1989). The HIV TAR RNA binds an Arg residue of Tat; it also binds the free amino acid, blocking the interaction of the RNA with Tat (Tao and Frankel, 1992). RNA aptamers can also be selected on the basis of their binding Arg (Geiger et al., 1996). There is already precedent for the direct inhibition of ribosomal peptidyl transferase activity by Arg (Palacián and Vazquez, 1979). However, that inhibitory effect was elicited by either D-Arg or L-Arg, but D-Arg does not elicit AAP-mediated control in *N. crassa* extracts (Wang and Sachs, 1997a) or *S. cerevisiae* extracts (data not shown).

Finally, Arg might exert its effect by interacting directly with the AAP. The AAP-Arg complex would stall the ribosome, and thus the AAP would function as a *cis*-acting "argometer" from within the ribosome. This is possibly the simplest model consistent with the data available thus far, but there is yet no direct evidence supporting it relative to the other models.

That ribosomes which have translated the AAP are sensitive to stalling by Arg in extracts is clear. This effect could explain the translational response to Arg observed *in vivo* in *N. crassa*, in which Arg reduces the average number of ribosomes associated with *arg-2* mRNA (Luo et al., 1995). But, in addition to reduced translation, the steady state level of *N. crassa arg-2* mRNA is also reduced by growth in Arg (Freitag et al., 1996; Luo et al., 1995; Luo and Sachs, 1996; Orbach et al., 1990; Sachs and Yanofsky, 1991). Similarly, Arg affects the level of *CPA1* transcript (Crabeel et al., 1990). Could there be a role for the uORF-encoded AAP in regulating the level of transcript in response to Arg in these systems, perhaps as a consequence of its function to modulate ribosome stalling? In *N. crassa* continuously grown in the presence of Arg, a reporter gene containing the wild-type *arg-2* uORF shows a reduction in both the level of translation and the level of mRNA, as does the endogenous *arg-2* gene. Introduction of the D12N mutation into the uORF of the reporter gene causes loss of regulation at both translation and mRNA levels *in vivo* , while the endogenous *arg-2* gene remains regulated (Freitag et al., 1996). The wild-type *CPA1* mRNA is known to be destabilized by growth in Arg (Crabeel et al., 1990). One hypothesis – which remains to be tested – that could link our observations on stalling *in vitro* in *S. cerevisiae* and *N. crassa* systems with observations *in vivo* in these

fungi on regulation at the level of mRNA is that ribosome stalling at the wild-type AAP termination codon in response to Arg triggers RNA destabilization. Consistent with this possibility, links between uORF termination codons and RNA stability are observed in *S. cerevisiae* (Hilleren and Parker, 1999; Vilela et al., 1999).

In summary, translation of the evolutionarily conserved AAP in the presence of high concentrations of Arg causes ribosomes to stall. In *S. cerevisiae*, *N. crassa*, and other fungi, the AAP is encoded by a uORF in the 5'-leader of the transcript. The data are consistent with a model for regulation in which the AAP-mediated stalling of ribosomes at the uORF termination codon in response to Arg blocks downstream initiation. Another uORF whose sequence is evolutionarily conserved, the second uORF of cytomegalovirus gpUL4 (gp48) (Alderete et al., 1999), also causes ribosomes to stall after they have translated it (Cao and Geballe, 1996b; Cao and Geballe, 1998). The existence of other uORFs whose peptide sequences are known to be important for regulation (Geballe, 1996), such as the uORF in S- adenosylmethionine decarboxylase (Mize et al., 1998), as well as the existence of evolutionarily conserved uORFs of unknown regulatory function such as are present in transcripts specifying mammalian HER2/*neu* (Child et al., 1999b), *bcl-2* (Harigai et al., 1996; Salomons et al., 1998), CCAAT/enhancer binding protein (Lincoln et al., 1998), and plant bZIP proteins (Martinez-Garcia et al., 1998), suggest that other conserved uORF-encoded peptides may prove to have special roles in regulating translation.

2.4 EXPERIMENTAL PROCEDURES

2.4.1 Templates for RNA Synthesis

To ensure that equal amounts of synthetic RNA were added to reactions, trace amounts of [α-^{32}P] UTP were added to *in vitro* transcription reactions and the incorportated ^{32}P-UTP was quantiated to determine the concentrations of synthetic RNAs. Linearized plasmid templates were designed to produce capped and polyadenylated synthetic RNAs encoding firefly LUC and contained a poly(T) tract (approximately 50 nucleotides) for *in vitro* transcription of poly(A) tails. The first type of RNA was designed to contain the entire *S. cerevisiae CPA1* uORF and intercistronic region in its 5'-

leader (pAG101; Figure 2.1B, Table 2.1). The second type of RNA was designed to have the *CPA1* uORF coding sequence fused directly in-frame with the LUC ORF (pAG102; Figure 2.1C, Table 2.1). Also constructed were mutant variants of each type containing either an Asp to Asn codon change at codon 13 of the *CPA1* uORF (pAG103 and pAG104) or a Met to Leu codon change (AUG->UUG) at the predicted *CPA1* uORF translation initiation codon (pAG105) by using PCR-based procedures (Freitag et al., 1996). PCR products were placed into the pHLUC+NFS4 vector (Wang and Sachs, 1997a). Primers for PCR reactions were: AG1 (5′-TGTTGAAGATCTACCCTTTTT-GCAGATTTG-3′), which includes a 5′-*Bgl*II site, used for pAG101, pAG102, pAG103, and pAG104; AG3 (5′-ATCTGACCATGGTTGAAATATTTTTAGGAGTGGTT-3′), which includes a 3′-*Nco*I site, used for pAG101, pAG103, and pAG105; AG4 (5′-ATA-GATGGTGACCTGGTGGGAGCTAGTTTTCCA-3′), which includes a 3′-*BstE*II site, used for pAG102, pAG104, and pAG106; AG5 (5′-CAGATATGTAGTTTTGGCAGG-3′), which contains the Asp to Asn codon change at codon 13 of the *CPA1* uORF, used for pAG103 and pAG104; and AG6 (5′-TGTTGAAGATCTACCCTTTTTGCAGATTT-GAAATAAAAAAAACATTATTTGTTTAGCTTAT-3′), which contains a 5′-*Bgl*II site and changes the predicted uORF translation initiation codon, used for pAG105 and pAG106. Corresponding templates for the synthesis of RNA containing the *N. crassa arg-2* AAP in the 5'-leader region (Table 2.1) were described previously (Wang et al., 1998; Wang and Sachs, 1997a; Wang and Sachs, 1997b), 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 by equilibrium centrifugation or by using a plasmid purification kit from Qiagen; capped, polyadenylated RNA was synthesized with T7 RNA polymerase from *Eco*RI linearized plasmid DNA templates, and the yield of RNA was quantified (Wang and Sachs, 1997a).

2.4.2 Cell-Free Translation and Primer Extension Inhibition (Toeprint) Analyses

The preparation of translation extracts was as described (Tarun and Sachs, 1995) from *S. cerevisiae* strain YAS1874 (*MAT***a** *MAK10::URA3 PEP4::HIS3 prb1 prc1 ade2 leu2 trp1 his3 ura3*) (Kessler and Sachs, 1998) with two modifications: buffer A was pH

7.6 instead of pH 7.4, and extracts were treated with micrococcal nuclease immediately after recovery from the Sephadex G-25 column, prior to freezing and storage. Nuclease-treated yeast extracts were used because nuclease treatment did not significantly affect amino acid-dependence or Arg-specific regulation under our assay conditions, but in initial comparative studies greatly increased the absolute level of reporter RNA translation and yielded superior toeprints (data not shown). The preparation of translation extracts from *N. crassa* (with no nuclease treatment) was as described (Wang and Sachs, 1997a).

The reaction conditions for *in vitro* translation using *S. cerevisiae* and *N. crassa* extracts were essentially as described previously (Tarun and Sachs, 1995; Wang and Sachs, 1997a). For translation in *S. cerevisiae* extracts, the final concentrations of K^+ and Mg^{2+} were 230 mM and 3.4 mM respectively. Translation reaction conditions using nuclease-treated wheat germ extracts (Promega) were essentially those specified by the supplier, except that, to achieve maximum activity, K^+ and Mg^{2+} final concentrations were adjusted to 100 mM and 2.1 mM, respectively. All reaction mixtures were incubated at 25°C; for LUC assays, translation was halted by freezing in liquid nitrogen after 30 min of incubation, and 5-µl aliquots of the ice-thawed mixtures were used for analysis (Wang et al., 1998; Wang and Sachs, 1997a).

A wide range of conditions were examined to find those which were optimal for toeprinting in *S. cerevisiae*-derived reactions, including pH, heat-pretreatment, reaction temperature and Mg^{2+} concentration (Kozak, 1998). These were similar to those earlier determined to be optimal for *N. crassa*-derived reactions (data not shown). Therefore, the toeprint assays of both *S. cerevisiae*- and *N. crassa*-derived reaction mixtures were accomplished after incubation as described in the text using primer ZW4 and the previously established method (Wang and Sachs, 1997b). All toeprint data shown are representative of multiple experiments.

2.4.3 Measurement of tRNA Aminoacylation

The assays for tRNA aminoacylation were adapted from a previously described procedure (Varshney et al., 1991). Translation reaction mixtures with total volumes of 100 µl (*S. cerevisiae* and *N. crassa*) or 60 µl (wheat germ) containing different

concentrations of Arg were incubated for 10 min. Then aliquots of 90 µl (*S. cerevisiae* and *N. crassa*) or 54 µl (wheat germ) were removed (the remainder of translation reaction mixtures were incubated to the 30-min time-point, then used for LUC assays) and immediately added to ice-cold tubes containing a mixture of 300 µl phenol (pH 4.5) and 200 µl sodium acetate (pH 4.5). Tubes were vortexed for 60 s and then centrifuged for 20 min. The aqueous layers were transferred to new tubes and mixed with 2.5 volumes of ethanol. Tubes were frozen at -80°C for at least 15 min and then the total nucleic acids were recovered by centrifugation for 20 min. The nucleic acid pellet was dissolved in 20 µl of 10 mM sodium acetate -1 mM EDTA (pH 4.5). An aliquot (2.0 µl) was used to measure A_{260}; immediately prior to gel electrophoresis, nucleic acids were adjusted to a final concentration of 2.5 µg/µl (assuming 40 µg nucleic acid per A_{260}) by the addition of acid gel loading buffer (Varshney et al., 1991).

The level of tRNA charging initially present in extracts at time T_0 was determined by processing as described above of 50 µl extract (*S. cerevisiae* and *N. crassa*) or 30 µl extract (wheat germ) with no addition of other reaction-mixture components (e.g., additional salt, synthetic mRNA and amino acids in all three cases, plus an energy regenerating system for *S. cerevisiae* and *N. crassa*). As an additional control, tRNAs in T_0 extracts were deacylated by alkali treatment. First, aliquots of T_0 extracts (50 or 30 µl) were mixed with 250 µl 0.2M Tris-HCl (pH 8.0) and extracted with 300 µl of phenol (pH 8.0). Nucleic acids in the aqueous phase were precipitated with salt and ethanol; the precipitates were dissolved in 100 µl 0.1 M Tris-HCl (pH 8.8) and incubated at 37°C for 20-min to deacylate the tRNAs. After another ethanol precipitation, the pellet was dissolved in 20 µl of 10 mM sodium acetate - 1 mM EDTA (pH 4.5) prior to dilution with acid gel loading buffer.

The procedures for acid/urea gel electrophoresis, electrophoretic transfer, and northern blot hybridization to identify charged and uncharged tRNAs were essentially as described (Varshney et al., 1991), except that denatured salmon sperm DNA was not included in the prehybridization and hybridization solutions, and the membranes after hybridization were exposed to screens of a Molecular Dynamics PhosphorImager for approximately 4-h. DNA probes are the reverse complements of the these tRNA regions: A11: 5'-TCGGTTTCGATCCGAGGACATCAGGGTTATGA-3', complement to 32-63

of *S. cerevisiae* $tRNA_i^{Met}$ (Anderson et al., 1998; Cigan and Donahue, 1986`; James Anderson, personal communication); ZW32: 5'- ACGATGGGGGTCGAACCC -3', complement to 50-67of *S. cerevisiae* Arg-tRNA 3a and 3b (Keith and Dirheimer, 1980); ZW33: 5'- TGGTTCGCAGCCAGACGC -3', complement to 24-41of *S. cerevisiae* Arg-tRNA 2 (Weissenbach et al., 1975); ZW34: 5'-ATCTTCTGGTTCGCAGCC -3', complement to 30-47 of *S. cerevisiae* Arg-tRNA 2; ZW38: 5'- ACCACGCTGGGAGT-CGAACC -3', complement to 52-71 of wheat germ tRNA-Arg (CCG) (Baum and Beier, 1998); ZW39: 5'- ACTCCGCTGGGGATCGAACC -3', complement to 52-71 of wheat germ tRNA-Arg (ICG) ; (Barciszewska et al., 1986); ZW40: 5'- TGGGACCTGTGGG-TTATGGG -3', complement to 31-50 of wheat germ $tRNA_i^{Met}$ (Ghosh et al., 1982); ZW41: 5'- TCGATCCTGGGACCTGTGG -3', complement to 39-57 of wheat germ $tRNA_i^{Met}$; ZW42: 5'- ACCTCCGGGTTATGAGCCC -3', complement to 28-46 of *N. crassa* $tRNA_i^{Met}$ (Gillum et al., 1977); ZW43: 5'- TCGAGTGACCTCCGGGTT -3', complement to 36-53 of *N. crassa* $tRNA_i^{Met}$; ZW44: 5'- CTTCAGTCTGACGCTCTCCC -3', complement to 18-37 of *N. crassa* tRNA-Phe (GAA) (Alzner-DeWeerd et al., 1980); ZW45: 5'- TGCGGTTTGTGTGGATCG -3', complement to 56-73 of *N. crassa* tRNA-Phe (GAA).

Note: The author's work in this chapter was in collaboration with Dr. Zhong Wang. The author was responsible for all of the pAG plasmids and performed the experiments for Table 2.1. Dr. Wang made the plasmids pPR101, pPRS101, pRF107, and pSF104. We prepared the cell-free extracts together; we individually prepared synthetic RNAs. We worked together for the experiments in Figs. 2.2, 2.3, 2.4, 2.5A, and 2.6. The author is responsible for Fig. 2.5B.

CHAPTER 3

PHYSICAL EVIDENCE FOR DISTINCT MECHANISMS OF TRANSLATIONAL CONTROL BY UPSTREAM OPEN READING FRAMES*

3.1 INTRODUCTION

Upstream open reading frames (uORFs) in mRNAs regulate translation in prokaryotes and eukaryotes (Geballe and Sachs, 2000; Gong and Yanofsky, 2001; Lovett and Rogers, 1996; Morris and Geballe, 2000). 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 *S. 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; Hinnebusch, 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; Hinnebusch, 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

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Gaba, A., Wang, Z., Krishnamoorthy, T., Hinnebusch, A. G., and Sachs, M. S. (2001). Physical evidence for distinct mechanisms of translational control by upstream open reading frames. *EMBO J.* **20**, 6453-6463.

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.

S. 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 (Delbecq et al., 2000; Delbecq et al., 1994; Wang et al., 1999; Werner et al., 1987), 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 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, 1997b). However, it has been pointed out that the situation could potentially be different in *S. cerevisiae* (Delbecq et al., 2000; McCarthy, 1998). 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 uORFs. Thus the *GCN4* and *CPA1* uORFs regulate translation in different ways.

3.2 RESULTS

3.2.1 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 3.1 and Table 3.1). Equivalent molar amounts of each mRNA were used to program *S. cerevisiae* cell-free extracts and the synthesis of LUC measured enzymatically (Table 3.2). 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 3.2). 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 3.2). 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 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

Figure 3.1 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 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.

Table 3.1

Constructs Used in This Study

Construct	5′-leader
pPG101	*GCN4* uORFs 1-4
pPG102	*GCN4* uORF1
pPG103	*GCN4* uORF4
pPG104	*GCN4* no uORFs
pPG105	*GCN4* uORFs 1 and 4
pAG112	*GCN4* uORFs 3 and 4
pAG101	*CPA1* uORF
pPR101	*arg-2* uORF
pPT101	↑*arg-2* uORF[a]

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

Table 3.2

Effects of *GCN4* uORFs *in vitro* and *in vivo*

uORFs[a]	LUC[b]	Ribosomes at *GCN4* 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

[a]Constructs used for analyses of LUC synthesis *in vitro* are described in Table 3.1.

[b]The 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 9 independent experiments employing 5 different RNA preparations and 7 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.

[c]The measurement of ribosomes at the LUC AUG codon were obtained using ImageQuant to analyze the data in Figure 3.6 (Lanes 12, 14, 16, 18 and 20), with values normalized as for the LUC assays.

[d]The *GCN4-lacZ* expression data are from (Hinnebusch, 1996); expression values were normalized as for the LUC assays.

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; Hinnebusch, 1997). We therefore examined this directly using the toeprinting assay in which reverse transcriptase is used to extend a radiolabeled 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). First, 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 3.2) and *N. crassa* (Fang et al., 2000; Wang et al., 1998) 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 [^{35}S]Met-labeling, data not shown). There appear to be two steady-state phases of LUC accumulation in *S. cerevisiae* (10-20 minutes and 20-50 minutes) 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 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 3.3). That each of these signals corresponded to a uORF initiation codon was established 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 mRNA constructs lacking that initiation codon was used to program extracts (Figure 3.3, Figure 3.4, and data not shown). For example, toeprints

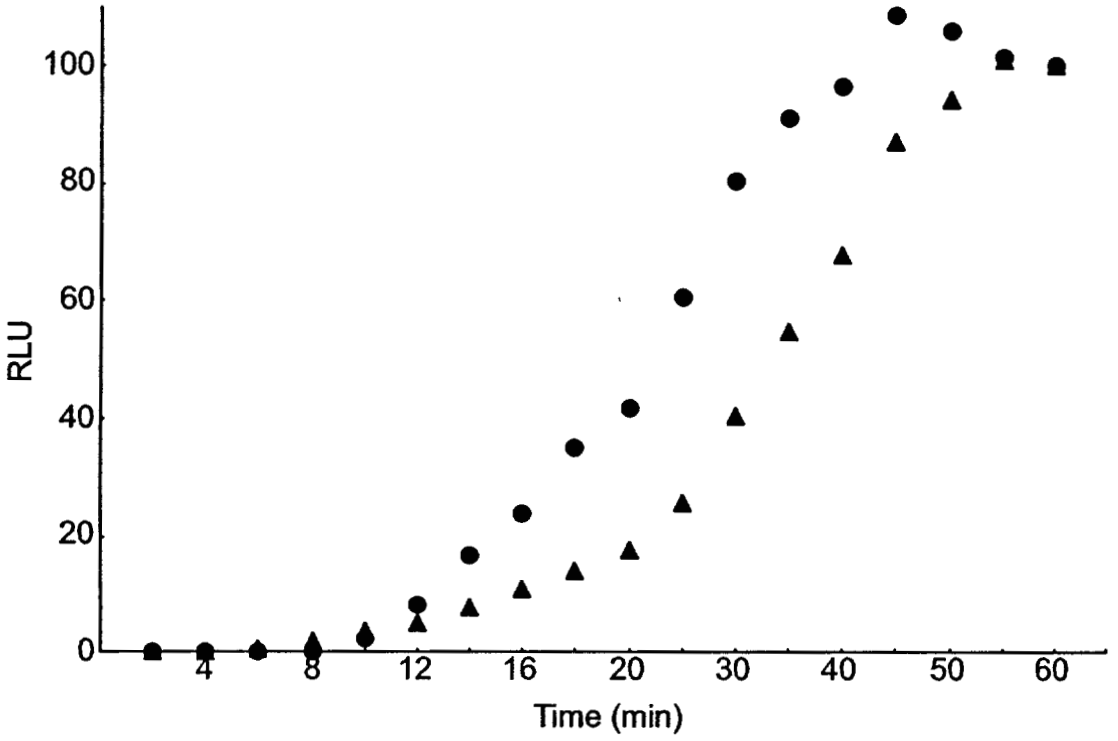

Figure 3.2 Time-course of translation in *S. cerevisiae* programmed with RNAs encoding firefly LUC and sea pansy LUC. A translation reaction (150 µl) was initiated using a mixture of 90 ng of GCN4-LUC RNA lacking uORFs and 9 ng of sea pansy LUC RNA and incubated at 25°C for the indicated periods when 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.

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 3.4A). 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, 1997b). 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 3.5, lane 18 and Figure 3.6, lane 23) but not in primer extension analyses of extract in the absence of exogenously added mRNA (e.g., Figure 3.5, lane 6 and 12, and Figure 3.6, 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 3.5A, 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 (Fang et al., 2000; Wang et al., 1998; Wang et al., 1999; Wang and Sachs, 1997b). Mapping of *GCN4* uORF1 in *N. crassa* extracts showed ribosomes at the initiation codon, the two sense codons, and the termination codon (Figure 3.4B). 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, 1997b) 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 1and 2 (Figure 3.3) or uORFs 1, 2 and 3 (Figure 3.3) resulted in the appearance of a much stronger

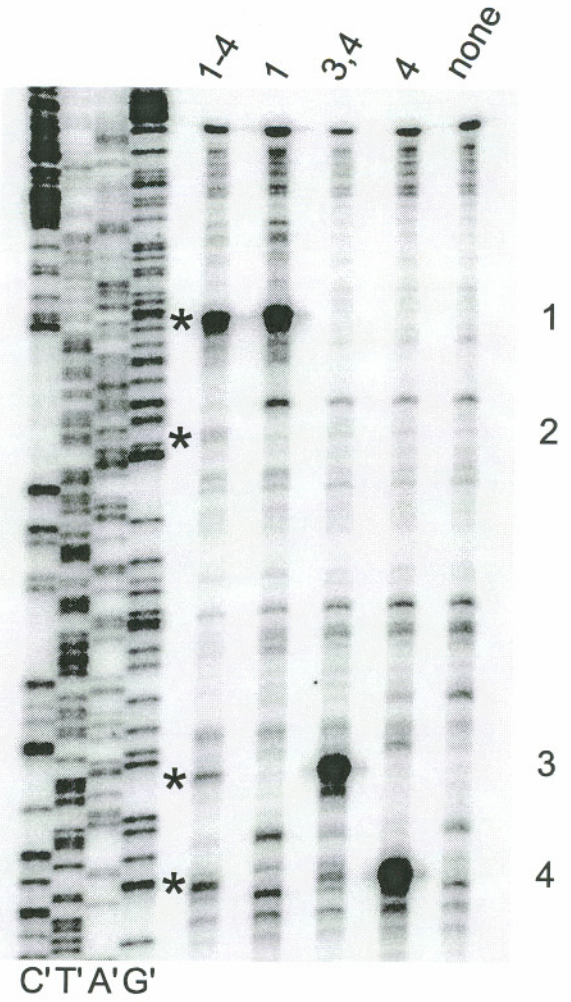

Figure 3.3 Detection of *S. cerevisiae* ribosomes at *GCN4* uORF initiation codons. Equal amounts (120 ng) of synthetic RNA transcripts were used to program translation mixtures derived from *S. cerevisiae*. The *GCN4-LUC* transcripts encoded uORFs 1-4, uORF1, uORFs 3 and 4, uORF4, or no uORFs in the 5' leader as indicated. Transcripts were translated in reaction mixtures at 25 °C for 10 min then supplemented with cycloheximide followed by additional 10 min incubation at 25 °C. Radiolabeled 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.

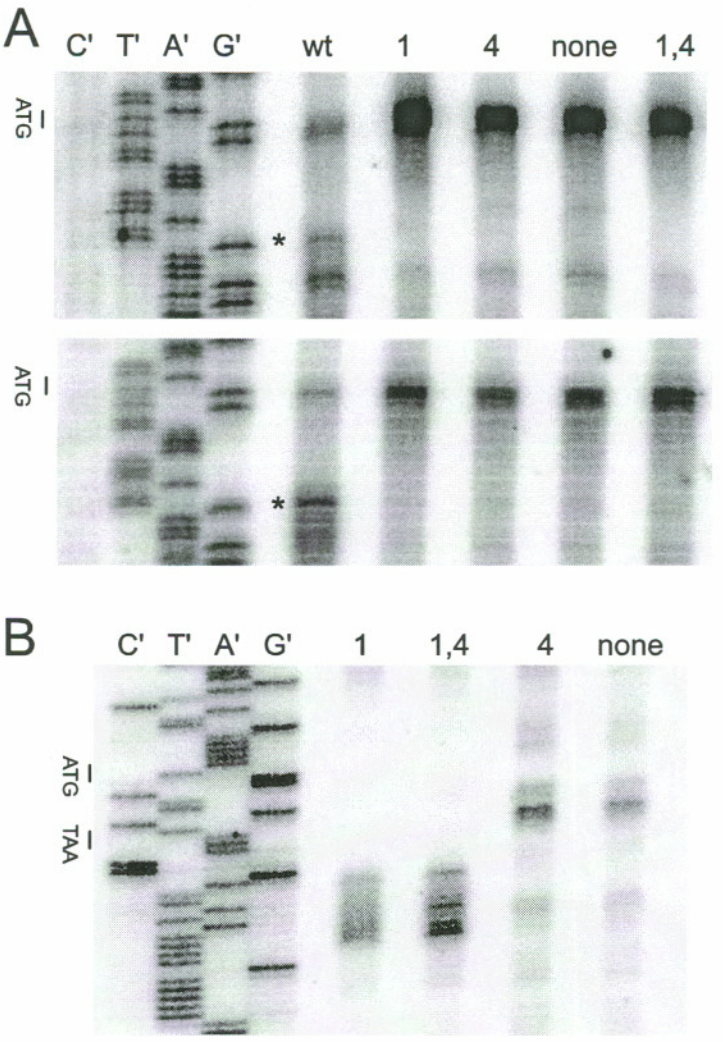

Figure 3.4 Detection of *S. cerevisiae* and *N. crassa* ribosomes translating uORFs 1 and 2 in *S. cerevisiae GCN4* mRNA. Equal amounts (120 ng) of synthetic RNA transcripts were used to program translation mixtures derived from *S. cerevisiae* and *N. crassa*. The *GCN4-LUC* transcripts encoded all 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 3.3 except that primer ZW18 was used. (**B**) Transcripts were translated in *N. crassa* reaction mixtures at 25 °C for 15 min and toeprints obtained in the absence of translational inhibitors. The positions of the uORF1 start and stop triplets marked by vertical bars.

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 3.5, 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 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 3.2). 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.

3.2.2 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 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 3.5). 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 3.8).

When cycloheximide was added at T_0, toeprints corresponding predominantly to the start codon nearest the 5'-end of the mRNA were observed (Figure 3.5A, lanes 7-11). Events at the uORF1, uORF4 and Gcn4p start codons from Figure 3.5A are shown magnified Figure 3.5B. When uORF1 was present, ribosomes loaded predominantly at its start codon at T_0 (Figure 3.5A, lanes 7, 8 and 11; Figure 3.5B). 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 3.5A, Lane 7); when they were absent, a signal corresponding to the initiation site of uORF4 was detected (Figure 3.5A, lanes 9 and 11; Figure 3.5B). When uORFs 2, 3 or 4 were present, few ribosomes were observed at downstream Gcn4p start codon (Figure 3.5A, lanes 7, 9 and 11; Figure 3.5B). When uORF1, but not the other uORFs, was present, some ribosomes were observed at the Gcn4p start codon (Figure 3.5A, Lane 8; Figure 3.5B). When no uORFs were present, substantial loading of ribosomes occurred at the *GCN4* start codon (Figure 3.5A, Lane 10; Figure 3.5B). These results indicated 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 3.5A, compare lanes 13, 14 and 17 to lanes 7, 8, and 11; Figure 3.5B). 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 3.5A, 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 3.5A, compare lane 10 and lane 16; Figure 3.5B). 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 3.5, compare lane 9 with lane 15; Figure 3.5B).

3.2.3 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.

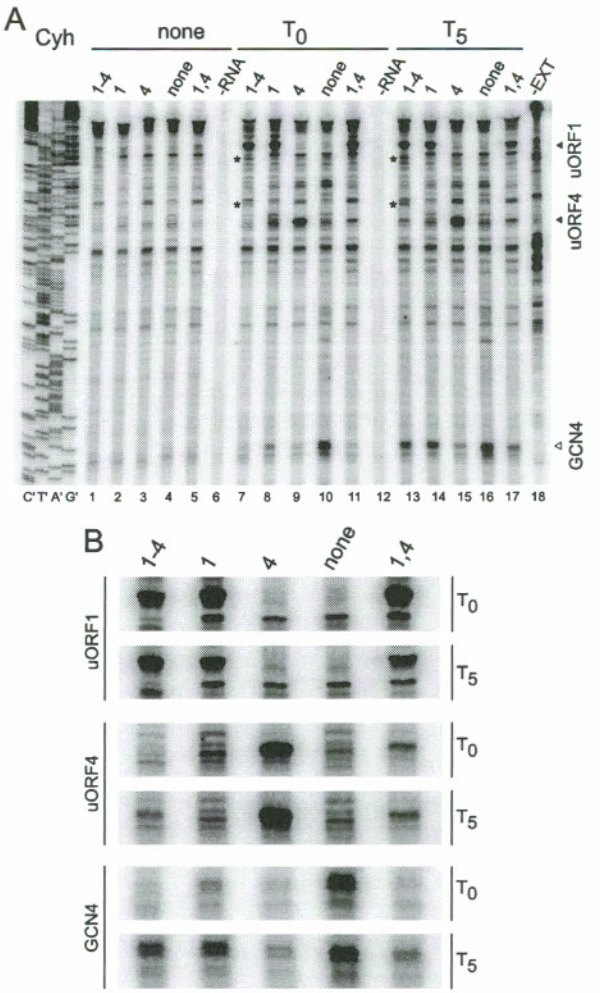

Figure 3.5 Reinitiation following GCN4 uORF1 translation. Equal amounts (120 ng) of synthetic *GCN4-LUC* RNA transcripts encoding either uORFs 1-4, uORF1, uORF4, uORFs 1 and 4, or no uORFs in the 5' leader were used to program translation mixtures derived from *S. cerevisiae*. (**A**) Transcripts were 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 (T_0) or after 5 min of incubation at 25 °C (T_5). Toeprinting and analysis with controls were accomplished as described in the legend to Figure 3.3. 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 T_0 and T_5.

We measured the amount of eIF2γ 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 3.6, 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 3.6, compare lanes 20 and 21). These effects were small (less than 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.

3.2.4 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 3.7A, 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 3.5), 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

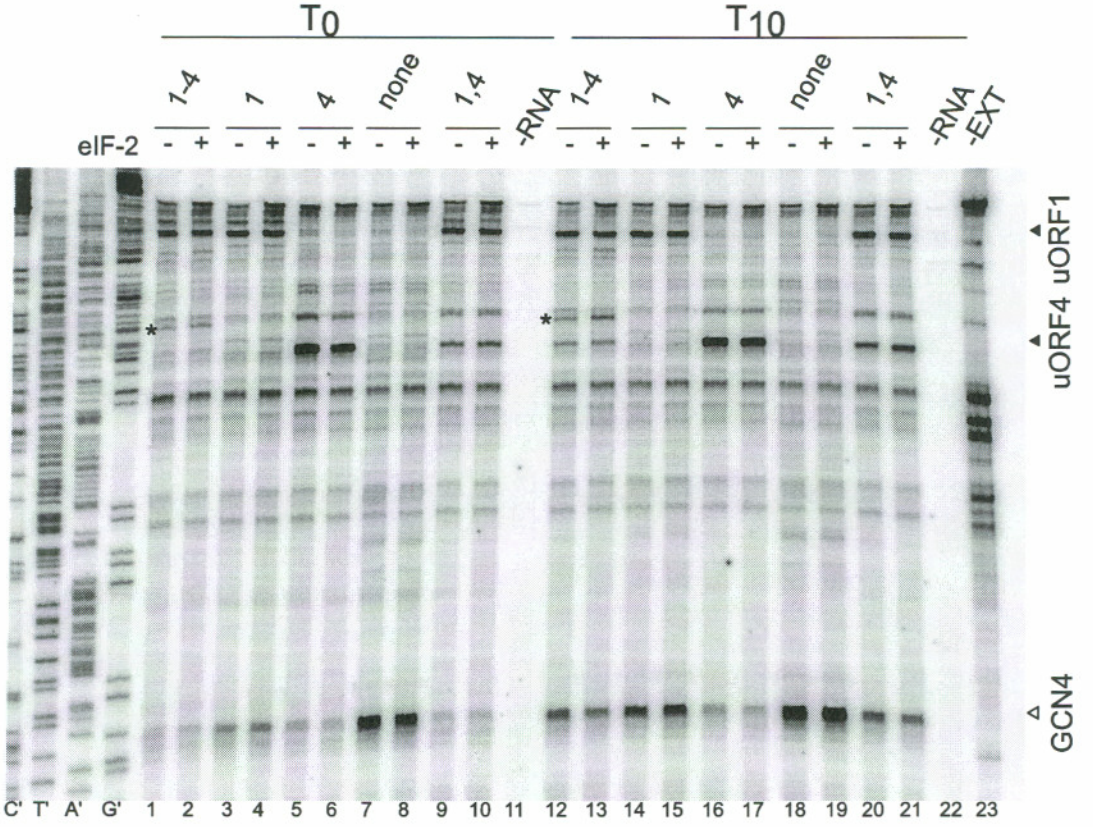

Figure 3.6 Adding eIF2 increases initiation at uORF3 and uORF4 and decreases it at *GCN4*. Equal amounts (60 ng) of synthetic *GCN4-LUC* RNA transcripts encoding uORFs 1-4, uORF1, uORF4, uORFs 1 and 4, or no uORFs in the 5' leader were used to program translation mixtures derived from *S. cerevisiae*. Transcripts were incubated at 25 °C for 20 min in reaction mixtures supplemented with cycloheximide prior to incubation (T_0) or after 10 min of incubation at 25°C (T_{10}). 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 3.3. 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}.

level of initiation at the LUC start codon was detected in low or high Arg (Figure 3.7A, 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 3.7A, 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 termination 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 et al., 1998; Wang and Sachs, 1997b).

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 3.3). 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 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 Cpa1p 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, 1997b). Figure 3.7B shows an analysis of the effects of initiation context in both *N. crassa* and *S. cerevisiae* extracts, with cycloheximide added at T_0 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

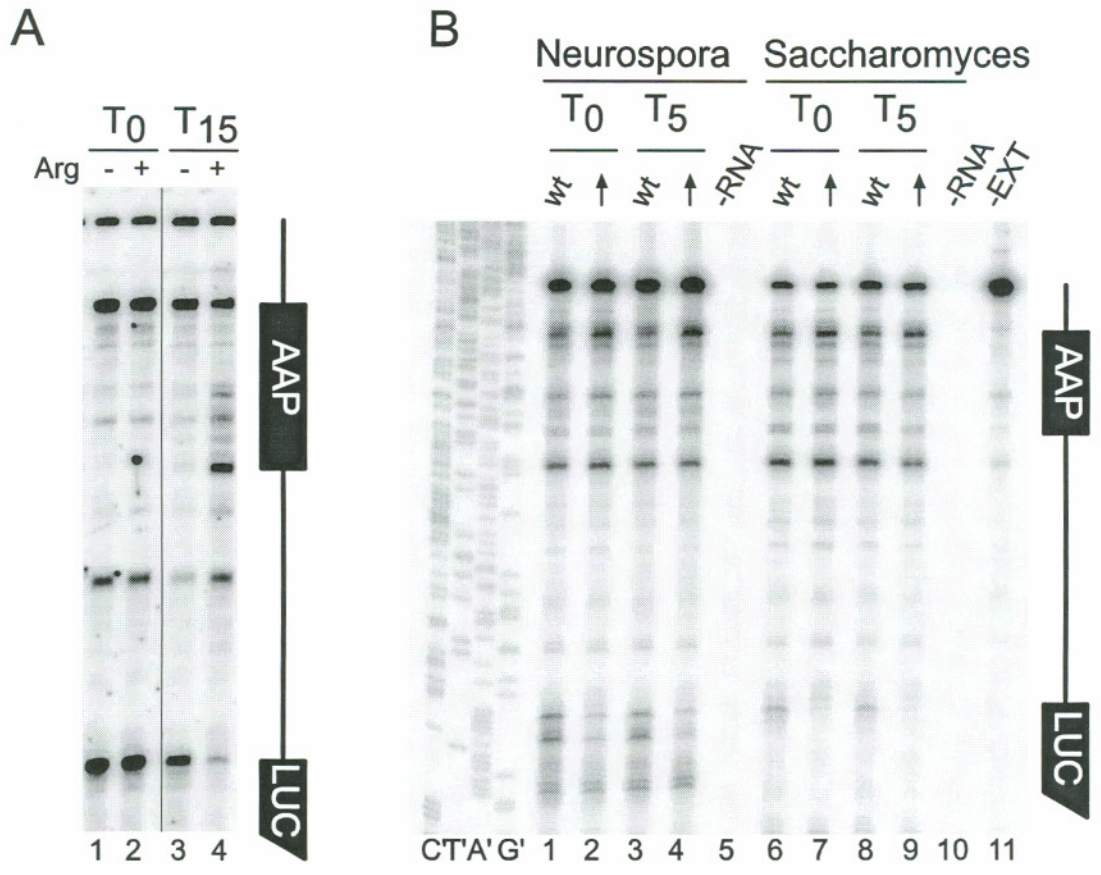

Figure 3.7 Leaky scanning past the *CPA1* and *arg-2* uORFs. Equal amounts (120 ng) of synthetic RNA transcripts were used to program translation mixtures derived from *S. cerevisiae* or *N. crassa*. (**A**) *S. cerevisiae CPA1-LUC* RNA containing the wild-type *CPA1* uORF and intergenic region in the 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 (T_0) or after 15 min of incubation at 25 °C (T_{15}), 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 (T_0) or after 5 min of incubation (T_5). Toeprinting and analysis with controls was performed as described in Figure 3.3. 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.

Table 3.3

Predicted Strengths of Initiation Codons

Initiation codon	Sequence Context	A_{UG} CAI[a]
CPA1 AAP	CATTAT ATG TTT	0.287
arg-2 AAP	GCCCTT ATG AAC	0.174
improved *arg-2* AAP	GCCACA ATG AAC	0.424
GCN4 uORF1	TGAAAA ATG GCT	0.751
GCN4 uORF4	TTCAAG ATG TTT	0.493
*GCN4*Gcn4p	ATAAAA ATG TCC	0.707

[a]The AUG codon adaptation index was calculated as described (Miyasaka, 1999).

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 3.7A (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 T_0.

3.3 DISCUSSION

We 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 3.8A). 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 3.8C). This behavior is what we observed for transcripts containing *GCN4* uORF1 and downstream coding regions (Figure 3.5 and Figure 3.6), 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 3.8B). Therefore, whether cycloheximide is added to extracts at T_0 or under steady-state conditions, ribosomes will collect at both upstream and downstream start codons (Figure

3.8C). This pattern was observed for ribosomes loaded on transcripts containing the *CPA1* or *arg-2* uORFs (Figure 3.7). 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 3.7B). 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 3.7A). 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 et al., 1998; Wang and Sachs, 1997b).

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 (Geballe and Sachs, 2000; Gong and Yanofsky, 2001; Law et al., 2001; Lovett and Rogers, 1996; Morris and Geballe, 2000).

Considerable genetic and biochemical evidence indicate that the derepression of *GCN4* translation *in vivo* is triggered by a decreased 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

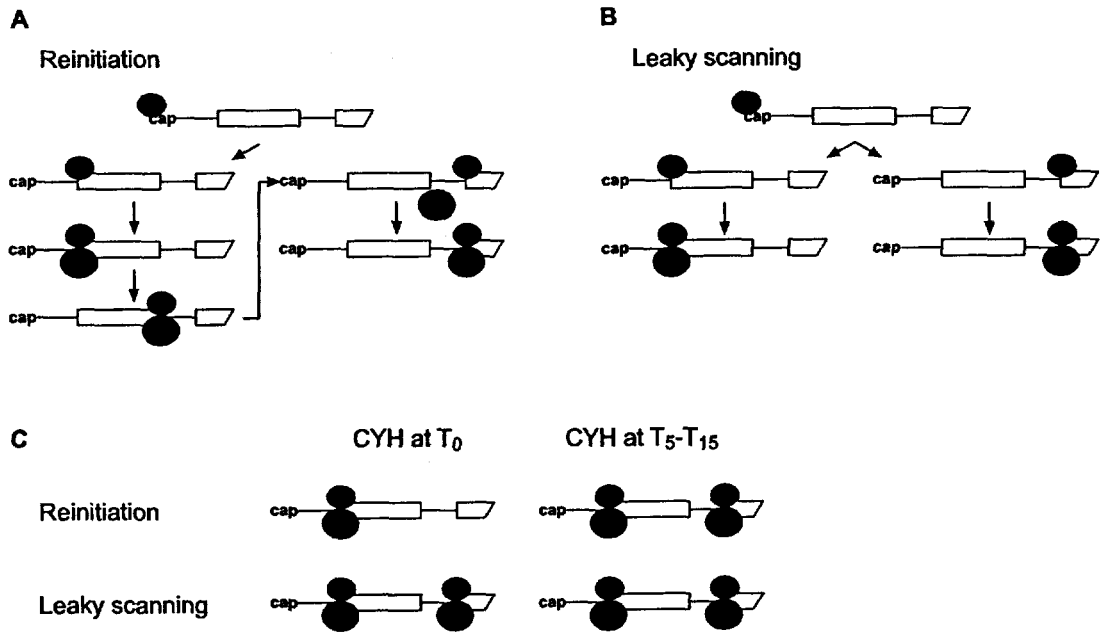

Figure 3.8 uORF control of reinitiation and leaky scanning. (**A**) Reinitiation. Scanning ribosomes load at the uORF start codon and initiate translation there; subsequently, they reinitiate translation downstream. (**B**) Leaky scanning. Ribosomes scan and load at either the upstream or downstream start codons. (**C**) Discriminating reinitiation from leaky scanning. Adding cycloheximide (CYH) at T_0 will trap ribosomes at the positions where they first load on the mRNA; adding it during steady state translation (T_5-T_{15}) will trap ribosomes where they are loaded following the primary initiation event and subsequent reinitiation events.

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-$tRNA_i^{Met}$, 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 nonstarvation 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 3.2). 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 re-bind 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. 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), *A. niger cpcA* (Wanke et al., 1997) and *Candida albicans GCN4* (Genbank 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 (Calkhoven et al., 2000; Lincoln et al., 1998). 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 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; Cao and Geballe, 1996a; Cao and Geballe, 1996b) 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.

3.4 EXPERIMENTAL PROCEDURES

3.4.1 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 *BamHI*- and *XhoI*-digested pV101 vector which is essentially pHLUC + NFS4 (Wang and Sachs, 1997a) with an introduced *BamHI* site 3′ of the *XhoI* site. Primers for PCR reactions were: ZW10 (5′-CGCGGATCCACAAAACAAAACAAAAC-3′), which includes a 5′-*BamHI* site and ZW12 (5′-CGGCTCGAGGCTGATATTCGGACATTT-3′), which includes a 3′-*XhoI* site. Templates for the synthesis of RNAs containing the *CPA1* and *arg-2* 5′-leaders were described previously (Wang et al., 1999; Wang and Sachs, 1997b), as was the template used to produce capped and adenylated synthetic mRNA encoding sea pansy LUC to serve as a internal control for translation reactions (Wang et al., 1998).

Plasmid DNA templates were purified as described (Wang and Sachs, 1997b). 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, 1997a).

3.4.2 Cell-Free Translation and Primer Extension Inhibition (Toeprint) Analyses

The preparation of translation extracts and reaction conditions were as described in chapter 2. 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, 1997b) except that for GCN4 primer extension incubation at 37 °C was for 35 min instead of 30 min.

The preparation of 5′–^{32}P-labeled ZW4 and ZW18 primers was simplified from the previously described procedure (Wang and Sachs, 1997b). 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 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

radiolabeled oligonucleotides was adjusted with either TE or DEPC treated water to approximately 0.1 μM based on a presumed 70% recovery from the spin column. The eluted material was predominantly radiolabeled primer as determined by checking for inorganic phosphate and unreacted ATP by polyethylenimine thin-layer chromatography (Wang and Sachs, 1997b).

Note: The author's work in this chapter was in collaboration with Dr. Zhong Wang. We worked together on the experiments for Figs. 3.4B, 3.5, and 3.7B. The author performed all experiments for the other figures. The eIF2 used for toeprinting was from Drs. T. Krishnamoorthy and A. G. Hinnebusch.

CHAPTER 4
RIBOSOME OCCUPANCY OF THE *CPA1* UPSTREAM OPEN READING FRAME TERMINATION CODON MODULATES NONSENSE-MEDIATED mRNA DECAY

4.1 INTRODUCTION

The *Saccharomyces cerevisiae CPA1* mRNA specifies the small subunit of arginine-specific carbamoyl phosphate synthetase. Classical genetic analyses in the 1970s demonstrated that *CPA1* was under the control of a regulatory system that reduced its expression when the growth medium was supplemented with arginine (Arg) (Thuriaux et al., 1972). Based on the then-current understanding of gene regulation, linked *cis*-dominant mutations leading to constitutive expression were proposed to affect an operator site (*cpa1O* mutations) and an unlinked recessive *trans*-acting mutation was thought to affect a repressor (*cpaR*). Subsequent sequencing of the *cis*-acting mutations showed that they did not affect an operator but could alter the translation of a predicted upstream open reading frame (uORF) in the mRNA 5'-leader (Werner et al., 1987). Measurement of β-galactosidase produced by *CPA1-lacZ* reporters was consistent with the hypothesis that the uORF had a role in controlling gene expression (Werner et al., 1987). A similar uORF is found in the *Neurospora crassa* homolog, *arg-2* (Orbach et al., 1990). Pulse-chase experiments, analyses of the distribution of *arg-2* mRNA on polysomes, and characterization of an *arg-2* uORF missense mutation established that this gene is translationally controlled in response to Arg and that this regulatory mechanism is dependent on the uORF (Luo et al., 1995). Experiments with cell-free translation systems from both *S. cerevisiae* and *N. crassa* provided direct evidence that the peptide specified by the *CPA1* and *arg-2* uORFs, named the arginine attenuator peptide (AAP), translationally regulates gene expression in response to Arg (Fang et al.,

2000; Gaba et al., 2001; Wang et al., 1998; Wang et al., 1999; Wang and Sachs, 1997a; Wang and Sachs, 1997b). These studies showed that the AAP causes Arg-specific stalling of ribosomes at the uORF termination codon, thereby reducing their access to the downstream open reading frame (ORF). The corresponding uORF missense mutations, D13N in *CPA1* and D12N in *arg-2*, eliminate this Arg-specific translational control, a consequence of altering the AAP nascent peptide, not the RNA sequence that specifies it (Fang et al., 2000). While this mode of regulation has been principally studied in lower eukaryotes, the mechanisms that enable it are evolutionarily conserved since the AAP also causes stalling of plant and animal ribosomes in response to Arg (Fang et al., 2004).

Although the control of mRNA stability does not appear to be a primary component of AAP-mediated regulation *in vitro* (Wang and Sachs, 1997a; Wang and Sachs, 1997b), studies *in vivo* have demonstrated *CPA1* mRNA sensitivity to the nonsense-mediated mRNA decay (NMD) pathway (He et al., 2003; Ruiz-Echevarria and Peltz, 2000). In yeast, NMD targets mRNAs containing premature termination codons for rapid degradation in a process dependent on the regulatory factors Upf1p, Nmd2p (Upf2p), and Upf3p (Jacobson and Peltz, 2000). The *CPA1* transcript half-life is 3 min in a wild-type strain and approximately 18 min in an isogenic *upf1Δ* strain (Ruiz-Echevarria and Peltz, 2000), providing direct evidence that this mRNA is a substrate for NMD. Consistent with NMD control of *CPA1* mRNA, the *cpaR* mutation is an allele of *UPF1* (Messenguy et al., 2002).

Here we show that the CPA1 uORF plays a critical role in modulating NMD of *CPA1* mRNA. By analysis of protein and RNA levels derived from reporter gene expression in wild-type and NMD-deficient strains, we have dissected the translational and NMD components of CPA1 uORF-mediated regulation. The experimental data provide the basis for a regulatory model in which the nascent uORF-encoded peptide acts in concert with Arg to reduce CPA1 expression by affecting both translation efficiency and mRNA stability. This represents a paradigmatic example in which the synthesis of a eukaryotic peptide directly controls the level of the mRNA template that encodes it.

4.2 RESULTS

4.2.1 The Wild-type *CPA1* uORF Mediates Arg-specific Regulation With an *in vivo* Reporter System

In a *S. cerevisiae* cell-free system, *CPA1* uORF-mediated Arg-specific negative regulation of downstream translation requires the ability of the uORF-encoded AAP to act as a nascent peptide to stall ribosomes at the uORF termination codon in response to Arg (Wang et al., 1999). An AAP missense mutation (D13N) that eliminates the AAP's ability to stall ribosomes also eliminates Arg-specific regulation of the synthesis of a luciferase (*LUC*) reporter initiating from a downstream start codon (Wang et al., 1999). To investigate the *in vivo* effects of the *CPA1* uORF on gene expression in cells affected in pathways governing post-transcriptional control of mRNA levels, we exploited these observations and employed a reporter system in which isogenic strains harboring different mutations affecting mRNA degradation, and an isogenic unaffected strain ("wild-type"), were transformed with centromeric plasmids designed for the expression of *CPA1-LUC* chimeric transcripts. These reporters contained wild-type or mutated *CPA1* mRNA 5'-leader regions downstream of the *PGK1* promoter and upstream of the firefly *LUC* cistron and *PGK1* 3' sequences (Figure 4.1, Table 4.1, Table 4.2).

To demonstrate Arg-regulation with this reporter system, luciferase activity was measured in whole-cell extracts prepared from wild-type cells grown in minimal medium that was or was not supplemented with 1 mg/ml Arg (-Arg and +Arg, respectively) (Werner et al., 1987). Levels of luciferase were compared in terms of relative enzyme activity per mg of extract protein. Cells expressing *WT-CPA1-LUC* mRNA, which contained the wild-type *CPA1* uORF, showed an approximate 2-fold reduction in luciferase activity in response to Arg (Figure 4.2). This effect was absent from wild-type cells expressing *ΔAUG-CPA1-LUC* mRNA, which contained an AUG to UUG point mutation in the *CPA1* uORF translation initiation codon, indicating that the presence of the uORF was crucial for Arg-regulation *in vivo* (Figure 4.2). Luciferase activity from wild-type cells expressing *D13N-CPA1-LUC* mRNA, which contained the D13N missense mutation that eliminates ribosome stalling *in vitro*, also showed no regulation in response to arginine supplementation (Figure 4.2). Consistent with observations *in vitro*

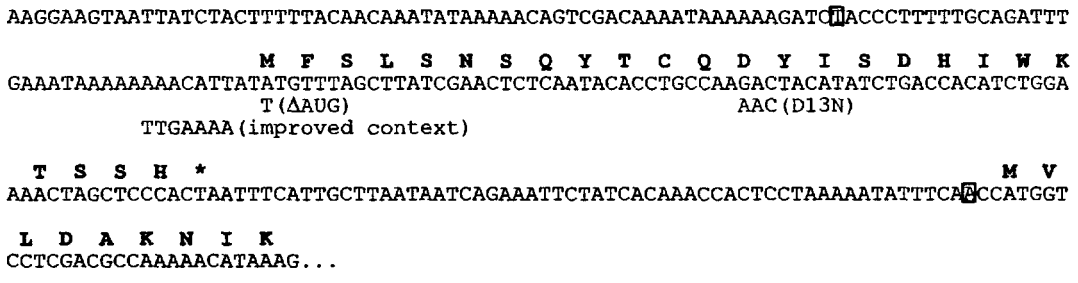

Figure 4.1 Sequences of the 5′-leader regions of *CPA1-LUC* genes used in this study. The sequence begins with the *PGK1* transcription start site and ends within the *LUC* coding region. The 5′ and 3′ boundaries of the wild-type *CPA1* sequence are boxed. Mutations are shown below the wild-type sequence.

Table 4.1

CPA1-LUC Reporters Used in This Study

Plasmid	Initiation context	uORF	*CPA1-LUC* transcript
pAG121	Native	Wild-type	*WT-CPA1-LUC*
pAG122	Native	D13N	*D13N-CPA1-LUC*
pAG123	Native	ΔAUG	*ΔAUG-CPA1-LUC*
pAG134	*GCN4* uORF1	Wild-type	*WT-CPA1-LUC*[a]
pAG135	*GCN4* uORF1	D13N	*D13N-CPA1-LUC*[a]

[a]Transcripts containing uORFs in improved initiation contexts are indicated appropriately in the text and figures.

that, in its native context, the *CPA1* uORF initiation codon does not capture ribosomes efficiently (leading to efficient initiation at the downstream start codon (Gaba et al., 2001)), the levels of expression of the *ΔAUG-CPA1-LUC* and *D13N-CPA1-LUC* reporters were similar under all conditions. Thus, the *CPA1* uORF faithfully mediated sequence-dependent negative regulation of the *LUC* reporter in response to Arg.

Comparisons of the levels of luciferase produced by each of the three constructs in minimal medium (Figure 4.2) indicated that the wild-type AAP construct had an inhibitory effect on gene expression relative to the ΔAUG and D13N controls, even in the absence of Arg. One explanation for this effect is that the level of Arg present in cells growing in minimal medium is already sufficient to produce a partial regulatory response. This result is not unanticipated since supplementation of cell-free extracts with 150 μM Arg promotes an AAP-mediated regulatory response in translation assays *in vitro*, and the cytoplasmic Arg concentration is estimated to be near this value (Davis, 1986; Kitamoto et al., 1988). To avoid complications in interpretation arising from the use of minimal medium, all further studies utilized rich medium (YPD) containing Arg, conditions under which the regulatory effects of the wild-type uORF appeared maximal.

4.2.2 Translation of the Wild-type *CPA1* uORF Induces NMD of *CPA1-LUC* Transcripts

Isogenic wild-type and *nmd2Δ* cells containing either *WT-CPA1-LUC*, *D13N-CPA1-LUC*, or *ΔAUG-CPA1-LUC* were grown in YPD and the corresponding mRNA steady-state levels were assessed by northern blotting. As a control, we examined the abundance of the *CYH2* pre-mRNA, an endogenous cytoplasmic substrate of the NMD pathway that is targeted because its unspliced intron includes an in-frame premature termination codon (He et al., 1993). As shown in Figure 4.3, the level of the *CYH2* pre-mRNA was substantially elevated in the *nmd2Δ* strains, thus confirming that these strains were defective for NMD. The level of the mature *CYH2* transcript, which is not an NMD substrate, was unaffected in *nmd2Δ* cells, but the level of the endogenous *CPA1* transcript in the same cells was elevated approximately 4.5-fold (Figure 4.3A and Figure 4.4B). These observations confirm earlier studies demonstrating that the level of the endogenous

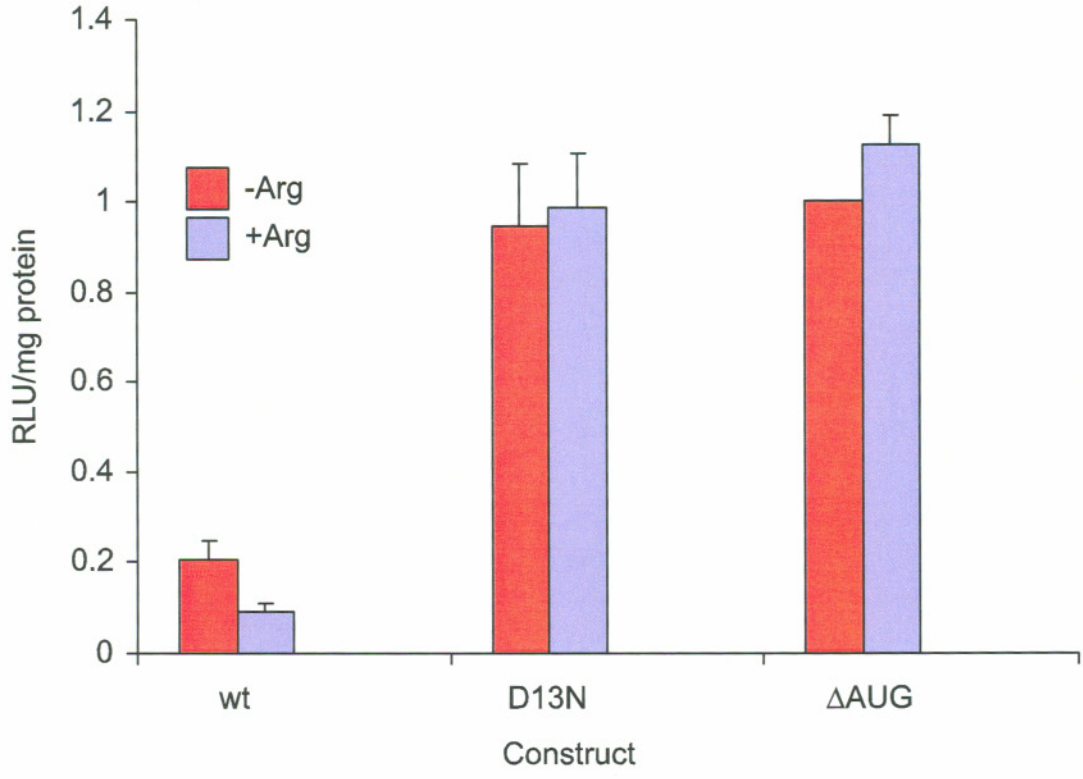

Figure 4.2 Production of luciferase in NMD$^+$ cells containing plasmids expressing different *CPA1-LUC* reporters. Cells from strains yAG201, yAG202, and yAG203 contained *CPA1-LUC* constructs with the wild-type uORF (wt), the uORF containing a missense mutation previously demonstrated to lack regulation (D13N), or no uORF (ΔAUG), as indicated. Luciferase activity was measured as described in Experimental Procedures and is expressed as relative light units (RLU) per mg of protein. Extracts utilized for luciferase activity measurements were prepared from cells grown in minimal medium lacking (-Arg) or containing 1 mg/ml arginine (+Arg). For comparative purposes, all values are normalized to the results obtained for the strain containing the *ΔAUG CPA1-LUC* construct in -Arg medium. The results shown represent the combined data from analyses of two independent growth experiments with each of two transformants.

CPA1 transcript is controlled by NMD (He et al., 2003; Messenguy et al., 1983; Ruiz-Echevarria and Peltz, 2000).

Figure 4.3A and the quantitative data in Figure 4.4A show that the levels of *ΔAUG-* and *D13N-CPA1-LUC* transcripts were similar in the wild-type and *nmd2Δ* strains, indicating that these mRNAs are not subject to control by NMD. However, in the NMD^+ strain, the *WT-CPA1-LUC* mRNA was approximately 2-fold reduced compared to the *ΔAUG-CPA1-LUC* and *D13N-CPA1-LUC* transcripts. In the *nmd2Δ* strain, the *WT-CPA1-LUC* transcript was approximately 3.5-fold more abundant than *ΔAUG-CPA1-LUC* and *D13N-CPA1-LUC* transcripts (Figure 4.3A and Figure 4.4A). Thus, the steady-state abundance of the *WT-CPA1-LUC* transcript was elevated approximately 7-fold in the *nmd2Δ* strain relative to the NMD^+ strain. The increased level of *WT-CPA1-LUC* mRNA, but not the *ΔAUG-CPA1-LUC* and *D13N-CPA1-LUC* transcripts in the *nmd2Δ* strain, indicated that the level of the *WT-CPA1-LUC* reporter is controlled by NMD. The increased levels of the *ΔAUG-CPA1-LUC* and *D13N-CPA1-LUC* transcripts relative to the *WT-CPA1-LUC* mRNA in the NMD^+ strain was also consistent with this interpretation.

4.2.3 Modulation of NMD by the Extent of Ribosomal Occupancy of the *CPA1* uORF Termination Site

That the wild-type uORF, but not the D13N uORF, triggered NMD suggested that it might not simply be the termination of uORF translation that triggered NMD. Rather, in light of the stalling effects exerted on terminating ribosomes by the wild-type AAP but not the D13N AAP (Gaba et al., 2001; Wang et al., 1999), it appeared that the extent of ribosome occupancy at the termination site was a critical determinant of *CPA1* NMD. If this hypothesis were correct, then increasing ribosomal occupancy by means other than wild-type AAP-mediated stalling should trigger NMD. We tested this possibility by improving the uORF initiation context to obtain higher levels of uORF translation without affecting the efficiency with which the nascent peptide sequences could cause stalling. When the D13N uORF is in an improved initiation context, its termination site is predicted to be occupied by ribosomes to a greater extent than in the native context, even though the intrinsic efficiency of termination should remain unchanged.

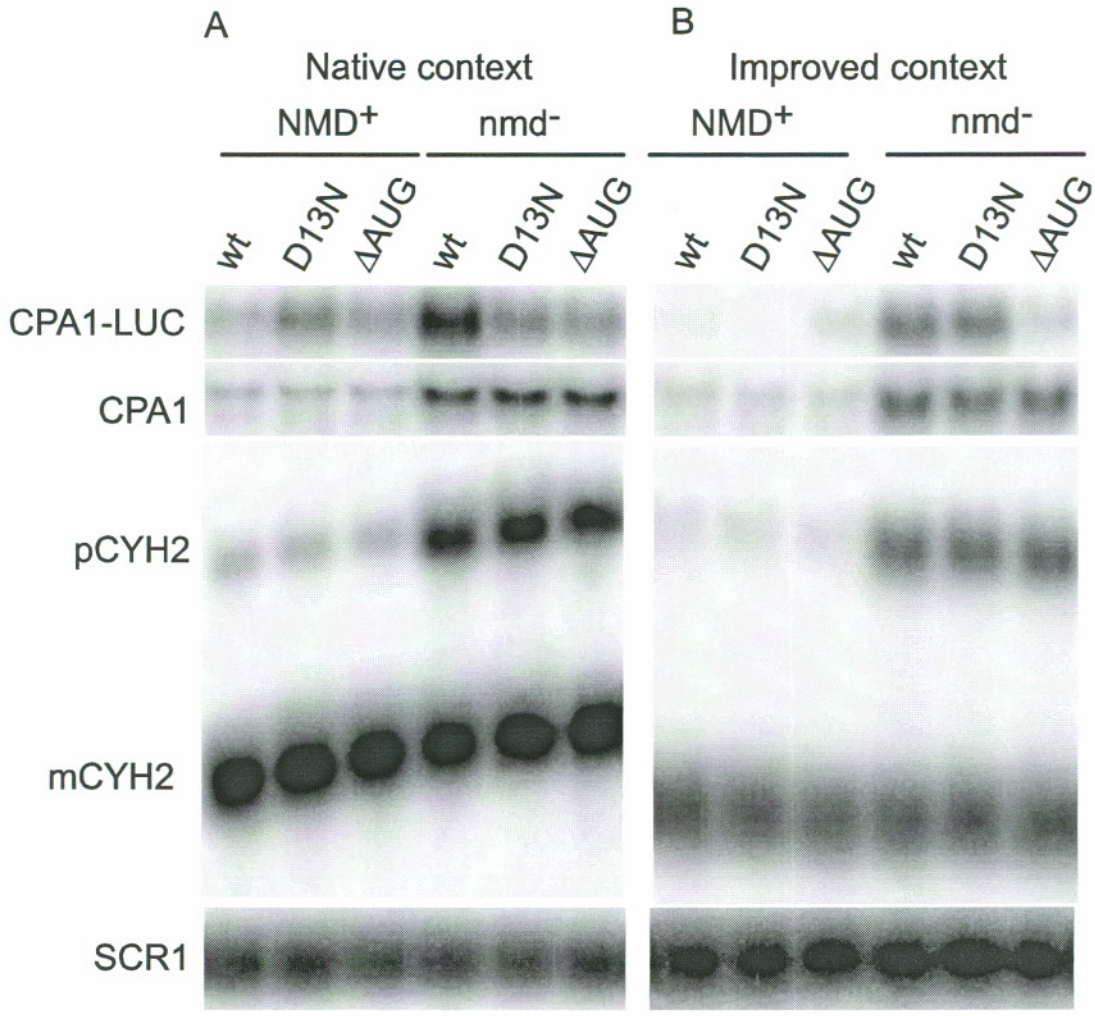

Figure 4.3 Northern blot analysis of *CPA1-LUC*, *CPA1*, *CYH2*, and *SCR1* transcripts in isogenic NMD$^+$ and *nmd2Δ* strains containing plasmids expressing different *CPA1-LUC* reporters. Strains were grown in YPD as described in Experimental Procedures and contained *CPA1-LUC* reporters with the wild-type uORF (wt), the uORF containing a missense mutation previously demonstrated to lack regulation (D13N), or no uORF (ΔAUG). When present, uORFs were in either (**A**) the native context or (**B**) the improved context, as indicated. Lanes represent blots of 8 μg RNA prepared from each strain and the identified transcripts are marked on the left. *SCR1* is the control used for normalizing RNA loading across lanes for quantitative analyses. pCYH2 denotes the *CYH2* pre-mRNA that is stabilized in the *nmd2Δ* strain. mCYH2 denotes the mature *CYH2* transcript.

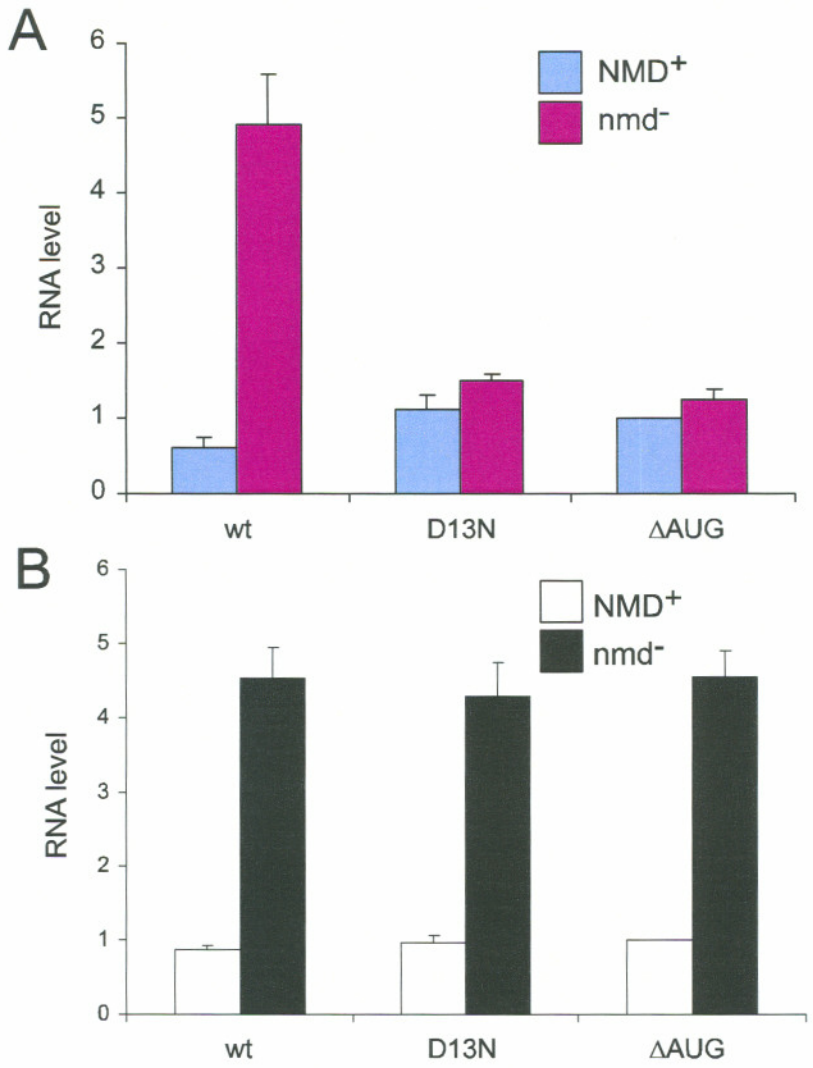

Figure 4.4 Relative *CPA1-LUC* and *CPA1* transcript levels in isogenic NMD$^+$ and *nmd2Δ* strains expressing *CPA1-LUC* reporters with the uORF initiation codon in its native context. Cells used for RNA preparation contained *CPA1-LUC* reporters with the wild-type uORF (wt), the D13N uORF (D13N), or no uORF (ΔAUG). Northern blotting of multiple samples was performed as described for Figure 4.3. The amounts of (**A**) *CPA1-LUC* and (**B**) *CPA1* transcripts were determined relative to the *SCR1* transcript as described in Experimental Procedures. The levels of the *ΔAUG CPA1-LUC* and *CPA1* transcripts in the NMD$^+$ strain were used to normalize relative transcript levels in panels A and B, respectively. For both NMD$^+$ and *nmd2Δ* strains, each bar represents the results of analyses of two independent growth experiments with one transformant, and an independent growth experiment with a second transformant, with all RNA samples analyzed in triplicate using three separate blots.

To evaluate the effect of increasing occupancy at the uORF termination site by ribosomes, we transformed isogenic wild-type and *nmd2Δ* strains with expression plasmids encoding *WT-CPA1-LUC-* and *D13N-CPA1-LUC* transcripts which contained wild-type and D13N *CPA1* uORFs, respectively, in the more efficient *GCN4* uORF1 initiation context (Figure 4.1 and Table 4.1) (Gaba et al., 2001). In the wild-type strain, the steady-state levels of both the improved-context *WT-* and *D13N-CPA1-LUC* transcripts were reduced, approximately 2- and 5-fold, respectively, relative to that of the *ΔAUG-CPA1-LUC* transcript (Figure 4.3B and Figure 4.5). In the *nmd2Δ* strain, the abundance of the improved-context *WT-* and *D13N-CPA1-LUC* transcripts was elevated, approximately 6- and 5-fold, respectively, relative to the level of the *ΔAUG-CPA1-LUC* transcript (Figure 4.3B). These data show that improved-context *WT-* and *D13N-CPA1-LUC* transcripts were both NMD substrates, which is in contrast to what occurs with the native uORF AUG context, in which the *WT-* but not the *D13N-CPA1-LUC* transcript was a substrate for NMD (Figure 4.3, Figure 4.4A and Figure 4.5A). The response of the endogenous *CPA1* transcript to the presence of a functional NMD pathway was similar in each strain examined, as expected (Figure 4.5B). These analyses identify two modes for the induction of NMD of the *CPA1* mRNA. When the native *CPA1* uORF initiation context is present, ribosome stalling at the uORF termination codon is required for the *CPA1* uORF to induce NMD of *CPA1-LUC* mRNA, but when the initiation context is improved, ribosome stalling is no longer required for the *CPA1* uORF to induce NMD.

4.2.4 Inactivation of Xrn1p, but not Ski2p, Alters Steady-state Levels of CPA1 mRNAs

To characterize further the components of *CPA1* uORF-mediated modulation of steady-state mRNA abundance, we determined the effects of mutations that eliminated either Xrn1p, an exoribonuclease required for 5′-to-3′ mRNA decay (Hsu and Stevens, 1993), or Ski2p, a putative RNA helicase, required for normal 3′-to-5′ exosome-mediated mRNA decay (Anderson and Parker, 1998). The steady-state levels of *CPA1* and *CPA1-LUC* transcripts in isogenic wild-type, *xrn1Δ*, and *ski2Δ* strains were assessed by northern blotting (Figure 4.6A and data not shown) and the results of analyzing these data (Figure 4.6B and C) showed that, relative to the isogenic wild-type strain, *CPA1* and *CPA1-LUC*

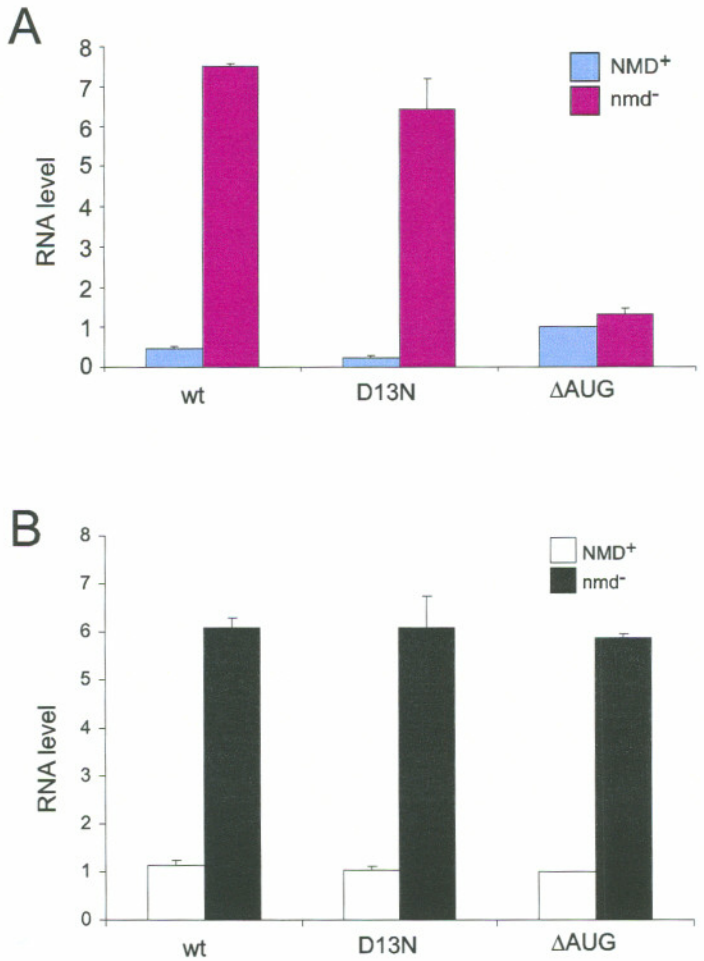

Figure 4.5 Relative *CPA1-LUC* and *CPA1* transcript levels in isogenic NMD$^+$ and *nmd2Δ* strains expressing different *CPA1-LUC* reporters with the uORF initiation codon in an improved context. Cells used for RNA preparation contained *CPA1-LUC* reporters with the wild-type uORF (wt), the D13N uORF (D13N), or no uORF (ΔAUG). Northern blotting was performed as described for Figure 4.3 and the amounts of (**A**) *CPA1-LUC* and (**B**) *CPA1* transcripts were determined relative to the *SCR1* transcript as described in Experimental Procedures. The levels of the *ΔAUG CPA1-LUC* and *CPA1* transcripts in the NMD$^+$ strain were used to normalize relative transcript levels in panels A and B, respectively. For the NMD$^+$ strain, each bar represents the results of analyses of two independent growth experiments with one transformant, and an independent growth experiment with a second transformant. For the *nmd2Δ* strain, each bar represents the results of two independent growth experiments, each using an independent transformant. All RNA samples were analyzed in duplicate using two separate blots.

transcript levels were approximately 6-fold higher in the *xrn1Δ* strain and only slightly elevated (1.2-fold) in the *ski2Δ* strain. Consistent with earlier conclusions drawn from microarray analyses (He et al., 2003), these results indicated that the 5′-to-3′ decay pathway, and not the 3′-to-5′ decay pathway, is primarily involved in determining the steady-state levels of *CPA1* and *CPA1-LUC* transcripts. Similarly, since the steady-state abundance of the *WT-CPA1-LUC* transcript was reduced approximately 2-fold relative to the *ΔAUG-CPA1-LUC* and *D13N-CPA1-LUC* transcripts in the wild-type and *ski2*Δ strains, but not in the *xrn1*Δ strain (Figure 4.6B), uORF-mediated NMD of the *WT-CPA1-LUC* transcript was largely dependent on Xrn1p activity but not Ski2p activity.

4.2.5 *CPA1* uORF-mediated Translational Controls Function Independently of Effects on mRNA levels

To investigate the role of the *CPA1* uORF on gene expression at the level of translation, we measured luciferase activity in wild-type and *nmd2Δ* cells, and controlled for differences in mRNA levels by normalizing luciferase activity to the amount of *CPA1-LUC* transcript (expressed as LUC/RNA). Wild-type and *nmd2Δ* strains expressing the *ΔAUG-CPA1-LUC* and native-context *D13N-CPA1-LUC* transcripts showed similar LUC/RNA values (Figure 4.7A), indicating that the translation efficiency of these reporters is similar in both strains. In the wild-type strain, however, expression of native-context *WT-CPA1-LUC* mRNA resulted in LUC/RNA levels that were approximately 4-fold less than those obtained from the *ΔAUG-* and *D13N-CPA1-LUC* mRNAs. Since all three mRNAs shared the same *LUC* reporter sequences and differed only in the status of their respective uORFs, these effects cannot be attributed to post-translational events and most likely represent uORF-mediated translational control of gene expression. Inasmuch as the reduction of LUC/RNA levels by the wild-type uORF in its native context was also evident in *xrn1Δ* and *ski2Δ* strains (Figure 4.7A and C), this effect must be largely independent of degradative events simultaneously occurring on the mRNA.

Further evidence that the observed differences in LUC/RNA levels in wild-type cells represented *CPA1* uORF-mediated inhibition of downstream translation was

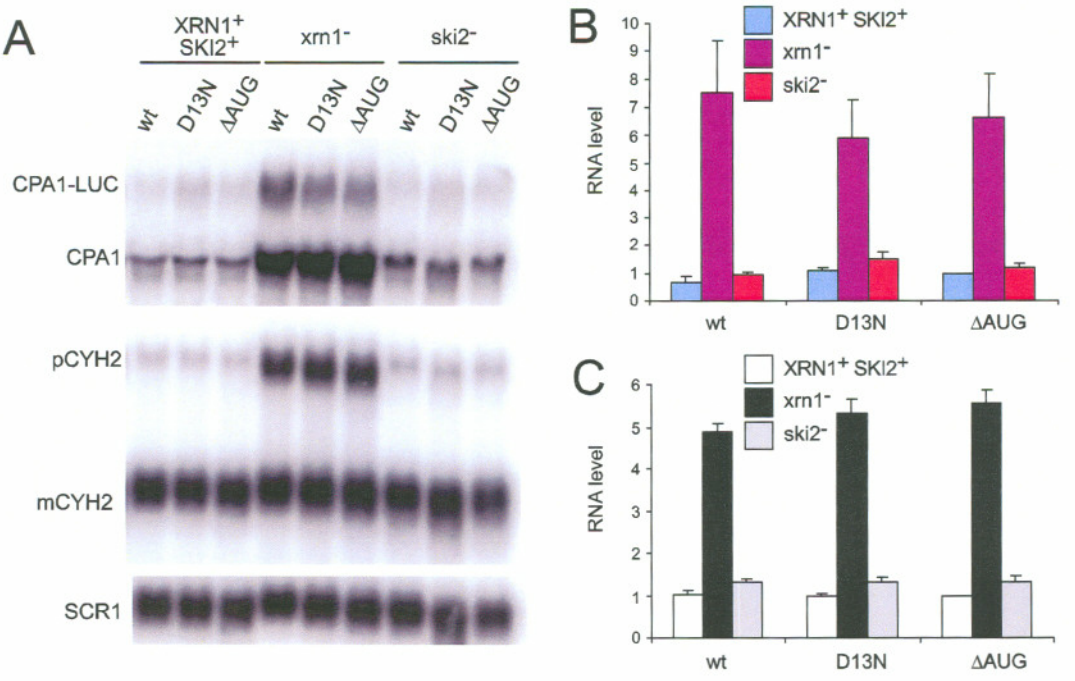

Figure 4.6 Relative *CPA1-LUC* and *CPA1* transcript levels in isogenic *XRN1 SKI2*, *xrn1Δ*, and *ski2Δ* strains containing plasmids expressing different *CPA1-LUC* reporters. The strains used for RNA preparation expressed *CPA1-LUC* reporters containing the wild-type uORF (wt), the D13N uORF (D13N), or no uORF (ΔAUG). The uORF, when present, was in its native initiation context. Northern blot analyses were performed as described for Figure 4.3. (**A**) Representative northern blot results. Strains and reporter constructs were as indicated and the conditions for blotting were as described in Figure 4.3; (**B**, **C**) The amounts of *CPA1-LUC* (panel B) and *CPA1* (panel C) transcripts were determined relative to the *SCR1* transcript as described in Experimental Procedures. The levels of the *ΔAUG-CPA1-LUC* and *CPA1* transcripts in the wild-type strain were used to normalize relative transcript levels in panels B and C, respectively. For wild-strains, each bar represents the results of analyses of three independent growth experiments with one transformant, and two independent growth experiments with a second transformant. Each bar represents the results of analyses of at least two independent growth experiments with one transformant and at least one independent growth experiment with a second transformant. All RNA samples were analyzed in duplicate using two separate blots.

obtained from analyses of reporter mRNAs with improved initiation contexts. When initiation contexts were improved, LUC/RNA levels indicated that the wild-type and D13N uORFs were significantly more inhibitory to LUC synthesis than when the uORFs were in their native initiation contexts (Figure 4.7A and B). More specifically, when the wild-type uORF was in its native context or in the improved initiation context, the LUC/RNA levels in wild-type cells were 20% and 2%, respectively, of the level observed for the *ΔAUG-CPA1-LUC* transcript. In the same cells, the D13N uORF in native and improved initiation contexts showed LUC/RNA levels that were 96% and 12%, respectively, of the level obtained from the *ΔAUG-CPA1-LUC* transcript. These results are consistent with a model for *CPA1* translational control in which the context of the uORF initiation codon governs the extent of scanning to the downstream start codon regardless of the uORF peptide's sequence, and in which the wild-type uORF but not the D13N uORF confers Arg-specific control on scanning by blocking scanning ribosomes in the presence of high Arg (Gaba et al., 2001).

An additional regulatory effect of the *CPA1* uORF was detected by comparing mRNA translatability in wild-type cells to that in strains defective in the degradation of NMD substrates. In the *nmd2Δ* strain, *CPA1-LUC* mRNA containing the wild-type uORF in native and improved initiation contexts showed LUC/RNA levels that were 6% and 0.2%, respectively, of the level observed with the *ΔAUG-CPA1-LUC* transcript, whereas the *D13N-CPA1-LUC* mRNA in native and improved initiation contexts showed LUC/RNA levels that were 80% and 0.6%, respectively, of those obtained with the *ΔAUG-CPA1-LUC* transcript in the same strain. Thus, independent of uORF-effects on the stalling of ribosomes, uORF-containing mRNAs that were normally substrates for NMD but were not degraded in *nmd2Δ* cells were less translatable in the mutant than the corresponding mRNA in the NMD^+ strain. This phenomenon was specific to NMD substrates and the NMD pathway because it was not detected with the *D13N-CPA1-LUC* mRNA harboring a native initiation context (Figure 4.7A), nor was it manifested in *xrn1Δ* cells. The reduction in LUC/RNA levels observed in the latter cells (Figure 4.7C) affected all three test transcripts uniformly and most likely reflected the decreased translatability characteristic of the deadenylated and decapped transcripts known to accumulate in such cells (Hsu and Stevens, 1993).

4.3 DISCUSSION

4.3.1 Ribosome Stalling at the *CPA1* uORF Termination Codon Controls Gene Expression at Multiple Levels

The results presented here demonstrate that the *CPA1* uORF can negatively regulate gene expression in *cis* by both reducing downstream translation and by triggering rapid mRNA degradation. Analyses of the function of the *CPA1* uORF-encoded AAP in cell-free extracts show that it decreases downstream translation in response to Arg by stalling ribosomes that have just translated the uORF. In turn, these stalled ribosomes block access of scanning ribosomes to the initiation codon of the principal *CPA1* ORF (Gaba et al., 2001; Wang et al., 1999). The evolutionarily conserved AAPs present in the *N. crassa arg-2* gene, and homologous genes from *Aspergillus nidulans* and *Magnaporthe grisea,* act similarly *in vitro* (Wang et al., 1999 and references therein). In *N. crassa*, pulse-chase analyses of polypeptide synthesis and analyses of the distribution of *arg-2*-uORF containing transcripts on polysomes showed that the AAP also regulated *arg-2* translation in response to Arg *in vivo* (Freitag et al., 1996; Luo et al., 1995; Luo and Sachs, 1996).

Our experiments utilizing reporter genes to examine *S. cerevisiae CPA1* uORF control indicate that the aforementioned translational regulatory effects of the wild-type uORF are accompanied *in vivo* by additional effects on mRNA levels. To distinguish the two classes of effects, translational regulation was also evaluated in mutant strains defective in specific mRNA decay activities and was also reassessed after normalization for differences in mRNA levels. Since reporter genes containing the wild-type uORF always showed reduced levels of reporter expression per unit of mRNA when compared to reporter genes containing the D13N or ΔAUG mutations (Figure 4.7), regardless of the capacity of the host strain to degrade mRNA, the effects of the uORF on translation were clearly independent of its effects on transcript stability.

It was previously established that the steady-state level and the half-life of the *CPA1* transcript were regulated by the NMD pathway (He et al., 2003; Ruiz-Echevarria and Peltz, 2000). While it was presumed that the NMD effects required the presence of

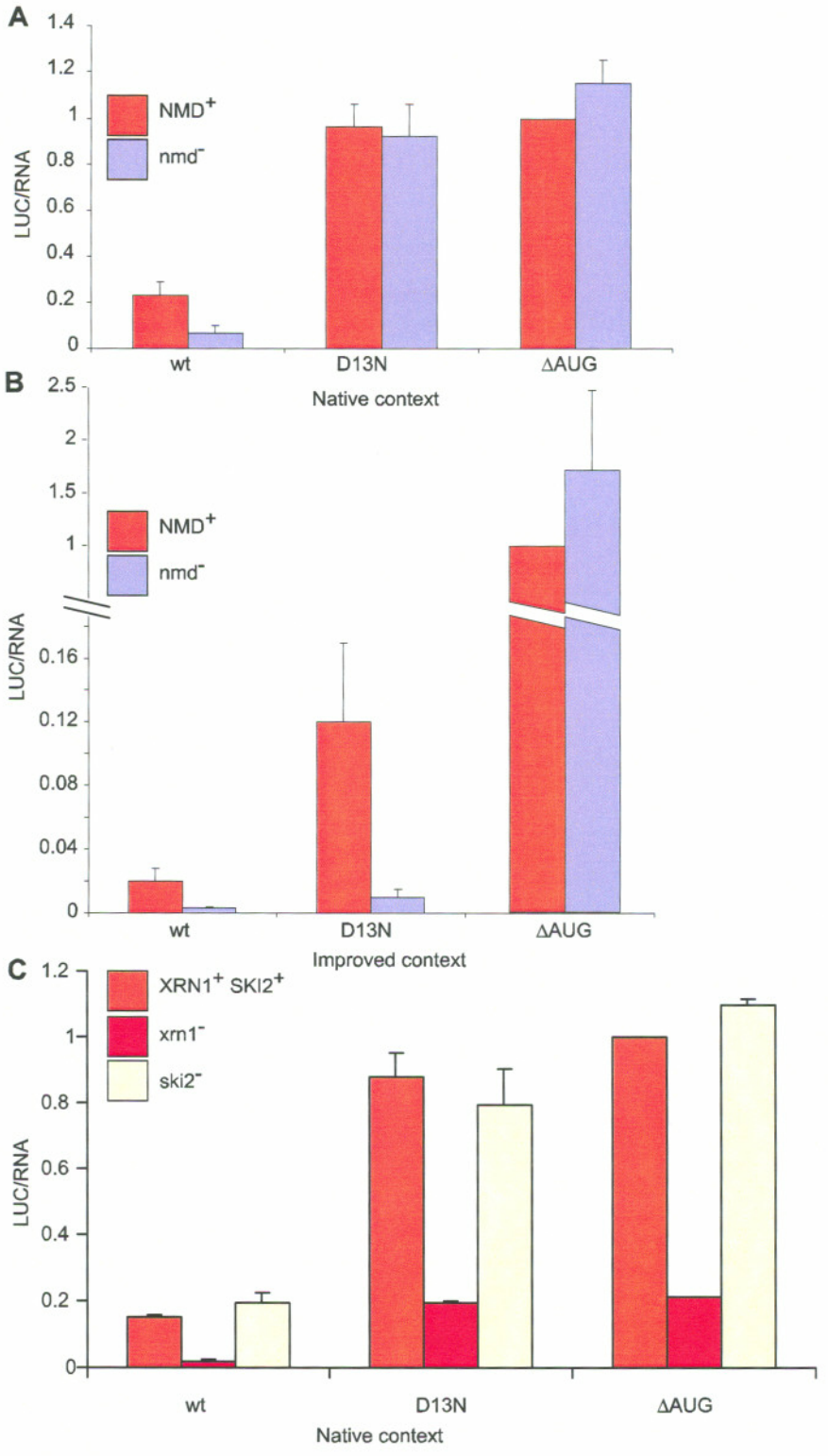
A
1.4
1.2
1
0.8
0.6
0.4
0.2
0
LUC/RNA
NMD+
nmd-
wt
D13N
ΔAUG
Native context
B
2.5
2
1.5
1
0.16
0.12
0.08
0.04
0
LUC/RNA
NMD+
nmd-
wt
D13N
ΔAUG
Improved context
C
1.2
1
0.8
0.6
0.4
0.2
0
LUC/RNA
XRN1+ SKI2+
xrn1-
ski2-
wt
D13N
ΔAUG
Native context

Figure 4.7 Luciferase production normalized to mRNA levels. Luciferase enzyme activity was measured in extracts prepared from the same batches of cells grown in Arg-containing medium and used for obtaining RNA for the experiments in Figures 4.4, 4.5, and 4.6. Levels of luciferase enzyme activity were normalized in each growth experiment relative to the level observed for the *ΔAUG-CPA1-LUC* reporter in the wild-type strain, as were the levels of reporter RNA. The data represent the relative level of luciferase enzyme activity/mg protein/RNA (LUC/RNA). (**A**) *CPA1-LUC* reporters containing the wild-type uORF (wt) and the D13N uORF (D13N) in their native contexts, or lacking a uORF (ΔAUG), expressed in NMD^+ and *nmd2Δ* strains; (**B**) *CPA1-LUC* reporters containing the wt and D13N uORFs in their improved contexts, and the *ΔAUG-CPA1-LUC* reporter, expressed in NMD^+ and *nmd2Δ* strains. The Y-axis is discontinuous because the uORFs in improved contexts strongly inhibited luciferase expression; (**C**) *CPA1-LUC* reporters containing the wt and D13N uORFs in their native contexts, and the *ΔAUG-CPA1-LUC* reporter, expressed in *XRN1 SKI2*, *xrn1Δ*, and *ski2Δ* strains.

the uORF, it was unknown whether NMD was triggered by AAP-specific ribosome stalling. Here we elucidated how the *CPA1* uORF controls NMD by independently altering: (i) the capacity of the uORF to stall ribosomes, (ii) the efficiency with which translation of the uORF was initiated, and (iii) the activity of specific cellular mRNA decay factors. The data obtained are consistent with the notion that occupancy of the *CPA1* uORF termination codon by ribosomes modulates NMD and that, like other NMD substrates (He et al., 2003; Ruiz-Echevarria and Peltz, 2000), degradation of the *CPA1* mRNA largely requires the 5' to 3' decay pathway but not its 3' to 5' counterpart. In the case of the wild-type transcript, nascent peptide mediated stalling of the ribosome at the uORF termination codon in response to Arg regulates both the stability of the transcript as well its translation; this is the first example of multifaceted control of eukaryotic gene expression in which the synthesis of a nascent peptide controls these two post-transcriptional regulatory events. These data provide a mechanistic explanation for earlier results, obtained by classical ^{3}H-uridine pulse-chase analysis, showing that the *CPA1* mRNA is destabilized in Arg-containing medium (Crabeel et al., 1990) and also demonstrate an additional mode by which mRNA destabilization by NMD can be a regulated phenomenon (Lee and Schedl, 2004; Wollerton et al., 2004).

4.3.2 Destabilization of Nonsense-containing mRNAs Requires Their Translation

The presence of a premature nonsense codon within a transcript is not sufficient to trigger its degradation. Destabilization of nonsense-containing mRNAs also requires their translation, a conclusion that follows from observations that decay can be antagonized by drugs, mutations, or RNA structures that interfere with protein synthesis (Belgrader et al., 1993; Ruiz-Echevarria et al., 1998; Zhang et al., 1997) or by tRNAs that suppress termination (Belgrader et al., 1993; Gozalbo and Hohmann, 1990; Losson and Lacroute, 1979). Our studies provide direct insight into how translation of an mRNA is involved in controlling its stability through NMD. With reporter transcripts containing the *CPA1* uORF in its native context, the wild-type uORF stalls ribosomes efficiently and is an efficient trigger of NMD, while the D13N uORF, which stalls ribosomes poorly or not at all, is an inefficient trigger (Figure 4.3A and Figure 4.4). When the initiation context of the uORF is improved, both the wild-type and D13N uORFs efficiently trigger

NMD (Figure 4.3B and Figure 4.5). These data indicate that it is the extent of ribosomal occupancy of an early stop codon that governs NMD (Figure 4.8), not downstream events affecting the association of bound factors (Jacobson and Peltz, 2000; Maquat, 2004). In turn, this implies that early termination of translation on the *CPA1* mRNA and/or the subsequent triggering of NMD must be inefficient. In this regard, it should be noted that an independent approach has recently shown that premature termination is considerably less efficient than normal termination (Amrani et al., 2004). As suggested by the *faux*-UTR model (Amrani et al., 2004; Jacobson and Peltz, 2000), inefficient termination might promote NMD by allowing the ribosome to associate with decay-inducing factors routinely precluded from interaction by the events of normal termination.

The *C. elegans gna-2* mRNA provides another example of a transcript in which NMD is regulated by the efficiency of uORF translation that is consistent with this model (Lee and Schedl, 2004). The RNA binding protein GLD-1 protects the *gna-2* mRNA from NMD by binding to the *gna-2* mRNA 5′-leader and reducing translation of two uORFs. Interestingly, both GLD-1 binding to *gna-2* mRNA and AAP-mediated ribosome stalling on *CPA1* mRNA reduce translation, yet induce opposing effects on NMD. Whereas GLD-1 binding to *gna-2* mRNA reduces NMD, AAP-mediated ribosome stalling on the *CPA1* mRNA induces NMD. A notable similarity in the regulation of *CPA1* uORF-mediated NMD and *gna-2* uORF-mediated NMD is that both involve *trans*-acting factors (i.e., the GLD-1 protein for *gna-2* and the amino acid Arg for *CPA1*) that act at the level of translation to regulate ribosome occupancy at uORF termination codons. A critical difference in these uORF-mediated systems is the specificity of the *trans*-acting factor. Whereas Arg appears to regulate NMD of the *CPA1* transcript by acting in concert with the nascent AAP that is synthesized from the transcript, GLD-1 appears to act more globally, because in addition to reducing NMD of the *gna-2* mRNA, it protects other nonsense-containing mRNAs from NMD (Lee and Schedl, 2004).

Although the uORF-containing *CPA1* and *gna-2* mRNAs are NMD substrates, the presence of a uORF does not automatically confer such status. Microarray analyses demonstrated that only a portion of the cellular uORF-containing mRNAs accumulate in

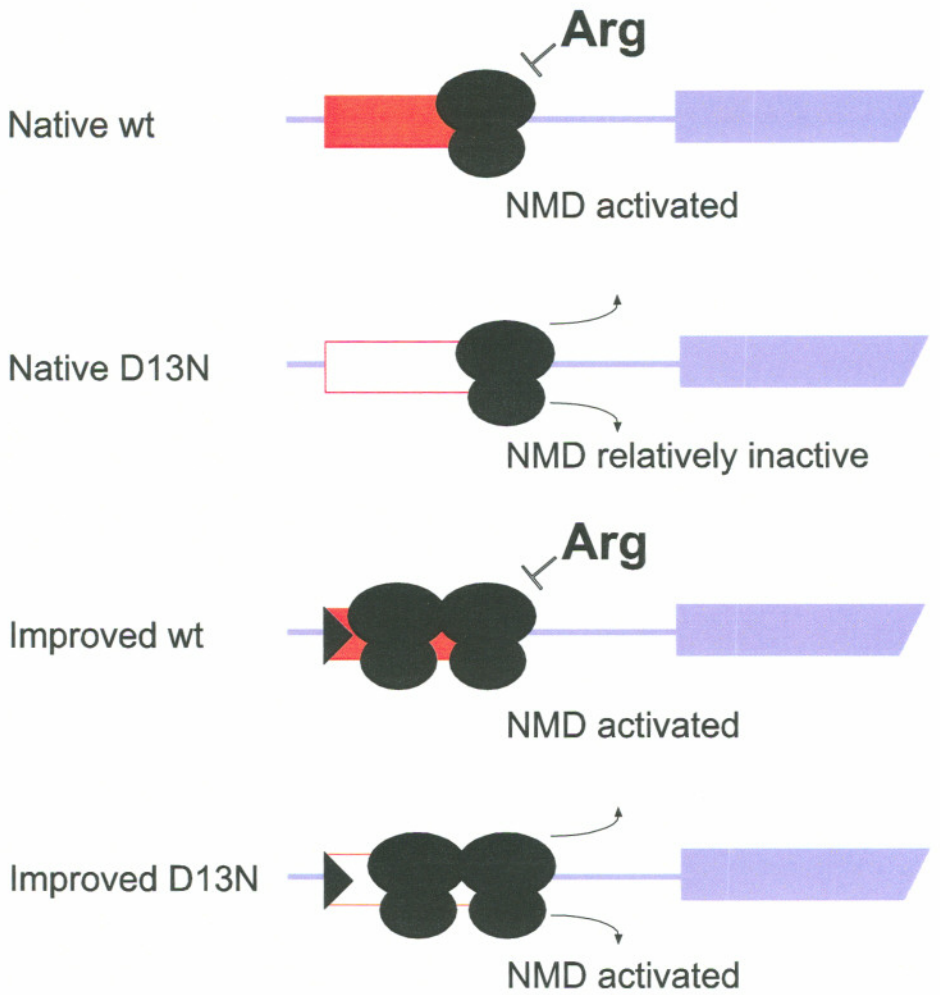

Figure 4.8 Model for control of NMD by ribosomal occupancy of a uORF termination codon. The wild-type *CPA1* uORF (solid orange box) in its native initiation context stalls ribosomes at the termination codon in response to Arg, inducing NMD of the transcript. The D13N *CPA1* uORF (open box) in its native initiation context does not stall ribosomes in response to Arg. The occupancy of the termination site by ribosomes for the D13N uORF is, therefore, low because initiation is inefficient. NMD is not induced because the occupancy of the termination codon is low. Improving the uORF initiation context (black arrowhead) increases uORF translation and causes NMD of both wild-type and D13N *CPA1* uORF-containing transcripts because the occupancy of the termination site by ribosomes is high in both cases.

yeast *upf1Δ, nmd2Δ*, or *upf3Δ* strains (He et al., 2003; Ruiz-Echevarria and Peltz, 2000) and directed studies of the uORFs in the *YAP1* and *GCN4* mRNAs showed that these uORFs neither mediate NMD nor destabilize their respective mRNAs (Ruiz-Echevarria et al., 1998; Vilela et al., 1998). The inability of the latter ORFs to trigger NMD was attributed to the presence of flanking stabilizer elements, *cis*-acting sequences that appeared to counteract the NMD-inducing effects of early termination. Given that premature termination appears to be functionally distinct from normal termination, and that this difference appears to play a role in promoting NMD (Amrani et al., 2004), such stabilizer elements may also play a role in regulating the qualitative nature of the termination event in the uORF.

Regardless of whether a premature termination codon is derived from a uORF, a nonsense mutation, a processing error, or another source, our data indicate that its role as an inducer of NMD depends on the extent to which it is occupied by the translation apparatus. A corollary of this conclusion is that events and factors that alter mRNA translatability should also affect NMD. In this regard, it should be noted that NMD in metazoans has been thought to be dependent on mRNA association with specific components of the exon junction complex (EJC) (Maquat, 2004; Singh and Lykke-Andersen, 2003). Since recent studies have shown that these factors can influence the efficiency with which an mRNA is translated (Nott et al., 2004; Wiegand et al., 2003), it is possible that the role of EJC proteins as NMD regulators is, at least in part, related to their ability to ensure efficient mRNA translation.

4.3.3 Decreased Translatability of *CPA1* mRNA in *nmd2Δ* Strains: Negative Regulation by Defective Termination?

The efficiency of luciferase production from mRNAs shown to be NMD substrates, but not from mRNAs unaffected by NMD, was selectively reduced in *nmd2Δ* cells relative to wild-type cells (Figure 4.7). This effect appeared independent of AAP-mediated ribosome stalling because it was also observed in mRNA containing the D13N uORF in an improved initiation context. This indicates that, while mRNA affected by NMD can be present at an increased level in nmd$^-$ strains, its translatability can nevertheless be impaired relative to its translatability in an NMD$^+$ strain. For example,

when the *CPA1-LUC* reporter contained the wild-type uORF in its native initiation context, its mRNA level was 7-fold higher in the *nmd2Δ* strain relative to the NMD^+ strain (Figure 4.4). However, due to reduced translatability of this reporter in the *nmd2Δ* strain, the absolute amount of luciferase in *nmd2Δ* cells was only approximately 2-fold greater than the levels in NMD^+ cells (data not shown). This two-fold increase in reporter activity did not correlate well with the change in mRNA levels, but is nevertheless significant since genetic selection for *trans*-acting factors that increase expression of the endogenous *CPA1* gene led to the isolation of mutants affected in NMD (Messenguy et al., 2002; Thuriaux et al., 1972).

The diminished translation activity of nonsense-containing mRNAs in *nmd2Δ* cells is not likely to be attributable to reduced translation initiation on those mRNAs because *in vitro* translation and primer-extension inhibition assays that mapped the positions of stalled ribosomes, in the absence of cycloheximide (Gaba et al., 2001; Sachs et al., 2002; Wang et al., 1999), demonstrated that *nmd2Δ* extracts exhibited the same Arg-specific ribosome stalling activity at the uORF termination codon as wild-type extracts (data not shown). These assays suggest that a comparable number of ribosomes initiate translation and reach the uORF termination codon in wild-type and *nmd2Δ* cells. Events subsequent to termination codon recognition, however, have been shown to differ markedly in the two types of cells. *In vivo*, several different approaches have shown that nmd^- cells promote nonsense suppression, i.e., nonsense codon read-through (Keeling et al., 2004; Maderazo et al., 2000; Weng et al., 1996a; Weng et al., 1996b) and, *in vitro*, recent toeprinting analyses indicate that extracts from nmd^- cells eliminate an aberrant toeprint characteristic of premature termination (Amrani et al., 2004). Since ribosomes that have translated the *CPA1* uORF do not normally appear to reinitiate translation at the downstream *LUC* initiation codon (Gaba et al., 2001; Wang et al., 1999) these observations suggest that, in *nmd2Δ* cells, ribosomes engaged in translation elongation downstream of the uORF termination codon may create an additional impediment to scanning ribosomes otherwise capable of downstream initiation. These effects may not, however, be universally indicative of all prematurely terminated mRNAs since measurements of the specific translation activity of chimeric *PGK1/CUP1* nonsense-containing mRNAs showed that these mRNAs were translationally defective in wild-type

cells but were restored to normal translational efficiencies when the *UPF1* gene was deleted (Muhlrad and Parker, 1999).

4.4 EXPERIMENTAL PROCEDURES

4.4.1 Yeast Strains, Growth Conditions, and General Methods

The yeast strains used in this study are listed in Table 4.2 and were created by transforming the plasmids listed in Table 4.1 into isogenic strains HFY1200 (*MATa ade2-1 his3-11, 15 leu2-3, 112 trp1-1 ura3-1 can1-100 NMD2 XRN1 SKI2*), HFY1300 (*MATa ade2-1 his3-11, 15 leu2-3, 112 trp1-1 ura3-1 can1-100 nmd2::HIS3 XRN1 SKI2*) (He and Jacobson, 1995), HFY1080 (*MATa ade2-1 his3-11, 15 leu2-3, 112 trp1-1 ura3-1 can1-100 NMD2 xrn1::ADE2 SKI2*) (He and Jacobson, 2001), and HFY1170 (*MATa ade2-1 his3-11, 15 leu2-3, 112 trp1-1 ura3-1 can1-100 NMD2 XRN1 ski2::URA3*) (He, F., personal comm.). Synthetic medium lacking tryptophan was used to select for and maintain *CPA1-LUC* expression plasmids (Table 4.2). For all experiments, overnight starter cultures (3 ml) were grown in synthetic medium lacking tryptophan and used to inoculate fresh cultures (40 ml) which were grown to an OD_{600} of 0.6 – 0.8 at harvest. All cultures were grown at 30°C. For arginine regulation experiments, cultures were grown in appropriately supplemented, selective liquid minimal medium (Werner et al., 1987) that either lacked arginine supplementation (-Arg) or contained 1 mg/ml arginine (+Arg). For all subsequent luciferase assay experiments and for all RNA analysis experiments, cells were grown in liquid YPD media. Preparation of standard yeast media, methods of cell culture, genomic DNA purification, and transformations were as described (Burke et al., 2000).

4.4.2 Plasmids

All *in vivo CPA1-LUC* expression plasmids are derivatives of pAG119 which was generated by removing the *Bgl*II site of YCplac22 (Gietz and Sugino, 1988), introducing at the multiple cloning site a 3-kb *BamH*I-*Hin*dIII *PGK1-LUC* fragment fromYCplac22-ΔPGKORF-LUC, (kindly provided by R. Ganesan, University of Massachusetts Medical School) which contains the *PGK1* promoter followed by a firefly luciferase cistron that

has its translation initiation codon replaced with a *Sal*I site, and introducing *Bgl*II-*Nco*I sites in the *PGK1-LUC* fragment at the *Sal*I site. *Bgl*II-*Nco*I fragments from pAG101, pAG103, and pAG105 (Wang et al., 1999) were subcloned into pAG119 to produce pAG121, pAG122, and pAG123 respectively. Plasmids pAG101 and pAG103 (Wang et al., 1999) were used for PCR with primers AG35 (5′-TGTTGAAGATCTACCCTTTTT-GCAGATTTGAAATAAAAAAATTGAAAAAT GTTTAGCTTATCGAAC-3′) and AG3 (5′-ATCTGACCATGGTTGAAATATTTTTAGGAGTGGTT-3′) to produce 200-bp PCR products that were digested with *Bgl*II and *NcoI*, and subcloned between the *Bgl*II and *NcoI* sites of pAG119 to create plasmids pAG134 and pAG135, respectively. Genomic DNA isolated from HFY1200 cells and primers AG31 (5′-CTGGATTCTAGA-TTCACGCAACCCTTGATTGG-3′) and AG32 (5′-TGTTGAAGATCTGACCACCTTT-TGCCTCTGGG-3′) were used to PCR amplify a 900-bp *CPA1* DNA fragment that was digested with *Bgl*II and *Xba*I, and subcloned between the *Bgl*II and *XbaI* sites of pAG119 to create plasmid pAG133.

4.4.3 RNA Analyses

Total RNA was isolated by the hot phenol method as described previously (Herrick et al., 1990). Equal amounts (8 μg) of each RNA sample were fractionated on 1.0% formaldehyde agarose gels and analyzed by northern blotting with probes prepared by random priming, using [α-^{32}P]dCTP and a kit from Roche. Relative mRNA levels were determined by quantitation of northern blots with a Molecular Dynamics PhosphorImager. The *CPA1* and *CPA1-LUC* blots were normalized for loading to *SCR1*, a stable RNA polymerase III transcript. The following DNA fragments were gel-purified and used for the random priming reactions to generate radiolabeled probes for northern blotting: a 900-bp *Bgl*II-*Xba*I fragment of *CPA1* from pAG133 (to detect endogenous *CPA1* mRNA), a 1.6-kb *Eco*RI-*Sal*I fragment of *PGK1-LUC* from pDM345; kindly provided by Robin Ganesan, University of Massachusetts Medical School, which contains luciferase coding sequence and 3′-*PGK1* sequences (to detect *CPA1-LUC* mRNAs), a 600-bp *Eco*RI-*Hind*III fragment from pGEM4Z-CYH2 (Herrick et al., 1990) (to detect both the pre-*CYH2* mRNA and the *CYH2* mRNA), and a 400-bp *Eco*RI

Table 4.2

Yeast Strains Used in This Study

Strain	mRNA decay background	Genotype
yAG201	Wild-type	*MATa ade2-1 his3-11, 15 leu2-3, 112 trp1-1 ura3-1 can1-100 NMD2 XRN1 SKI2* [pAG121 *WT-CPA1-LUC*]
yAG202	Wild-type	*MATa ade2-1 his3-11, 15 leu2-3, 112 trp1-1 ura3-1 can1-100 NMD2 XRN1 SKI2* [pAG122 *D13N-CPA1-LUC*]
yAG203	Wild-type	*MATa ade2-1 his3-11, 15 leu2-3, 112 trp1-1 ura3-1 can1-100 NMD2 XRN1 SKI2* [pAG123 *ΔAUG-CPA1-LUC*]
yAG213	Wild-type	*MATa ade2-1 his3-11, 15 leu2-3, 112 trp1-1 ura3-1 can1-100 NMD2 XRN1 SKI2* [pAG134 *↑WT-CPA1-LUC*]
yAG214	Wild-type	*MATa ade2-1 his3-11, 15 leu2-3, 112 trp1-1 ura3-1 can1-100 NMD2 XRN1 SKI2* [pAG135 *↑D13N-CPA1-LUC*]
yAG204	*nmd2Δ*	*MATa ade2-1 his3-11, 15 leu2-3, 112 trp1-1 ura3-1 can1-100 nmd2::HIS3 XRN1 SKI2* [pAG121 *WT-CPA1-LUC*]
yAG205	*nmd2Δ*	*MATa ade2-1 his3-11, 15 leu2-3, 112 trp1-1 ura3-1 can1-100 nmd2::HIS3 XRN1 SKI2* [pAG122 *D13N-CPA1-LUC*]
yAG206	*nmd2Δ*	*MATa ade2-1 his3-11, 15 leu2-3, 112 trp1-1 ura3-1 can1-100 nmd2::HIS3 XRN1 SKI2*

		[pAG123 *ΔAUG-CPA1-LUC*]
yAG215	*nmd2Δ*	*MATa ade2-1 his3-11, 15 leu2-3, 112 trp1-1 ura3-1 can1-100 nmd2::HIS3 XRN1 SKI2* [pAG134 *↑WT-CPA1-LUC*]
yAG216	*nmd2Δ*	*MATa ade2-1 his3-11, 15 leu2-3, 112 trp1-1 ura3-1 can1-100 nmd2::HIS3 XRN1 SKI2 [pAG135 ↑D13N-CPA1-LUC]*
yAG207	*xrn1Δ*	*MATa ade2-1 his3-11, 15 leu2-3, 112 trp1-1 ura3-1 can1-100 NMD2 xrn1::ADE2 SKI2 [pAG121 WT-CPA1-LUC]*
yAG208	*xrn1Δ*	*MATa ade2-1 his3-11, 15 leu2-3, 112 trp1-1 ura3-1 can1-100 NMD2 xrn1::ADE2 SKI2 [pAG122 D13N-CPA1-LUC]*
yAG209	*xrn1Δ*	*MATa ade2-1 his3-11, 15 leu2-3, 112 trp1-1 ura3-1 can1-100 NMD2 xrn1::ADE2 SKI2 [pAG123 ΔAUG-CPA1-LUC]*
yAG210	*ski2Δ*	*MATa ade2-1 his3-11, 15 leu2-3, 112 trp1-1 ura3-1 can1-100 NMD2 XRN1 ski2::URA3 [pAG121 WT-CPA1-LUC]*
yAG211	*ski2Δ*	*MATa ade2-1 his3-11, 15 leu2-3, 112 trp1-1 ura3-1 can1-100 NMD2 XRN1 ski2::URA3 [pAG122 D13N-CPA1-LUC]*
yAG212	*ski2Δ*	*MATa ade2-1 his3-11, 15 leu2-3, 112 trp1-1 ura3-1 can1-100 NMD2 XRN1 ski2::URA3 [pAG123 ΔAUG-CPA1-LUC]*

fragment of *SCR1* from pCR2.1-SCR1 (provided by R. Ganesan), in which the 400-bp *Eco*RI fragment of *SCR1* was obtained by PCR with genomic DNA and primers SCR1-1 and SCR1-2 (He and Jacobson, 2001) (to detect *SCR1* mRNA).

4.4.4 Luciferase Enzyme Activity Analysis

Duplicate aliquots (3 ml) from each culture were collected by centrifugation at 4°C and the cell pellets were stored at -80°C for later steps. Subsequently, the cell pellets, on ice, were resuspended in 100 µl of freshly prepared luciferase extraction buffer (0.1 M KPO_4, pH 7.8; 1.0 mM DTT; 1.0 mM PMSF), followed by the addition of 100 µl acid-washed 0.5 mm glass beads, and successive vortexing for 15 sec followed by 30 sec on ice, for a total of 225 sec. Samples were then centrifuged for 5 min at 4°C and the supernatants were transferred to fresh tubes, frozen at -80°C, and later thawed for measurement of luciferase activity, which was accomplished using 5 µl of extract, as described previously (Wang and Sachs, 1997a). Relative light units (RLU) were normalized to the concentration of total protein in the extracts, which was determined using BioRad's Protein Dye Assay Reagent and following the microassay procedure for microtiter plates provided by the manufacturer. All standards and extract samples were assayed in triplicate.

CHAPTER 5
CONCLUSIONS AND FUTURE DIRECTIONS

5.1 SUMMARY OF RESEARCH

This research has focused on understanding the *cis*-acting regulation mediated by the *S. cerevisiae CPA1* uORF-encoded arginine attenuator peptide (AAP). Studies with the *CPA1* homolog in *N. crassa*, *arg-2*, had shown that in a cell-free *N. crassa* translation system, the wild-type uORF-encoded AAP functioned to stall ribosomes at the *arg-2* uORF termination codon in response to Arg. In addition to ribosome stalling, these studies detected fewer ribosomes at the downstream reporter start codon in response to Arg, consistent with reduced reporter activity in response to Arg. Although genetic studies in *S. cerevisiae* strongly suggested that *CPA1* AAP function is analogous to that of the *N. crassa* AAP, this prediction had not been supported by biochemical evidence. To further investigate the regulatory activity of the *S. cerevisiae* AAP, this thesis work began with studies that used synthetic transcripts containing wild-type or mutated *CPA1* mRNA 5′-leaders fused to firefly luciferase (*CPA1-LUC*) for *in vitro* translation in cell-free *S. cerevisiae*, *N. crassa*, and wheat germ translation systems, which reconstituted AAP-mediated negative regulation of reporter activity in response to Arg, as previously demonstrated with the *N. crassa in vitro* system. Primer extension inhibition (toeprinting) analyses showed that ribosomes on reporter transcripts were regulated similarly by the *CPA1* and *arg-2* AAPs in response to Arg. The toeprinting assay was then utilized to demonstrate that *CPA1* and *GCN4* uORFs regulate ribosomes through distinct mechanisms. Lastly, *in vivo* analyses showed that, in addition to translational regulation, the *CPA1* uORF also regulates mRNA stability. These studies provide evidence for a mechanism in which Arg-induced ribosome stalling at the uORF termination codon regulates downstream translation initiation and the level of *CPA1* mRNA.

5.1.1 The *S. cerevisiae* AAP Induces Ribosome Stalling in Response to Arg in Cell-Free Translation Systems

In *S. cerevisiae*, *N. crassa*, and wheat germ cell-free translation systems, the *CPA1* and *arg-2* uORF-encoded arginine attenuator peptides (AAPs) negatively regulated downstream cistron expression in response to Arg, indicating that factors required for fungal AAP function are also present in plant systems. Primer extension inhibition (toeprinting) was used to detect ribosomes on synthetic *CPA1-LUC* mRNA and showed that the AAPs functioned to stall *S. cerevisiae* and *N. crassa* ribosomes at the uORF termination codons in response to Arg. Stalling was also observed when the AAPs were fused directly to the N terminus of firefly luciferase, demonstrating that the AAPs can function to stall ribosomes engaged in translation elongation. As Arg concentrations increased, AAP-LUC activity decreased and the extent of ribosome stalling increased. Increasing Arg concentrations did not affect the Arg-tRNA charge status because three of the five Arg-tRNAs appeared fully charged at all Arg concentrations (the other two Arg-tRNAs were not analyzed because their sequences were unknown). These studies indicated that Arg levels regulate Cpa1p translation by regulating AAP-mediated ribosome stalling at the *CPA1* uORF termination codon, and that this regulation lacks an apparent role for the level of charged tRNA, which is in contrast to previous examples of translational regulation for amino acid biosynthetic genes.

5.1.2 The *CPA1* and *GCN4* uORFs Control Translation by Distinct Mechanisms

Toeprinting was used in combination with the translation elongation inhibitor, cycloheximide, to map the positions of initiating ribosomes on synthetic *CPA1-LUC* transcripts to determine where ribosomes loaded first and where they loaded during steady-state translation. When cycloheximide was added to translation reactions containing low Arg prior to the addition of cell extract (T_0), toeprint analyses detected similar levels of ribosomes at the uORF and *LUC* initiation codons, indicating that translation was not required for initiation downstream of the *CPA1* uORF. Addition of cycloheximide after 5 – 10 min. of translation (steady-state) did not alter the distribution of ribosomes at the *CPA1* uORF and *LUC* initiation codons. By contrast, in translation reactions with *GCN4-LUC* mRNA, addition of cycloheximide at T_0 resulted in detection

of ribosomes at 5′-proximal *GCN4* uORF start codons, and the levels of these ribosomes were significantly greater than those levels detected at the *GCN4* initiation codon. When cycloheximide was added at steady-state, however, similar levels of ribosomes were detected at the uORF1 and *GCN4* start codons, indicating that translation increased the level of ribosomes at the downstream *GCN4* start codon. These results are consistent with the predicted mechanisms of the *CPA1* and *GCN4* uORF systems. As the *CPA1* uORF initiation context is predicted to inefficiently initiate translation, ribosomes should scan past the uORF (leaky-scanning) and initiate translation downstream, which is consistent with similar ribosome levels detected at uORF and *LUC* initiation codons when cycloheximide was added at T_0. Ribosome stalling at the *CPA1* uORF termination codon in response to Arg was shown to reduce the steady-state level of ribosomes at the *LUC* initiation codon, as would be expected based on AAP function to stall ribosomes at the uORF termination codon in response to Arg, which in turn, appears to block the access of leaky scanning ribosomes to the *LUC* initiation codon.

Unlike the inefficient *CPA1* uORF initiation context, the *GCN4* uORF initiation contexts are predicted to efficiently initiate translation, consistent with increased levels of ribosomes detected at the *GCN4* uORFs relative to those levels detected at the *GCN4* initiation codon when cycloheximide was added at T_0. Translation of *GCN4* uORF1, but not uORF4, resulted in detection of increased ribosome levels at downstream initiation codons. These results are consistent with the *GCN4* uORF model, which predicts that translation of uORF1, but not uORF4, allows ribosomes to reinitiate translation downstream. The *GCN4* model also predicts that Gcn4p translation is repressed in response eIF2-induced translation reinitiation at the start codons of uORFs 3 and 4, which is consistent with increased steady-state levels of ribosomes detected at the uORF3 initiation codon and reduced steady-state levels of ribosomes detected at the *GCN4* initiation codon in response to addition of purified eIF2 to translation reactions. These analyses provide physical evidence for distinct uORF-mediated mechanisms that regulate Cpa1p and Gcn4p translation. Whereas the *CPA1* uORF-encoded AAP regulates leaky-scanning ribosomes in response to Arg levels, the *GCN4* uORFs regulate reinitiation in response to eIF2 levels.

5.1.3 Translational Control Mediated by the *CPA1* uORF Regulates Nonsense Mediated mRNA Decay

In a wild-type *S. cerevisiae* strain grown in rich Arg-containing medium, the wild-type uORF, but not the D13N uORF, reduced reporter activity per mRNA levels, consistent with wild-type AAP-mediated effects *in vitro* to stall ribosomes at the uORF and reduce ribosome levels at the downstream start codon. Sequence-dependent *CPA1* uORF-mediated regulation of steady-state reporter mRNA abundance was also observed in wild-type cells, but not in *nmd2Δ* cells, which were defective for the nonsense mediated mRNA decay (NMD) pathway that rapidly degrades nonsense-containing transcripts. These results indicated that the wild-type *CPA1* uORF, in addition to its effects on translation, controls steady-state levels of *CPA1* mRNA by affecting transcript susceptibility to the NMD pathway. In *ski2Δ* cells, which were defective in 3′ to 5′ decay, but not in *xrn1Δ* cells defective in 5′ to 3′ decay, sequence-dependent uORF-mediated reduction of steady-state reporter mRNA abundance was observed, indicating that NMD of *CPA1* mRNA predominantly involves 5′ to 3′ decay, and not 3′ to 5′ decay. In *nmd2Δ*, *xrn1Δ*, and *ski2Δ* strains, sequence-dependent uORF-mediated reduction in the levels of reporter activity per mRNA was observed, indicating that translational control mediated by the *CPA1* uORF functions independently of these mRNA decay activities. Improvement of the uORF initiation context resulted in wild-type and D13N uORF-mediated NMD of reporter transcripts, indicating that ribosome stalling activity is not essential for NMD. These analyses identified two modes for the induction of NMD of the *CPA1* mRNA. When the native *CPA1* uORF initiation context is present, ribosome stalling at the uORF termination codon is required for the *CPA1* uORF to induce NMD of reporter mRNA, but when the initiation context is improved, ribosome stalling is no longer required for the *CPA1* uORF to induce NMD. AAP-mediated ribosome stalling at the uORF termination codon in response to Arg thus appears to regulate both the stability of the transcript as well its translation, the first example of multifaceted control of eukaryotic gene expression in which the synthesis of a nascent peptide controls these two post-transcriptional regulatory events.

5.2 FUTURE DIRECTIONS

The *CPA1* and *arg-2* uORF-encoded AAPs clearly respond to Arg to stall ribosomes at their uORF termination codons. How Arg induces the AAP to stall ribosomes however, has been a long-standing question. The rapid translational effects exerted by Arg (Luo et al., 1995; Wang et al., 1999; Wang and Sachs, 1997a), suggests that Arg acts directly on the translational machinery. Considering the complexity of the ribosome, a variety of possible scenarios can be envisioned for how the AAP senses Arg. Possibilities include a direct interaction between Arg and the AAP or the AAP may sense Arg via interactions with ribosomal RNA(s), ribosomal protein(s), and/or translation factor(s). In addition to Arg sensing, it is unknown how the AAP actually "stalls" ribosomes. Direct or Arg-mediated interactions between ribosomal factors and the AAP may contribute to the stalling effect, which presumably involves AAP function within the ribosome given that AAP regulatory sequences act in *cis* to stall ribosomes when encoded as an internal domain (Fang et al., 2004). Furthermore, a regulatory effect was not observed in response to synthetic *N. crassa* AAP supplementation to cell-free translation reactions that contained or lacked Arg (data not shown), consistent with AAP function occurring within the ribosome.

To investigate possible Arg-induced AAP interactions, experiments could be designed to cross-link *in vitro* translation reactions that contain synthetic transcripts encoding either wild-type or D13N AAPs fused at their N-terminus to a tag. The cross-linked translation reaction could be resolved on native polyacrylamide gels and transferred to membranes for western blot analysis with anti-tag antibody. If the detected molecular weight of the wild-type AAP-tag, but not the D13N AAP-tag, increases when translation reactions are supplemented with Arg, the AAP-tag may have complexed with a potential factor involved in AAP function to stall ribosomes. This complex could be further characterized to ultimately identify the associated products. The genes encoding the identified products could then be targets of site directed mutagenesis to create mutant strains that could be used to assay for *CPA1* uORF-mediated translation control to evaluate the effects of these mutations on AAP function. Mutant strains that show altered uORF-mediated translational control would provide evidence that the mutagenized gene

encodes a factor that plays a role in AAP-mediated ribosome stalling in response to Arg. Studies suggest that factors such as ribosomal proteins that line the peptide exit tunnel could be involved in AAP function (see below).

An alternative approach to investigate for factors that interact with the AAP to stall ribosomes could be to examine AAP-mediated ribosome stalling in translation extracts derived from yeast strains that contain mutations in specific ribosomal proteins that affect ribosome sensitivity to inhibitors of protein synthesis. For example, mutations in *CYH2* and *RIM-C* genes, which encode ribosomal proteins L29 and L41, respectively, confer resistance to the elongation inhibitor, cycloheximide (Fried and Warner, 1982; Kawai et al., 1992), while resistance to the elongation inhibitor cryptopleurine results from mutations in the *CRY1* and *CRY2* genes which encode ribosomal protein 59 (Bucher and Skogerson, 1976; Paulovich et al., 1993). Similar analyses could also be done with yeast strains that contain mutations in ribosomal RNA. Mutations in 25S rRNA for example, induce nonsense suppression (Liu and Liebman, 1996), while mutations in 18S rRNA inhibit nonsense suppression and appear to affect translational fidelity (Chernoff et al., 1996; Chernoff et al., 1994). Currently, the most compelling evidence indicating the involvement of ribosomal factor(s) in AAP-mediated ribosome stalling comes from recent studies with *secM* and *trpC* mRNAs in *E. coli* indicating that ribosomal proteins L22 and L4 are critical for nascent peptide-mediated ribosome stalling (Nakatogawa et al., 2004) (personal communication, Yanofsky C.). It is conceivable that a nascent peptide could interact with L22 and L4 because a domain of these ribosomal proteins is located on the surface of the large ribosomal subunit and contains an extended loop that is positioned at the surface of the peptide exit tunnel (Ban et al., 2000; Nakatogawa and Ito, 2002; Nissen et al., 2000). These studies and experiments showing that the *N. crassa* AAP functions to stall ribosomes in *E. coli* (personal communication, Yanofsky C.), raise the interesting possibility that ribosomal proteins L22 and L4 are highly conserved factors in prokaryotic and eukaryotic ribosomes that serve critical functions for posttranscriptional control mechanisms that regulate ribosome movement.

Given the conservation in translational control mediated by the *CPA1* and *arg-2* systems, the *arg-2* uORF is predicted to act similarly to its yeast counterpart to regulate NMD. However, a significant difference in the *S. cerevisiae* and *N. crassa* systems is

that genes from the former genome mostly lack introns, whereas genes from the latter genome typically contain introns. Since studies in higher eukaryotes indicate a relationship between splicing events, translation, and NMD (Kim et al., 2001; Lykke-Andersen et al., 2001; Maquat, 2002; Shibuya et al., 2004), it is conceivable that splicing events play a role in *arg-2* uORF-mediated regulation of translation and mRNA stability. To investigate the roles of the *arg-2* uORF and splicing activity on translation and NMD, a mutant *N. crassa* strain that appears defective for NMD (nmd$^-$) (Sachs lab personal communication) could be exploited. Using *arg-2* fusion reporter mRNAs analogous to those described in chapter 4, steady-state mRNA levels could be measured in wild-type and nmd$^-$ *N. crassa* cells to investigate for (i) *arg-2* mRNA sensitivity to NMD, (ii) *arg-2* uORF-dependence for NMD, and (iii) uORF-dependent effects on translation efficiency in the two strains. In addition, if intron-splicing factor activities could be altered in wild-type and nmd$^-$ strains, then splicing, translational, and mRNA decay effects could be analyzed independently from their roles in *arg-2* uORF-mediated regulation of translation and/or NMD. RNA inhibition (RNAi)-based approaches are being pursued in *N. crassa* to inhibit the translation of specific mRNAs (Sachs lab, personal communication) and may serve as a suitable approach to target the expression of specific splicing and mRNA decay factors. These studies could identify *N. crassa* as a valuable model system for understanding pre-mRNA splicing, nuclear export, translational, and mRNA decay mechanisms in higher eukaryotes.

LITERATURE CITED

Alderete, J. P., Jarrahian, S., and Geballe, A. P. (1999). Translational effects of mutations and polymorphisms in a repressive upstream open reading frame of the human cytomegalovirus UL4 gene. J Virol *73*, 8330-8337.

Altamura, N., Dujardin, G., Groudinsky, O., and Slonimski, P. P. (1994). Two adjacent nuclear genes, *ISF1* and *NAM7/UPF1*, cooperatively participate in mitochondrial functions in *Saccharomyces cerevisiae*. Mol Gen Genet *242*, 49-56.

Alzner-DeWeerd, B., Hecker, L. I., Barnett, W. E., and RajBhandary, U. L. (1980). The nucleotide sequence of phenylalanine tRNA from the cytoplasm of *Neurospora crassa*. Nucleic Acids Res *8*, 1023-1032.

Amrani, N., Ganesan, R., Kervestin, S., Mangus, D. A., Ghosh, S., and Jacobson, A. (2004). A faux 3'-UTR promotes aberrant termination and triggers nonsense-mediated mRNA decay. Nature *432*, 112-118.

Anand, M., Chakraburtty, K., Marton, M. J., Hinnebusch, A. G., and Kinzy, T. G. (2003). Functional interactions between yeast translation eukaryotic elongation factor (eEF) 1A and eEF3. J Biol Chem *278*, 6985-6991.

Anderson, J., Phan, L., Cuesta, R., Carlson, B. A., Pak, M., Asano, K., Bjork, G. R., Tamame, M., and Hinnebusch, A. G. (1998). The essential Gcd10p-Gcd14p nuclear complex is required for 1- methyladenosine modification and maturation of initiator methionyl-tRNA. Genes Dev *12*, 3650-3662.

Anderson, J. S., and Parker, R. P. (1998). The 3' to 5' degradation of yeast mRNAs is a general mechanism for mRNA turnover that requires the SKI2 DEVH box protein and 3' to 5' exonucleases of the exosome complex. EMBO J *17*, 1497-1506.

Anthony, D. D., and Merrick, W. C. (1992). Analysis of 40 S and 80 S complexes with mRNA as measured by sucrose density gradients and primer extension inhibition. J Biol Chem *267*, 1554-1562.

Arkov, A. L., Freistroffer, D. V., Ehrenberg, M., and Murgola, E. J. (1998). Mutations in RNAs of both ribosomal subunits cause defects in translation termination. EMBO J *17*, 1507-1514.

Arkov, A. L., Hedenstierna, K. O., and Murgola, E. J. (2002). Mutational eidence for a functional connection between two domains of 23S rRNA in translation termination. J Bacteriol *184*, 5052-5057.

Arrick, B. A., Lee, A. L., Grendell, R. L., and Derynck, R. (1991). Inhibition of translation of transforming growth factor-beta 3 mRNA by its 5' untranslated region. Mol Cell Biol *11*, 4306-4313.

Baek, J.-M., and Kenerley, C. M. (1998). The *arg2* gene of *Trichoderma virens*: cloning and development of a homologous transformation system. Fungal Genet Biol *23*, 34-44.

Ban, N., Nissen, P., Hansen, J., Moore, P. B., and Steitz, T. A. (2000). The complete atomic structure of the large ribosomal subunit at 2.4 A resolution. Science *289*, 905-920.

Barciszewska, M. Z., Keith, G., Kubli, E., and Barciszewski, J. (1986). The primary structure of wheat germ tRNAArg--the substrate for arginyl- tRNAArg:protein transferase. Biochimie *68*, 319-323.

Bates, B., Hardin, J., Zhan, X., Drickamer, K., and Goldfarb, M. (1991). Biosynthesis of human fibroblast growth factor-5. Mol Cell Biol *11*, 1840-1845.

Baum, M., and Beier, H. (1998). Wheat cytoplasmic arginine tRNA isoacceptor with a U*CG anticodon is an efficient UGA suppressor in vitro. Nucleic Acids Res *26*, 1390-1395.

Beelman, C. A., Stevens, A., Caponigro, G., LaGrandeur, T. E., Hatfield, L., Fortner, D. M., and Parker, R. (1996). An essential component of the decapping enzyme required for normal rates of mRNA turnover. Nature *382*, 642-646.

Belgrader, P., Cheng, J., and Maquat, L. E. (1993). Evidence to implicate translation by ribosomes in the mechanism by which nonsense codons reduce the nuclear level of human triosephosphate isomerase mRNA. Proc Natl Acad Sci USA *90*, 482-486.

Belli, B. A., and Samuel, C. E. (1993). Biosynthesis of reovirus-specified polypeptides: identification of regions of the bicistronic reovirus S1 mRNA that affect the efficiency of translation in animal cells. Virology *193*, 16-27.

Benne, R., Edman, J., Traut, R. R., and Hershey, J. W. (1978). Phosphorylation of eukaryotic protein synthesis initiation factors. Proc Natl Acad Sci USA *75*, 108-112.

Benne, R., and Hershey, J. W. (1976). Purification and characterization of initiation factor IF-E3 from rabbit reticulocytes. Proc Natl Acad Sci USA *73*, 3005-3009.

Bergenhem, N. C., Venta, P. J., Hopkins, P. J., Kim, H. J., and Tashian, R. E. (1992). Mutation creates an open reading frame within the 5' untranslated region of macaque

erythrocyte carbonic anhydrase (CA) I mRNA that suppresses CA I expression and supports the scanning model for translation. Proc Natl Acad Sci USA *89*, 8798-8802.

Boring, L., Gosling, J., Cleary, M., and Charo, I. F. (1998). Decreased lesion formation in *CCR2-/-* mice reveals a role for chemokines in the initiation of atherosclerosis. Nature *394*, 894-897.

Brown, C. M., Stockwell, P. A., Trotman, C. N., and Tate, W. P. (1990). Sequence analysis suggests that tetra-nucleotides signal the termination of protein synthesis in eukaryotes. Nucleic Acids Res *18*, 6339-6345.

Brown, C. Y., Mize, G. J., Pineda, M., George, D. L., and Morris, D. L. (1999a). Role of two upstream open reading frames in the translational control of oncogene *mdm2*. Oncogene *18*, 5631-5637.

Brown, C. Y., Mize, G. J., Pineda, M., George, D. L., and Morris, D. R. (1999b). Role of two upstream open reading frames in the translational control of oncogene *mdm2*. Oncogene *18*, 5631-5637.

Bucher, K., and Skogerson, L. (1976). Cryptopleurine--an inhibitor of translocation. Biochemistry *15*, 4755-4759.

Burke, D., Dawson, D., and Stearns, T. (2000). Methods in yeast genetics: a Cold Spring Harbor Laboratory course manual (Plainview, NY: Cold Spring Harbor Laboratory Press).

Byrne, P. C., Sanders, P. G., and Snell, K. (1995). Translational control of mammalian serine hydroxymethyltransferase expression. Biochem Biophys Res Commun *214*, 496-502.

Calkhoven, C. F., Muller, C., and Leutz, A. (2000). Translational control of C/EBPalpha and C/EBPbeta isoform expression. Genes Dev *14*, 1920-1932.

Calogero, R. A., Pon, C. L., Canonaco, M. A., and Gualerzi, C. O. (1988). Selection of the mRNA translation initiation region by *Escherichia coli* ribosomes. Proc Natl Acad Sci USA *85*, 6427-6431.

Cao, D., and Parker, R. (2001). Computational modeling of eukaryotic mRNA turnover. RNA *7*, 1192-1212.

Cao, D., and Parker, R. (2003). Computational modeling and experimental analysis of nonsense-mediated decay in yeast. Cell *113*, 533-545.

Cao, J., and Geballe, A. P. (1995). Translational inhibition by a human cytomegalovirus upstream open reading frame despite inefficient utilization of its AUG codon. J Virol *69*, 1030-1036.

Cao, J., and Geballe, A. P. (1996a). Coding sequence-dependent ribosomal arrest at termination of translation. Mol Cell Biol *16*, 603-608.

Cao, J., and Geballe, A. P. (1996b). Inhibition of nascent-peptide release at translation termination. Mol Cell Biol *16*, 7109-7114.

Cao, J., and Geballe, A. P. (1998). Ribosomal release without peptidyl tRNA hydrolysis at translation termination in a eukaryotic system. RNA *4*, 181-188.

Capoulade, C., Bressac-de Paillerets, B., Lefrere, I., Ronsin, M., Feunteun, J., Tursz, T., and Wiels, J. (1998). Overexpression of *MDM2*, due to enhanced translation, results in inactivation of wild-type p53 in Burkitt's lymphoma cells. Oncogene *16*, 1603-1610.

Caskey, C. T., Beaudet, A. L., Scolnick, E. M., and Rosman, M. (1971). Hydrolysis of fMet-tRNA by peptidyl transferase. Proc Natl Acad Sci USA *68*, 3163-3167.

Castilho-Valavicius, B., Yoon, H., and Donahue, T. F. (1990). Genetic characterization of the *Saccharomyces cerevisiae* translational initiation suppressors *sui1*, *sui2* and *SUI3* and their effects on *HIS4* expression. Genetics *124*, 483-495.

Ceci, M., Gaviraghi, C., Gorrini, C., Sala, L. A., Offenhauser, N., Marchisio, P. C., and Biffo, S. (2003). Release of eIF6 (p27BBP) from the 60S subunit allows 80S ribosome assembly. Nature *426*, 579-584.

Chakravarti, D., and Maitra, U. (1993). Eukaryotic translation initiation factor 5 from Saccharomyces cerevisiae. Cloning, characterization, and expression of the gene encoding the 45,346-Da protein. J Biol Chem *268*, 10524-10533.

Chan, C. C., Dostie, J., Diem, M. D., Feng, W., Mann, M., Rappsilber, J., and Dreyfuss, G. (2004). eIF4A3 is a novel component of the exon junction complex. RNA *10*, 200-209.

Chang, K. S., Lee, S. H., Hwang, S. B., and Park, K. Y. (2000). Characterization and translational regulation of the arginine decarboxylase gene in carnation (*Dianthus caryophyllus* L.). Plant J *24*, 45-56.

Chang, M. J., and Blissard, G. W. (1997). Baculovirus *gp64* gene expression: negative regulation by a minicistron. J Virol *71*, 7448-7460.

Chaudhuri, J., Chowdhury, D., and Maitra, U. (1999). Distinct functions of eukaryotic translation initiation factors eIF1A and eIF3 in the formation of the 40 S ribosomal preinitiation complex. J Biol Chem *274*, 17975-17980.

Chaudhuri, J., Si, K., and Maitra, U. (1997). Function of eukaryotic translation initiation factor 1A (eIF1A) (formerly called eIF-4C) in initiation of protein synthesis. J Biol Chem *272*, 7883-7891.

Chen, C. Y., Gherzi, R., Ong, S. E., Chan, E. L., Raijmakers, R., Pruijn, G. J., Stoecklin, G., Moroni, C., Mann, M., and Karin, M. (2001). AU binding proteins recruit the exosome to degrade ARE-containing mRNAs. Cell *107*, 451-464.

Chen, J. J., Throop, M. S., Gehrke, L., Kuo, I., Pal, J. K., Brodsky, M., and London, I. M. (1991). Cloning of the cDNA of the heme-regulated eukaryotic initiation factor 2 alpha (eIF-2 alpha) kinase of rabbit reticulocytes: homology to yeast GCN2 protein kinase and human double-stranded-RNA-dependent eIF-2 alpha kinase. Proc Natl Acad Sci USA *88*, 7729-7733.

Cheng, J., Belgrader, P., Zhou, X., and Maquat, L. E. (1994). Introns are cis effectors of the nonsense-codon-mediated reduction in nuclear mRNA abundance. Mol Cell Biol *14*, 6317-6325.

Chernoff, Y. O., Newnam, G. P., and Liebman, S. W. (1996). The translational function of nucleotide C1054 in the small subunit rRNA is conserved throughout evolution: genetic evidence in yeast. Proc Natl Acad Sci USA *93*, 2517-2522.

Chernoff, Y. O., Vincent, A., and Liebman, S. W. (1994). Mutations in eukaryotic 18S ribosomal RNA affect translational fidelity and resistance to aminoglycoside antibiotics. EMBO J *13*, 906-913.

Child, S. J., Miller, M. K., and Geballe, A. P. (1999a). Cell type-dependent and -independent control of HER-2/neu translation. Int J Biochem Cell Biol *31*, 201-213.

Child, S. J., Miller, M. K., and Geballe, A. P. (1999b). Translational control by an upstream open reading frame in the HER-2/*neu* transcript. J Biol Chem *274*, 24335-24341.

Christensen, A. K., Kahn, L. E., and Bourne, C. M. (1987). Circular polysomes predominate on the rough endoplasmic reticulum of somatotropes and mammotropes in the rat anterior pituitary. Am J Anat *178*, 1-10.

Cigan, A. M., and Donahue, T. F. (1986). The methionine initiator tRNA genes of yeast. Gene *41*, 343-348.

Cigan, A. M., Pabich, E. K., Feng, L., and Donahue, T. F. (1989). Yeast translation initiation suppressor *sui2* encodes the alpha subunit of eukaryotic initiation factor 2 and shares sequence identity with the human alpha subunit. Proc Natl Acad Sci USA *86*, 2784-2788.

Coller, J., and Parker, R. (2004). Eukaryotic mRNA decapping. Annu Rev Biochem *73*, 861-890.

Crabeel, M., LaValle, R., and Glansdorff, N. (1990). Arginine-specific repression in *Saccharomyces cerevisiae*: kinetic data on *ARG1* and *ARG3* mRNA transcription and stability support a transcriptional control mechanism. Mol Cell Biol *10*, 1226-1233.

Cui, Y., Dinman, J. D., Kinzy, T. G., and Peltz, S. W. (1998). The Mof2/Sui1 protein is a general monitor of translational accuracy. Mol Cell Biol *18*, 1506-1516.
Cui, Y., Dinman, J. D., and Peltz, S. W. (1996). *Mof4-1* is an allele of the *UPF1/IFS2* gene which affects both mRNA turnover and -1 ribosomal frameshifting efficiency. EMBO J *15*, 5726-5736.

Cui, Y., Gonzalez, C. I., Kinzy, T. G., Dinman, J. D., and Peltz, S. W. (1999). Mutations in the *MOF2/SUI1* gene affect both translation and nonsense-mediated mRNA decay. RNA *5*, 794-804.

Cui, Y., Hagan, K. W., Zhang, S., and Peltz, S. W. (1995). Identification and characterization of genes that are required for the accelerated degradation of mRNAs containing a premature translational termination codon. Genes Dev *9*, 423-436.

Czaplinski, K., Ruiz-Echevarria, M. J., Paushkin, S. V., Han, X., Weng, Y., Perlick, H. A., Dietz, H. C., Ter-Avanesyan, M. D., and Peltz, S. W. (1998). The surveillance complex interacts with the translation release factors to enhance termination and degrade aberrant mRNAs. Genes Dev *12*, 1665-1677.

Czaplinski, K., Weng, Y., Hagan, K. W., and Peltz, S. W. (1995). Purification and characterization of the Upf1 protein: a factor involved in translation and mRNA degradation. RNA *1*, 610-623.

Dahlseid, J. N., Puziss, J., Shirley, R. L., Atkin, A. L., Hieter, P., and Culbertson, M. R. (1998). Accumulation of mRNA coding for the ctf13p kinetochore subunit of *Saccharomyces cerevisiae* depends on the same factors that promote rapid decay of nonsense mRNAs. Genetics *150*, 1019-1035.

Das, S., Maiti, T., Das, K., and Maitra, U. (1997). Specific interaction of eukaryotic translation initiation factor 5 (eIF5) with the beta-subunit of eIF2. J Biol Chem *272*, 31712-31718.

Dasmahapatra, B., and Chakraburtty, K. (1981). Protein synthesis in yeast. I. Purification and properties of elongation factor 3 from Saccharomyces cerevisiae. J Biol Chem *256*, 9999-10004.

Davis, R. H. (1986). Compartmental and regulatory mechanisms in the arginine pathways of *Neurospora crassa* and *Saccharomyces cerevisiae*. Microbiol Rev *50*, 280-313.
Davis, R. H. (2000). Neurospora: contributions of a model organism (Oxford, Great Britain: Oxford University Press).

Davuluri, R. V., Suzuki, Y., Sugano, S., and Zhang, M. Q. (2000). CART classification of human 5' UTR sequences. Genome Res *10*, 1807-1816.

De Pietri Tonelli, D., Mihailovich, M., Di Cesare, A., Codazzi, F., Grohovaz, F., and Zacchetti, D. (2004). Translational regulation of BACE-1 expression in neuronal and non-neuronal cells. Nucleic Acids Res *32*, 1808-1817.

de The, H., Marchio, A., Tiollais, P., and Dejean, A. (1987). A novel steroid thyroid hormone receptor-related gene inappropriately expressed in human hepatocellular carcinoma. Nature *330*, 667-670.

Decker, C. J., and Parker, R. (1993). A turnover pathway for both stable and unstable mRNAs in yeast: evidence for a requirement for deadenylation. Genes Dev *7*, 1632-1643.

Degnin, C. R., Schleiss, M. R., Cao, J., and Geballe, A. P. (1993). Translational inhibition mediated by a short upstream open reading frame in the human cytomegalovirus gpUL4 (gp48) transcript. J Virol *67*, 5514-5521.

Delbecq, P., Calvo, O., Filipkowski, R. K., Pierard, A., and Messenguy, F. (2000). Functional analysis of the leader peptide of the yeast gene *CPA1* and heterologous regulation by other fungal peptides. Curr Genet *38*, 105-112.

Delbecq, P., Werner, M., Feller, A., Filipkowski, R. K., Messenguy, F., and Piérard, A. (1994). A segment of mRNA encoding the leader peptide of the *CPA1* gene confers repression by arginine on a heterologous yeast gene transcript. Mol Cell Biol *14*, 2378-2390.

Dever, T. E., Feng, L., Wek, R. C., Cigan, A. M., Donahue, T. F., and Hinnebusch, A. G. (1992). Phosphorylation of initiation factor 2alpha by protein kinase GCN2 mediates gene-specific translational control of *GCN4* in yeast. Cell *68*, 585-596.

Diba, F., Watson, C. S., and Gametchu, B. (2001). 5'UTR sequences of the glucocorticoid receptor 1A transcript encode a peptide associated with translational regulation of the glucocorticoid receptor. J Cell Biochem *81*, 149-161.

Donahue, T. F., Cigan, A. M., Pabich, E. K., and Valavicius, B. C. (1988). Mutations at a Zn(II) finger motif in the yeast eIF-2beta gene alter ribosomal start-site selection during the scanning process. Cell *54*, 621-632.

Dontsova, M., Frolova, L., Vassilieva, J., Piendl, W., Kisselev, L., and Garber, M. (2000). Translation termination factor aRF1 from the archaeon Methanococcus jannaschii is active with eukaryotic ribosomes. FEBS Lett *472*, 213-216.

Donzé, O., and Spahr, P. F. (1992). Role of the open reading frames of Rous sarcoma virus leader RNA in translation and genome packaging. EMBO J *11*, 3747-3757.

Dorris, D. R., Erickson, F. L., and Hannig, E. M. (1995). Mutations in GCD11, the structural gene for eIF-2 gamma in yeast, alter translational regulation of GCN4 and the selection of the start site for protein synthesis. EMBO J *14*, 2239-2249.

Dunckley, T., and Parker, R. (1999). The DCP2 protein is required for mRNA decapping in *Saccharomyces cerevisiae* and contains a functional MutT motif. EMBO J *18*, 5411-5422.

Ebbole, D. J., Paluh, J. L., Plamann, M., Sachs, M. S., and Yanofsky, C. (1991). *cpc-1*, the general regulatory gene for genes of amino acid biosynthesis in *Neurospora crassa*, is differentially expressed during the asexual life cycle. Mol Cell Biol *11*, 928-934.

Eiznhamer, D. A., Ashburner, B. P., Jackson, J. C., Gardenour, K. R., and Lopes, J. M. (2001). Expression of the *INO2* regulatory gene of *Saccharomyces cerevisiae* is controlled by positive and negative promoter elements and an upstream open reading frame. Mol Microbiol *39*, 1395-1405.

Endemann, G., Stanton, L. W., Madden, K. S., Bryant, C. M., White, R. T., and Protter, A. A. (1993). CD36 is a receptor for oxidized low density lipoprotein. J Biol Chem *268*, 11811-11816.

Erickson, F. L., and Hannig, E. M. (1996). Ligand interactions with eukaryotic translation initiation factor 2: role of the gamma-subunit. EMBO J *15*, 6311-6320.

Etchison, D., Milburn, S. C., Edery, I., Sonenberg, N., and Hershey, J. W. (1982). Inhibition of HeLa cell protein synthesis following poliovirus infection correlates with the proteolysis of a 220,000-dalton polypeptide associated with eucaryotic initiation factor 3 and a cap binding protein complex. J Biol Chem *257*, 14806-14810.

Eurwilaichitr, L., Graves, F. M., Stansfield, I., and Tuite, M. F. (1999). The C-terminus of eRF1 defines a functionally important domain for translation termination in Saccharomyces cerevisiae. Mol Microbiol *32*, 485-496.

Fajardo, J. E., and Shatkin, A. J. (1990). Translation of bicistronic viral mRNA in transfected cells: regulation at the level of elongation. Proc Natl Acad Sci USA *87*, 328-332.

Fang, P., Spevak, C. C., Wu, C., and Sachs, M. S. (2004). A nascent polypeptide domain that can regulate translation elongation. Proc Natl Acad Sci USA *101*, 4059-4064.

Fang, P., Wang, Z., and Sachs, M. S. (2000). Evolutionarily conserved features of the arginine attenuator peptide provide the necessary requirements for its function in translational regulation. J Biol Chem *275*, 26710-26719.

Fiaschi, T., Marzocchini, R., Raugei, G., Veggi, D., Chiarugi, P., and Ramponi, G. (1997). The 5'-untranslated region of the human muscle acylphosphatase mRNA has an inhibitory effect on protein expression. FEBS Lett *417*, 130-134.

Fletcher, C. M., Pestova, T. V., Hellen, C. U., and Wagner, G. (1999). Structure and interactions of the translation initiation factor eIF1. EMBO J *18*, 2631-2637.

Fletcher, S. P., and Jackson, R. J. (2002). Pestivirus internal ribosome entry site (IRES) structure and function: elements in the 5' untranslated region important for IRES function. J Virol *76*, 5024-5033.

Franceschetti, M., Hanfrey, C., Scaramagli, S., Torrigiani, P., Bagni, N., Burtin, D., and Michael, A. J. (2001). Characterization of monocot and dicot plant S-adenosyl-l-methionine decarboxylase gene families including identification in the mRNA of a highly conserved pair of upstream overlapping open reading frames. Biochem J *353*, 403-409.

Freistroffer, D. V., Pavlov, M. Y., MacDougall, J., Buckingham, R. H., and Ehrenberg, M. (1997). Release factor RF3 in *E. coli* accelerates the dissociation of release factors RF1 and RF2 from the ribosome in a GTP-dependent manner. EMBO J *16*, 4126-4133.

Freitag, M., Dighde, N., and Sachs, M. S. (1996). A UV-induced mutation that affects translational regulation of the *Neurospora arg-2* gene. Genetics *142*, 117-127.

Fried, H. M., and Warner, J. R. (1982). Molecular cloning and analysis of yeast gene for cycloheximide resistance and ribosomal protein L29. Nucleic Acids Res *10*, 3133-3148.

Frischmeyer, P. A., van Hoof, A., O'Donnell, K., Guerrerio, A. L., Parker, R., and Dietz, H. C. (2002). An mRNA surveillance mechanism that eliminates transcripts lacking termination codons. Science *295*, 2258-2261.

Frolova, L., Le-Goff, X., Rasmussen, H. H., Cheperegin, S., Drugeon, G., Kress, M., Arman, I., Haenni, A. L., Celis, J. E., Philippe, M., and et, a. (1994). A highly conserved eukaryotic protein family possessing properties of polypeptide chain release factor [see comments]. Nature *372*, 701-703.

Frolova, L., Le-Goff, X., Zhouravleva, G., Davydova, E., Philippe, M., and Kisselev, L. (1996). Eukaryotic polypeptide chain release factor eRF3 is an eRF1- and ribosome-dependent guanosine triphosphatase. RNA *2*, 334-341.

Fütterer, J., and Hohn, T. (1992). Role of an upstream open reading frame in the translation of polycistronic mRNAs in plant cells. Nucl Acids Res *20*, 3851-3857.

Gaba, A., Wang, Z., Krishnamoorthy, T., Hinnebusch, A. G., and Sachs, M. S. (2001). Physical evidence for distinct mechanisms of translational control by upstream open reading frames. EMBO J *20*, 6453-6463.

Gangloff, J., Schutz, A., and Dirheimer, G. (1976). Arginyl-tRNA synthetase from baker's yeast. Purification and some properties. Eur J Biochem *65*, 177-182.

Gao, Q., Das, B., Sherman, F., and Maquat, L. E. (2005). Cap-binding protein 1-mediated and eukaryotic translation initiation factor 4E-mediated pioneer rounds of translation in yeast. Proc Natl Acad Sci USA *102*, 4258-4263.

Garlapati, S., and Wang, C. C. (2002). Identification of an essential pseudoknot in the putative downstream internal ribosome entry site in giardiavirus transcript. RNA *8*, 601-611.

Gaspar, N. J., Kinzy, T. G., Scherer, B. J., Humbelin, M., Hershey, J. W., and Merrick, W. C. (1994). Translation initiation factor eIF-2. Cloning and expression of the human cDNA encoding the gamma-subunit. J Biol Chem *269*, 3415-3422.

Gatfield, D., and Izaurralde, E. (2004). Nonsense-mediated messenger RNA decay is initiated by endonucleolytic cleavage in *Drosophila*. Nature *429*, 575-578.

Gatfield, D., Unterholzner, L., Ciccarelli, F. D., Bork, P., and Izaurralde, E. (2003). Nonsense-mediated mRNA decay in *Drosophila*: at the intersection of the yeast and mammalian pathways. EMBO J *22*, 3960-3970.

Geballe, A. P. (1996). Translational control mediated by upstream AUG codons, In Translational control, J. W. B. Hershey, M. B. Mathews, and N. Sonenberg, eds. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press), pp. 173-197.

Geballe, A. P., and Sachs, M. S. (2000). Translational control by upstream open reading frames, In Translational control of gene expression, N. Sonenberg, J. W. B. Hershey, and M. B. Mathews, eds. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press), pp. 595-614.

Geiger, A., Burgstaller, P., von der Eltz, H., Roeder, A., and Famulok, M. (1996). RNA aptamers that bind L-arginine with sub-micromolar dissociation constants and high enantioselectivity. Nucl Acids Res *24*, 1029-1036.

Ghosh, H. P., Ghosh, K., Simsek, M., and RajBhandary, U. L. (1982). Nucleotide sequence of wheat germ cytoplasmic initiator methionine transfer ribonucleic acid. Nucleic Acids Res *10*, 3241-3247.

Gietz, R. D., and Sugino, A. (1988). New yeast-*Escherichia coli* shuttle vectors constructed with *in vitro* mutagenized yeast genes lacking six-base pair restriction sites. Gene *74*, 527-534.

Gillum, A. M., Hecker, L. I., Silberklang, M., Schwartzbach, S. D., RajBhandary, U. L., and Barnett, W. E. (1977). Nucleotide sequence of *Neurospora crassa* cytoplasmic initiator tRNA. Nucleic Acids Res *4*, 4109-4131.

Gingras, A.-C., Raught, B., and Sonenberg, N. (1999). eIF4 initiation factors: effectors of mRNA recruitment to ribosomes and regulators of translation. Annu Rev Biochem *68*, 913-963.

Gingras, A. C., Svitkin, Y., Belsham, G. J., Pause, A., and Sonenberg, N. (1996). Activation of the translational suppressor 4E-BP1 following infection with encephalomyocarditis virus and poliovirus. Proc Natl Acad Sci USA *93*, 5578-5583.

Gong, F., and Yanofsky, Y. (2001). Reproducing *tna* operon regulation *in vitro* in a S-30 system: Tryptophan induction inhibits cleavage of TnaC peptidyl-tRNA. J Biol Chem *276*, 1974-1983.

Gonsky, R., Itamar, D., Harary, R., and Kaempfer, R. (1992). Binding of ATP and messenger RNA by the beta-subunit of eukaryotic initiation factor 2. Biochimie *74*, 427-434.

Gonzalez, C. I., Ruiz-Echevarria, M. J., Vasudevan, S., Henry, M. F., and Peltz, S. W. (2000). The yeast hnRNP-like protein Hrp1/Nab4 marks a transcript for nonsense-mediated mRNA decay. Mol Cell *5*, 489-499.

Goumans, H., Thomas, A., Verhoeven, A., Voorma, H. O., and Benne, R. (1980). The role of eIF-4C in protein synthesis initiation complex formation. Biochim Biophys Acta *608*, 39-46.

Gozalbo, D., and Hohmann, S. (1990). Nonsense suppressors partially revert the decrease of the mRNA level of a nonsense mutant allele in yeast. Curr Genet *17*, 77-79.

Grant, C. M., and Hinnebusch, A. G. (1994). Effect of sequence context at stop codons on efficiency of reinitiation in *GCN4* translational control. Mol Cell Biol *14*, 606-618.

Grant, C. M., Miller, P. F., and Hinnebusch, A. G. (1994). Requirements for intercistronic distance and level of eukaryotic initiation factor 2 activity in reinitiation on *GCN4* mRNA vary with the downstream cistron. Mol Cell Biol *14*, 2616-2628.

Grass, D. S., and Manley, J. L. (1987). Selective translation initiation on bicistronic simian virus 40 late mRNA. J Virol *61*, 2331-2335.

Green, R., and Noller, H. F. (1997). Ribosomes and translation. Annu Rev Biochem *66*, 679-716.

Grentzmann, G., Brechemier-Baey, D., Heurgue, V., Mora, L., and Buckingham, R. H. (1994). Localization and characterization of the gene encoding release factor RF3 in *Escherichia coli*. Proc Natl Acad Sci USA *91*, 5848-5852.

Griffin, E., Re, A., Hamel, N., Fu, C., Bush, H., McCaffrey, T., and Asch, A. S. (2001). A link between diabetes and atherosclerosis: glucose regulates expression of CD36 at the level of translation. Nat Med *7*, 840-846.

Haeuptle, M. T., Frank, R., and Dobberstein, B. (1986). Translation arrest by oligodeoxynucleotides complementary to mRNA coding sequences yields polypeptides of predetermined length. Nucleic Acids Res *14*, 1427-1448.

Hagan, K. W., Ruiz-Echevarria, M. J., Quan, Y., and Peltz, S. W. (1995). Characterization of *cis*-acting sequences and decay intermediates involved in nonsense-mediated mRNA turnover. Mol Cell Biol *15*, 809-823.

Haghighat, A., Mader, S., Pause, A., and Sonenberg, N. (1995). Repression of cap-dependent translation by 4E-binding protein 1: competition with p220 for binding to eukaryotic initiation factor-4E. Embo J *14*, 5701-5709.

Han, S., Navarro, J., Greve, R. A., and Adams, T. H. (1993). Translational repression of *brlA* expression prevents premature development in *Aspergillus*. EMBO J *12*, 2449-2457.

Hanfrey, C., Franceschetti, M., Mayer, M. J., Illingworth, C., and Michael, A. J. (2002). Abrogation of upstream open reading frame-mediated translational control of a plant S-adenosylmethionine decarboxylase results in polyamine disruption and growth perturbations. J Biol Chem *277*, 44131-44139.

Harashima, S., and Hinnebusch, A. G. (1986). Multiple *GCD* genes required for repression of *GCN4*, a transcriptional activator of amino acid biosynthetic genes in *Saccharomyces cerevisiae*. Mol Cell Biol *6*, 3990-3998.

Harding, H. P., Novoa, I., Zhang, Y., Zeng, H., Wek, R., Schapira, M., and Ron, D. (2000). Regulated translation initiation controls stress-induced gene expression in mammalian cells. Mol Cell *6*, 1099-1108.

Harding, H. P., Zhang, Y., and Ron, D. (1999). Protein translation and folding are coupled by an endoplasmic-reticulum- resident kinase [published erratum appears in Nature 1999 Mar 4;398(6722):90]. Nature *397*, 271-274.

Harigai, M., Miyashita, T., Hanada, M., and Reed, J. C. (1996). A cis-acting element in the *BCL-2* gene controls expression through translational mechanisms. Oncogene *12*, 1369-1374.

Hashimoto, N. N., Carnevalli, L. S., and Castilho, B. A. (2002). Translation initiation at non-AUG codons mediated by weakened association of eukaryotic initiation factor (eIF) 2 subunits. Biochem J *367*, 359-368.

Hatfield, L., Beelman, C. A., Stevens, A., and Parker, R. (1996). Mutations in *trans*-acting factors affecting mRNA decapping in *Saccharomyces cerevisiae*. Mol Cell Biol *16*, 5830-5838.

He, F., Brown, A. H., and Jacobson, A. (1996). Interaction between Nmd2p and Upf1p is required for activity but not for dominant-negative inhibition of the nonsense-mediated mRNA decay pathway in yeast. RNA *2*, 153-170.

He, F., Brown, A. H., and Jacobson, A. (1997). Upf1p, Nmd2p, and Upf3p are interacting components of the yeast nonsense-mediated mRNA decay pathway. Mol Cell Biol *17*, 1580-1594.

He, F., and Jacobson, A. (1995). Identification of a novel component of the nonsense-mediated mRNA decay pathway by use of an interacting protein screen. Genes Dev *9*, 437-454.

He, F., and Jacobson, A. (2001). Upf1p, Nmd2p, and Upf3p regulate the decapping and exonucleolytic degradation of both nonsense-containing mRNAs and wild-type mRNAs. Mol Cell Biol *21*, 1515-1530.

He, F., Li, X., Spatrick, P., Casillo, R., Dong, S., and Jacobson, A. (2003). Genome-wide analysis of mRNAs regulated by the nonsense-mediated and 5' to 3' mRNA decay pathways in yeast. Mol Cell *12*, 1439-1452.

He, F., Peltz, S. W., Donahue, J. L., Rosbash, M., and Jacobson, A. (1993). Stabilization and ribosome association of unspliced pre-mRNAs in a yeast *upf1*- mutant. Proc Natl Acad Sci USA *90*, 7034-7038.

Herrick, D., Parker, R., and Jacobson, A. (1990). Identification and comparison of stable and unstable mRNAs in *Saccharomyces cerevisiae*. Mol Cell Biol *10*, 2269-2284.

Hershey, J. W. B. (1991). Translational control in mammalian cells. Ann Rev Biochem *60*, 717-755.

Hershey, J. W. B., and Merrick, W. C. (2000). The Pathway and Mechanism of Initiation of Protein Synthesis, In Translational control of gene expression, N. Sonenberg, J. W. B. Hershey, and M. B. Mathews, eds. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press), pp. 33-88.

Hill, J. R., and Morris, D. R. (1992). Cell-specific translation of S-adenosylmethionine decarboxylase mRNA. Regulation by the 5' transcript leader. J Biol Chem *267*, 21886-21893.

Hill, J. R., and Morris, D. R. (1993). Cell-specific translational regulation of S-adenosylmethionine decarboxylase mRNA: dependence on translation and coding capacity of the *cis*-acting upstream open reading frame. J Biol Chem *269*, 726-731.

Hilleren, P., and Parker, R. (1999). mRNA surveillance in eukaryotes: kinetic proofreading of proper translation termination as assessed by mRNP domain organization? RNA *5*, 711-719.

Hinnebusch, A. G. (1996). Translational control of *GCN4*: gene-specific regulation by phosphorylation of eIF2, In Translational control, J. W. B. Hershey, M. B. Mathews, and N. Sonenberg, eds. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press), pp. 199-244.

Hinnebusch, A. G. (1997). Translational regulation of yeast *GCN4*: a window on factors that control initiator-tRNA binding to the ribosome. J Biol Chem *272*, 21661-21664.

Hinnebusch, A. G. (2000). Translational control of *GCN4*: gene-specific regulation by phosphorylation of eIF2, In Translational control of gene expression, N. Sonenberg, J. W. B. Hershey, and M. B. Mathews, eds. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press), pp. 185-243.

Holzmann, K., Ambrosch, I., Elbling, L., Micksche, M., and Berger, W. (2001). A small upstream open reading frame causes inhibition of human major vault protein expression from a ubiquitous mRNA splice variant. FEBS Lett *494*, 99-104.

Hoshino, S., Hosoda, N., Araki, Y., Kobayashi, T., Uchida, N., Funakoshi, Y., and Katada, T. (1999). Novel function of the eukaryotic polypeptide-chain releasing factor 3 (eRF3/GSPT) in the mRNA degradation pathway. Biochemistry (Mosc) *64*, 1367-1372.

Hsu, C. L., and Stevens, A. (1993). Yeast cells lacking 5'-->3' exoribonuclease 1 contain mRNA species that are poly(A) deficient and partially lack the 5' cap structure. Mol Cell Biol *13*, 4826-4835.

Huang, H. K., Yoon, H., Hannig, E. M., and Donahue, T. F. (1997). GTP hydrolysis controls stringent selection of the AUG start codon during translation initiation in *Saccharomyces cerevisiae*. Genes Dev *11*, 2396-2413.

Imataka, H., Nakayama, K., Yasumoto, K., Mizuno, A., Fujii-Kuriyama, Y., and Hayami, M. (1994). Cell-specific translational control of transcription factor BTEB expression. The role of an upstream AUG in the 5'-untranslated region. J Biol Chem *269*, 20668-20673.

Ishigaki, Y., Li, X., Serin, G., and Maquat, L. E. (2001). Evidence for a pioneer round of mRNA translation: mRNAs subject to nonsense-mediated decay in mammalian cells are bound by CBP80 and CBP20. Cell *106*, 607-617.

Jackson, R. J., and Wickens, M. (1997). Translational controls impinging on the 5'-untranslated region and initiation factor proteins. Curr Opin Genet Dev *7*, 233-241.
Jacobson, A., and Peltz, S. W. (2000). Destabilization of nonsense-containing transcripts in *Saccharomyces cerevisiae*, In Translational control of gene expression, N. Sonenberg,

J. W. B. Hershey, and M. B. Mathews, eds. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press), pp. 827-847.

Jin, X., Turcott, E., Englehardt, S., Mize, G. J., and Morris, D. R. (2003). The two upstream open reading frames of oncogene mdm2 have different translational regulatory properties. J Biol Chem *278*, 25716-25721.

Jousse, C., Bruhat, A., Carraro, V., Urano, F., Ferrara, M., Ron, D., and Fafournoux, P. (2001). Inhibition of CHOP translation by a peptide encoded by an open reading frame localized in the chop 5'UTR. Nucleic Acids Res *29*, 4341-4351.

Kadlec, J., Izaurralde, E., and Cusack, S. (2004). The structural basis for the interaction between nonsense-mediated mRNA decay factors UPF2 and UPF3. Nat Struct Mol Biol *11*, 330-337.

Kawai, S., Murao, S., Mochizuki, M., Shibuya, I., Yano, K., and Takagi, M. (1992). Drastic alteration of cycloheximide sensitivity by substitution of one amino acid in the L41 ribosomal protein of yeasts. J Bacteriol *174*, 254-262.

Keeling, K. M., Lanier, J., Du, M., Salas-Marco, J., Gao, L., Kaenjak-Angeletti, A., and Bedwell, D. M. (2004). Leaky termination at premature stop codons antagonizes nonsense-mediated mRNA decay in *S. cerevisiae*. RNA *10*, 691-703.

Keenan, K. A., and Weiss, R. L. (1997). Characterization of vacuolar arginine uptake and amino acid efflux in *Neurospora crassa* using cupric ion to permeabilize the plasma membrane. Fungal Genet Biol *22*, 177-190.

Keith, G., and Dirheimer, G. (1980). Reinvestigation of the primary structure of brewer's yeast tRNA 3 Arg. Biochem Biophys Res Commun *92*, 116-119.

Kessler, S. H., and Sachs, A. B. (1998). RNA recognition motif 2 of yeast Pab1p is required for its functional interaction with eukaryotic translation initiation factor 4G. Mol Cell Biol *18*, 51-57.

Kim, V. N., Kataoka, N., and Dreyfuss, G. (2001). Role of the nonsense-mediated decay factor hUpf3 in the splicing-dependent exon-exon junction complex. Science *293*, 1832-1836.

Kimball, S. R. (1999). Eukaryotic initiation factor eIF2. Int J Biochem Cell Biol *31*, 25-29.

Kimball, S. R., and L.S., J. (2000). Regulation of translational initiation in mammalian cells by amino acids, In Translational control of gene expression, N. Sonenberg, J. W. B. Hershey, and M. B. Mathews, eds. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press), pp. 561-579.

Kinney, D. M., and Lusty, C. J. (1989). Arginine restriction induced by d-N-(phosphonacetyl)-L-ornithine signals increased expression of *HIS3*, *TRP5*, *CPA1*, and *CPA2* in *Saccharomyces cerevisiae*. Mol Cell Biol *9*, 4882-4888.

Kitamoto, K., Yoshizawa, K., Ohsumi, Y., and Anraku, Y. (1988). Dynamic aspects of vacuolar and cytosolic amino acid pools of *Saccharomyces cerevisiae*. J Bacteriol *170*, 2683-2686.

Komar, A. A., and Hatzoglou, M. (2005). Internal ribosome entry sites in cellular mRNAs: The mystery of their existence. J Biol Chem, In press.

Konan, K. V., and Yanofsky, C. (1999). Role of ribosome release in regulation of *tna* operon expression in *Escherichia coli*. J Bacteriol *181*, 1530-1536.

Konecki, D. S., Aune, K. C., Tate, W., and Caskey, C. T. (1977). Characterization of reticulocyte release factor. J Biol Chem *252*, 4514-4520.

Koonin, E. V. (1992). A new group of putative RNA helicases. Trends Biochem Sci *17*, 495-497.

Kos, M., Denger, S., Reid, G., and Gannon, F. (2002). Upstream open reading frames regulate the translation of the multiple mRNA variants of the estrogen receptor alpha. J Biol Chem *277*, 37131-37138.

Kostura, M., and Mathews, M. B. (1989). Purification and activation of the double-stranded RNA-dependent eIF-2 kinase DAI. Mol Cell Biol *9*, 1576-1586.

Kovalchuke, O., Kambampati, R., Pladies, E., and Chakraburtty, K. (1998). Competition and cooperation amongst yeast elongation factors. Eur J Biochem *258*, 986-993.

Kozak, M. (1980). Role of ATP in binding and migration of 40S ribosomal subunits. Cell *22*, 459-467.

Kozak, M. (1987). An analysis of 5'-noncoding sequences from 699 vertebrate messenger RNAs. Nucl Acids Res *15*, 8125-8148.

Kozak, M. (1989). The scanning model for translation: an update. J Cell Biol *108*, 229-241.

Kozak, M. (1991a). An analysis of vertebrate mRNA sequences: intimations of translational control. J Cell Biol *115*, 887-903.

Kozak, M. (1991b). A short leader sequence impairs the fidelity of initiation by eukaryotic ribosomes. Gene Expr *1*, 111-115.

Kozak, M. (1996). Interpreting cDNA sequences: some insights from studies on translation. Mamm Genome *7*, 563-574.

Kozak, M. (1998). Primer extension analysis of eukaryotic ribosome-mRNA complexes. Nucleic Acids Res *26*, 4853-4859.

Kozak, M. (1999). Initiation of translation in prokaryotes and eukaryotes. Gene *234*, 187-208.

Kozak, M. (2002). Pushing the limits of the scanning mechanism for initiation of translation. Gene *299*, 1-34.

Kwak, S. H., and Lee, S. H. (2001). The regulation of ornithine decarboxylase gene expression by sucrose and small upstream open reading frame in tomato (*Lycopersicon esculentum* Mill). Plant Cell Physiol *42*, 314-323.

Kwon, H. S., Lee, D. K., Lee, J. J., Edenberg, H. J., Ahn, Y. H., and Hur, M. W. (2001). Posttranscriptional regulation of human ADH5/FDH and Myf6 gene expression by upstream AUG codons. Arch Biochem Biophys *386*, 163-171.

Kyrpides, N. C., and Woese, C. R. (1998). Universally conserved translation initiation factors. Proceedings of the National Academy of Sciences of the United States of America *95*, 224-228.

Lamphear, B. J., Kirchweger, R., Skern, T., and Rhoads, R. E. (1995). Mapping of functional domains in eukaryotic protein synthesis initiation factor 4G (eIF4G) with picornaviral proteases. Implications for cap-dependent and cap-independent translational initiation. J Biol Chem *270*, 21975-21983.

Landers, J. E., Cassel, S. L., and George, D. L. (1997). Translational enhancement of *mdm2* oncogene expression in human tumor cells containing a stabilized wild-type p53 protein. Cancer Res *57*, 3562-3568.

Landers, J. E., Haines, D. S., Strauss, J. F. r., and George, D. L. (1994). Enhanced translation: a novel mechanism of *mdm2* oncogene overexpression identified in human tumor cells. Oncogene *9*, 2745-2750.

Landick, R., Turnbough, C. L. J., and Yanofsky, C. (1996). Transcription attenuation, In *Escherichia coli* and *Salmonella.* Cellular and Molecular Biology, F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. Brooks Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger, eds. (Washington, D.C.: ASM Press), pp. 1263-1286.

Laurino, J. P., Thompson, G. M., Pacheco, E., and Castilho, B. A. (1999). The beta subunit of eukaryotic translation initiation factor 2 binds mRNA through the lysine repeats and a region comprising the C2-C2 motif. Mol Cell Biol *19*, 173-181.

Law, G. L., Raney, A., Heusner, C., and Morris, D. R. (2001). Polyamine regulation of ribosome pausing at the upstream open reading frame of S-adenosylmethionine decarboxylase. J Biol Chem *276*, 38036-38043.

Le Hir, H., Gatfield, D., Izaurralde, E., and Moore, M. J. (2001). The exon-exon junction complex provides a binding platform for factors involved in mRNA export and nonsense-mediated mRNA decay. EMBO J *20*, 4987-4997.

Le Hir, H., Izaurralde, E., Maquat, L. E., and Moore, M. J. (2000). The spliceosome deposits multiple proteins 20-24 nucleotides upstream of mRNA exon-exon junctions. EMBO J *19*, 6860-6869.

LeBlanc, J. J., and Beemon, K. L. (2004). Unspliced Rous sarcoma virus genomic RNAs are translated and subjected to nonsense-mediated mRNA decay before packaging. J Virol *78*, 5139-5146.

Lee, B. S., and Culbertson, M. R. (1995). Identification of an additional gene required for eukaryotic nonsense mRNA turnover. Proc Natl Acad Sci USA *92*, 10354-10358.

Lee, J., Park, E. H., Couture, G., Harvey, I., Garneau, P., and Pelletier, J. (2002a). An upstream open reading frame impedes translation of the huntingtin gene. Nucleic Acids Res *30*, 5110-5119.

Lee, J. H., Pestova, T. V., Shin, B. S., Cao, C., Choi, S. K., and Dever, T. E. (2002b). Initiation factor eIF5B catalyzes second GTP-dependent step in eukaryotic translation initiation. Proc Natl Acad Sci USA *99*, 16689-16694.

Lee, M. H., and Schedl, T. (2004). Translation repression by GLD-1 protects its mRNA targets from nonsense-mediated mRNA decay in C. elegans. Genes Dev *18*, 1047-1059.

Leeds, P., Peltz, S. W., Jacobson, A., and Culbertson, M. R. (1991). The product of the yeast *UPF1* gene is required for rapid turnover of mRNAs containing a premature translational termination codon. Genes Dev *5*, 2303-2314.

Leeds, P., Wood, J. M., Lee, B. S., and Culbertson, M. R. (1992). Gene products that promote mRNA turnover in *Saccharomyces cerevisiae*. Mol Cell Biol *12*, 2165-2177.

Lejeune, F., Ishigaki, Y., Li, X., and Maquat, L. E. (2002). The exon junction complex is detected on CBP80-bound but not eIF4E-bound mRNA in mammalian cells: dynamics of mRNP remodeling. EMBO J *21*, 3536-3545.

Lejeune, F., Li, X., and Maquat, L. E. (2003). Nonsense-mediated mRNA decay in mammalian cells involves decapping, deadenylating, and exonucleolytic activities. Mol Cell *12*, 675-687.

Lew, J. E., Enomoto, S., and Berman, J. (1998). Telomere length regulation and telomeric chromatin require the nonsense-mediated mRNA decay pathway. Mol Cell Biol *18*, 6121-6130.

Lincoln, A. J., Monczak, Y., Williams, S. C., and Johnson, P. F. (1998). Inhibition of CCAAT/enhancer-binding protein alpha and beta translation by upstream open reading frames. J Biol Chem *273*, 9552-9560.

Linz, B., Koloteva, N., Vasilescu, S., and McCarthy, J. E. (1997). Disruption of ribosomal scanning on the 5'-untranslated region, and not restriction of translational initiation per se, modulates the stability of nonaberrant mRNAs in the yeast Saccharomyces cerevisiae. J Biol Chem *272*, 9131-9140.

Liu, R., and Liebman, S. W. (1996). A translational fidelity mutation in the universally conserved sarcin/ricin domain of 25S yeast ribosomal RNA. RNA *2*, 254-263.

Lohmer, S., Maddaloni, M., Motto, M., Salamini, F., and Thompson, R. D. (1993). Translation of the mRNA of the maize transcriptional activator *Opaque-2* is inhibited by upstream open reading frames present in the leader sequence. Plant Cell *5*, 65-73.

Losson, R., and Lacroute, F. (1979). Interference of nonsense mutations with eukaryotic messenger RNA stability. Proc Natl Acad Sci USA *76*, 5134-5137.

Lovett, P. S., and Rogers, E. J. (1996). Ribosome regulation by the nascent peptide. Microbiol Rev *60*, 366-385.

Lu, P. D., Harding, H. P., and Ron, D. (2004). Translation reinitiation at alternative open reading frames regulates gene expression in an integrated stress response. J Cell Biol *167*, 27-33.

Lukaszewicz, M., Jerouville, B., and Boutry, M. (1998). Signs of translational regulation within the transcript leader of a plant plasma membrane H(+)-ATPase gene. Plant J *14*, 413-423.

Luo, Z., Freitag, M., and Sachs, M. S. (1995). Translational regulation in response to changes in amino acid availability in *Neurospora crassa*. Mol Cell Biol *15*, 5235-5245.

Luo, Z., and Sachs, M. S. (1996). Role of an upstream open reading frame in mediating arginine-specific translational control in *Neurospora crassa*. J Bacteriol *178*, 2172-2177.

Lykke-Andersen, J., Shu, M. D., and Steitz, J. A. (2000). Human Upf proteins target an mRNA for nonsense-mediated decay when bound downstream of a termination codon. Cell *103*, 1121-1131.

Lykke-Andersen, J., Shu, M. D., and Steitz, J. A. (2001). Communication of the position of exon-exon junctions to the mRNA surveillance machinery by the protein RNPS1. Science *293*, 1836-1839.

Maderazo, A. B., Belk, J. P., He, F., and Jacobson, A. (2003). Nonsense-containing mRNAs that accumulate in the absence of a functional nonsense-mediated mRNA decay pathway are destabilized rapidly upon its restitution. Mol Cell Biol *23*, 842-851.

Maderazo, A. B., He, F., Mangus, D. A., and Jacobson, A. (2000). Upf1p control of nonsense mRNA translation is regulated by Nmd2p and Upf3p. Mol Cell Biol *20*, 4591-4603.

Maglione, D., Guerriero, V., Rambaldi, M., Russo, G., and Persico, M. G. (1993). Translation of the placenta growth factor mRNA is severely affected by a small open reading frame localized in the 5' untranslated region. Growth Factors *8*, 141-152.

Manzella, J. M., and Blackshear, P. J. (1990). Regulation of rat ornithine decarboxylase mRNA translation by its 5'- untranslated region. J Biol Chem *265*, 11817-11822.

Maquat, L. E. (2002). NASty effects on fibrillin pre-mRNA splicing: another case of ESE does it, but proposals for translation-dependent splice site choice live on. Genes Dev *16*, 1743-1753.

Maquat, L. E. (2004). Nonsense-mediated mRNA decay: splicing, translation and mRNP dynamics. Nat Rev Mol Cell Biol *5*, 89-99.

Marth, J. D., Overell, R. W., Meier, K. E., Krebs, E. G., and Perlmutter, R. M. (1988). Translational activation of the *lck* proto-oncogene. Nature *332*, 171-173.

Martinez-Garcia, J. F., Moyano, E., Alcocer, M. J., and Martin, C. (1998). Two bZIP proteins from Antirrhinum flowers preferentially bind a hybrid C-box/G-box motif and help to define a new sub-family of bZIP transcription factors. Plant J *13*, 489-505.

Matthews, M. B., Sonenberg, N., and Hershey, J. W. B. (2000). Origins and principles of translational control, In Translational control of gene expression, N. Sonenberg, J. W. B. Hershey, and M. B. Mathews, eds. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press), pp. 1-31.

McCarthy, J. E. G. (1998). Posttranscriptional control of gene expression in yeast. Microbiol Mol Biol Rev *62*, 1492-1553.

Meijer, H. A., Dictus, W. J., Keuning, E. D., and Thomas, A. A. (2000). Translational control of the *Xenopus laevis* connexin-41 5'-untranslated region by three upstream open reading frames. J Biol Chem *275*, 30787-30307 30793.

Mendell, J. T., Medghalchi, S. M., Lake, R. G., Noensie, E. N., and Dietz, H. C. (2000). Novel Upf2p orthologues suggest a functional link between translation initiation and nonsense surveillance complexes. Mol Cell Biol *20*, 8944-8957.

Mendell, J. T., Sharifi, N. A., Meyers, J. L., Martinez-Murillo, F., and Dietz, H. C. (2004). Nonsense surveillance regulates expression of diverse classes of mammalian transcripts and mutes genomic noise. Nat Genet *36*, 1073-1078.

Merkulova, T. I., Frolova, L. Y., Lazar, M., Camonis, J., and Kisselev, L. L. (1999). C-terminal domains of human translation termination factors eRF1 and eRF3 mediate their *in vivo* interaction. FEBS Lett *443*, 41-47.

Merrick, W. C., and Hershey, J. W. B. (1996). The pathway and mechanism of eukaryotic protein synthesis, In Translational control, J. W. B. Hershey, M. B. Mathews, and N. Sonenberg, eds. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press), pp. 31-69.

Messenguy, F., Feller, A., Crabeel, M., and Piérard, A. (1983). Control mechanisms acting at the transcriptional and post-transcriptional levels are involved in the synthesis of the arginine pathway carbamoyl-phosphate synthetase of yeast. EMBO J *2*, 1249-1254.

Messenguy, F., Vierendeels, F., Piérard, A., and Delbecq, P. (2002). Role of RNA surveillance proteins Upf1/CpaR, Upf2 and Upf3 in the translational regulation of yeast CPA1 gene. Curr Genet *41*, 224-231.

Michelet, B., Lukaszewicz, M., Dupriez, V., and Boutry, M. (1994). A plant plasma membrane proton-ATPase gene is regulated by development and environment and shows signs of a translational regulation. Plant Cell *6*, 1375-1389.

Mignone, F., Gissi, C., Liuni, S., and Pesole, G. (2002). Untranslated regions of mRNAs. Genome Biol *3*, REVIEWS0004.

Mikuni, O., Ito, K., Moffat, J., Matsumura, K., McCaughan, K., Nobukuni, T., Tate, W., and Nakamura, Y. (1994). Identification of the *prfC* gene, which encodes peptide-chain-release factor 3 of *Escherichia coli*. Proc Natl Acad Sci USA *91*, 5798-5802.

Miller, P. F., and Hinnebusch, A. G. (1989). Sequences that surround the stop codons of upstream open reading frames in *GCN4* mRNA determine their distinct functions in translational control. Genes Dev *3*, 1217-1225.

Mitchell, P., and Tollervey, D. (2003). An NMD pathway in yeast involving accelerated deadenylation and exosome-mediated 3'-->5' degradation. Mol Cell *11*, 1405-1413.

Miyasaka, H. (1999). The positive relationship between codon usage bias and translation initiation AUG context in Saccharomyces cerevisiae. Yeast *15*, 633-637.

Mize, G. J., Ruan, H., Low, J. J., and Morris, D. R. (1998). The inhibitory upstream open reading frame from mammalian S- adenosylmethionine decarboxylase mRNA has a strict sequence specificity in critical positions. J Biol Chem *273*, 32500-32505.

Moldave, K. (1985). Eukaryotic protein synthesis. Annu Rev Biochem *54*, 1109-1149.
Moore, P. B., and Steitz, T. A. (2003). After the ribosome structures: how does peptidyl transferase work? RNA *9*, 155-159.

Morris, D. R. (1995). Growth control of translation in mammalian cells. Prog Nucleic Acid Res Mol Biol *51*, 339-363.

Morris, D. R., and Geballe, A. P. (2000). Upstream open reading frames as regulators of mRNA translation. Mol Cell Biol *20*, 8635-8642.

Moustakas, A., Sonstegard, T. S., and Hackett, P. B. (1993). Alterations of the three short open reading frames in the Rous sarcoma virus leader RNA modulate viral replication and gene expression. J Virol *67*, 4337-4349.

Muhlrad, D., Decker, C. J., and Parker, R. (1995). Turnover mechanisms of the stable yeast *PGK1* mRNA. Mol Cell Biol *15*, 2145-2156.

Muhlrad, D., and Parker, R. (1994). Premature translational termination triggers mRNA decapping. Nature *370*, 578-581.

Muhlrad, D., and Parker, R. (1999). Recognition of yeast mRNAs as "nonsense containing" leads to both inhibition of mRNA translation and mRNA degradation: implications for the control of mRNA decapping. Mol Biol Cell *10*, 3971-3978.

Muller, E. A., and Danner, D. J. (2004). Tissue-specific translation of murine branched-chain alpha-ketoacid dehydrogenase kinase mRNA is dependent upon an upstream open reading frame in the 5'-untranslated region. J Biol Chem *279*, 44645-44655.

Nakatogawa, H., and Ito, K. (2002). The ribosomal exit tunnel functions as a discriminating gate. Cell *108*, 629-636.

Nakatogawa, H., Murakami, A., and Ito, K. (2004). Control of SecA and SecM translation by protein secretion. Curr Opin Microbiol *7*, 145-150.

Ni, J., Sakanyan, V., Charlier, D., Glansdorff, N., and Van Duyne, G. D. (1999). Structure of the arginine repressor from *Bacillus stearothermophilus*. Nat Struct Biol *6*, 427-432.

Nissen, P., Hansen, J., Ban, N., Moore, P. B., and Steitz, T. A. (2000). The structural basis of ribosome activity in peptide bond synthesis. Science *289*, 920-930.

Nomura, A., Iwasaki, Y., Saito, M., Aoki, Y., Yamamori, E., Ozaki, N., Tachikawa, K., Mutsuga, N., Morishita, M., Yoshida, M., *et al.* (2001). Involvement of upstream open reading frames in regulation of rat V(1b) vasopressin receptor expression. Am J Physiol Endocrinol Metab *280*, E780-787.

Nott, A., Le Hir, H., and Moore, M. J. (2004). Splicing enhances translation in mammalian cells: an additional function of the exon junction complex. Genes Dev *18*, 210-222.

Nozaki, S., Kashiwagi, H., Yamashita, S., Nakagawa, T., Kostner, B., Tomiyama, Y., Nakata, A., Ishigami, M., Miyagawa, J., and Kameda-Takemura, K. (1995). Reduced uptake of oxidized low density lipoproteins in monocyte-derived macrophages from CD36-deficient subjects. J Clin Invest *96*, 1859-1865.

Ogle, J. M., Brodersen, D. E., Clemons, W. M., Jr., Tarry, M. J., Carter, A. P., and Ramakrishnan, V. (2001). Recognition of cognate transfer RNA by the 30S ribosomal subunit. Science *292*, 897-902.

Orbach, M. J., Sachs, M. S., and Yanofsky, C. (1990). The *Neurospora crassa arg-2* locus: structure and expression of the gene encoding the small subunit of arginine-specific carbamoyl phosphate synthetase. J Biol Chem *265*, 10981-10987.

Otero, L. J., Ashe, M. P., and Sachs, A. B. (1999). The yeast poly(A)-binding protein Pab1p stimulates in vitro poly(A)- dependent and cap-dependent translation by distinct mechanisms. EMBO J *18*, 3153-3163.

Palacián, E., and Vazquez, D. (1979). Interaction of arginine with the ribosomal peptidyl transferase centre. Eur J Biochem *101*, 469-473.

Palacios, I. M., Gatfield, D., St Johnston, D., and Izaurralde, E. (2004). An eIF4AIII-containing complex required for mRNA localization and nonsense-mediated mRNA decay. Nature *427*, 753-757.

Paluh, J. L., Orbach, M. J., Legerton, T. L., and Yanofsky, C. (1988). The cross-pathway control gene of *Neurospora crassa*, *cpc-1*, encodes a protein similar to *GCN4* of yeast and the DNA-binding domain of the oncogene *v-jun*-encoded protein. Proc Natl Acad Sci USA *85*, 3728-3732.

Parker, R., and Song, H. (2004). The enzymes and control of eukaryotic mRNA turnover. Nat Struct Mol Biol *11*, 121-127.

Parola, A. L., and Kobilka, B. K. (1994). The peptide product of a 5' leader cistron in the $ß_2$ adrenergic receptor mRNA inhibits receptor synthesis. J Biol Chem *269*, 4497-4505.

Parrott, L. A., and Templeton, D. J. (1999). Osmotic stress inhibits p70/85 S6 kinase through activation of a protein phosphatase. J Biol Chem *274*, 24731-24736.

Paulovich, A. G., Thompson, J. R., Larkin, J. C., Li, Z., and Woolford, J. L., Jr. (1993). Molecular genetics of cryptopleurine resistance in *Saccharomyces cerevisiae*: expression of a ribosomal protein gene family. Genetics *135*, 719-730.

Pause, A., Methot, N., Svitkin, Y., Merrick, W. C., and Sonenberg, N. (1994). Dominant negative mutants of mammalian translation initiation factor eIF-4A define a critical role for eIF-4F in cap-dependent and cap-independent initiation of translation. Embo J *13*, 1205-1215.

Pavitt, G. D., Ramaiah, K. V. A., Kimball, S. R., and Hinnebusch, A. G. (1998). eIF2 independently binds two distinct eIF2B subcomplexes that catalyze and regulate guanine-nucleotide exchange. Genes Dev *12*, 514-526.

Pavlov, M. Y., Freistroffer, D. V., Dincbas, V., MacDougall, J., Buckingham, R. H., and Ehrenberg, M. (1998). A direct estimation of the context effect on the efficiency of termination. J Mol Biol *284*, 579-590.

Peltz, S. W., Brown, A. H., and Jacobson, A. (1993). mRNA destabilization triggered by premature translational termination depends on at least three cis-acting sequence elements and one trans-acting factor. Genes Dev *7*, 1737-1754.

Perez, L., Wills, J. W., and Hunter, E. (1987). Expression of the Rous sarcoma virus *env* gene from a simian virus 40 late-region replacement vector: effects of upstream initiation codons. J Virol *61*, 1276-1281.

Pesole, G., Gissi, C., Grillo, G., Licciulli, F., Liuni, S., and Saccone, C. (2000). Analysis of oligonucleotide AUG start codon context in eukariotic mRNAs. Gene *261*, 85-91.

Pesole, G., Liuni, S., Grillo, G., and Saccone, C. (1997). Structural and compositional features of untranslated regions of eukaryotic mRNAs. Gene *205*, 95-102.

Pestova, T. V., Hellen, C. U. T., and Shatsky, I. N. (1996). Canonical eukaryotic initiation factors determine initiation of translation by internal ribosomal entry. Mol Cell Biol *16*, 6859-6869.

Pestova, T. V., and Kolupaeva, V. G. (2002). The roles of individual eukaryotic translation initiation factors in ribosomal scanning and initiation codon selection. Genes Dev *16*, 2906-2922.

Pestova, T. V., Lomakin, I. B., Lee, J. H., Choi, S. K., Dever, T. E., and Hellen, C. U. (2000). The joining of ribosomal subunits in eukaryotes requires eIF5B. Nature *403*, 332-335.

Pinto, I., Na, J. G., Sherman, F., and Hampsey, M. (1992). Cis- and trans-acting suppressors of a translation initiation defect at the *cyc1* locus of *Saccharomyces cerevisiae*. Genetics *132*, 97-112.

Podrez, E. A., Febbraio, M., Sheibani, N., Schmitt, D., Silverstein, R. L., Hajjar, D. P., Cohen, P. A., Frazier, W. A., Hoff, H. F., and Hazen, S. L. (2000). Macrophage scavenger receptor CD36 is the major receptor for LDL modified by monocyte-generated reactive nitrogen species. J Clin Invest *105*, 1095-1108.

Polymenis, M., and Schmidt, E. V. (1997). Coupling of cell division to cell growth by translational control of the G_1 cyclin *CLN3* in yeast. Genes Dev *11*, 2522-2531.

Pooggin, M. M., Hohn, T., and Futterer, J. (1998). Forced evolution reveals the importance of short open reading frame A and secondary structure in the cauliflower mosaic virus 35S RNA leader. J Virol *72*, 4157-4169.

Poole, E. S., Brown, C. M., and Tate, W. P. (1995). The identity of the base following the stop codon determines the efficiency of in vivo translational termination in *Escherichia coli*. EMBO J *14*, 151-158.

Preiss, T., and W., H. M. (2003). Starting the protein synthesis machine: eukaryotic translation initiation. Bioessays *25*, 1201-1211.

Qin, S. L., Xie, A. G., Bonato, M. C., and McLaughlin, C. S. (1990). Sequence analysis of the translational elongation factor 3 from Saccharomyces cerevisiae. J Biol Chem *265*, 1903-1912.

Rajavel, K. S., and Neufeld, E. F. (2001). Nonsense-mediated decay of human *HEXA* mRNA. Mol Cell Biol *21*, 5512-5519.

Ramakrishnan, V. (2002). Ribosome structure and the mechanism of translation. Cell *108*, 557-572.

Raney, A., Law, G. L., Mize, G. J., and Morris, D. R. (2002). Regulated translation termination at the upstream open reading frame in S-adenosylmethionine decarboxylase mRNA. J Biol Chem *277*, 5988-5994.

Raychaudhuri, P., Stringer, E. A., Valenzuela, D. M., and Maitra, U. (1984). Ribosomal subunit antiassociation activity in rabbit reticulocyte lysates. Evidence for a low molecular weight ribosomal subunit antiassociation protein factor (Mr = 25,000). J Biol Chem *259*, 11930-11935.

Reynolds, K., Zimmer, A. M., and Zimmer, A. (1996). Regulation of RARß2 mRNA expression: evidence for an inhibitory peptide encoded in the 5'-untranslated region. J Cell Biol *134*, 827-835.

Rodnina, M. V., and Wintermeyer, W. (2001). Fidelity of aminoacyl-tRNA selection on the ribosome: kinetic and structural mechanisms. Annu Rev Biochem *70*, 415-435.

Rogers, G. W., Jr., Richter, N. J., Lima, W. F., and Merrick, W. C. (2001). Modulation of the helicase activity of eIF4A by eIF4B, eIF4H, and eIF4F. J Biol Chem *276*, 30914-30922.

Roy, A. L., Chakrabarti, D., Datta, B., Hileman, R. E., and Gupta, N. K. (1988). Natural mRNA is required for directing Met-tRNA(f) binding to 40S ribosomal subunits in animal cells: involvement of Co-eIF-2A in natural mRNA-directed initiation complex formation. Biochemistry *27*, 8203-8209.

Rozen, F., Edery, I., Meerovitch, K., Dever, T. E., Merrick, W. C., and Sonenberg, N. (1990). Bidirectional RNA helicase activity of eucaryotic translation initiation factors 4A and 4F. Mol Cell Biol *10*, 1134-1144.

Ruan, H., Hill, J. R., Fatemie-Nainie, S., and Morris, D. R. (1994). Cell-specific translational regulation of S-adenosylmethionine decarboxylase mRNA: influence of the structure of the 5' transcript leader on regulation by the upstream open reading frame. J Biol Chem *269*, 17905-17910.

Ruiz-Echevarria, M. J., Czaplinski, K., and Peltz, S. W. (1996). Making sense of nonsense in yeast. Trends Biochem Sci *21*, 433-438.

Ruiz-Echevarria, M. J., Gonzalez, C. I., and Peltz, S. W. (1998). Identifying the right stop: determining how the surveillance complex recognizes and degrades an aberrant mRNA. EMBO J *17*, 575-589.

Ruiz-Echevarria, M. J., and Peltz, S. W. (2000). The RNA-binding protein Pub1 modulates the stability of transcripts containing upstream open reading frames. Cell *101*, 741-751.

Russell, D. W., and Spremulli, L. L. (1978). Identification of a wheat germ ribosome dissociation factor distinct from initiation factor eIF-3. J Biol Chem *253*, 6647-6649.

Russell, D. W., and Spremulli, L. L. (1979). Purification and characterization of a ribosome dissociation factor (eukaryotic initiation factor 6) from wheat germ. J Biol Chem *254*, 8796-8800.

Sachs, A. B., Sarnow, P., and Hentze, M. W. (1997). Starting at the beginning, middle, and end: translation initiation in eukaryotes. Cell *89*, 831-838.

Sachs, M. S. (1996). General and cross-pathway controls of amino acid biosynthesis, In The Mycota: Biochemistry and Molecular Biology, R. Brambl, and G. A. Marzluf, eds. (Heidelberg: Springer-Verlag), pp. 315-345.

Sachs, M. S., Wang, Z., Gaba, A., Fang, P., Belk, J., Ganesan, R., Amrani, N., and Jacobson, A. (2002). Toeprint analysis of the positioning of translational apparatus components at initiation and termination codons of fungal mRNAs. Methods *26*, 105-114.

Sachs, M. S., and Yanofsky, C. (1991). Developmental expression of genes involved in conidiation and amino acid biosynthesis in *Neurospora crassa*. Dev Biol *148*, 117-128.

Salomons, G. S., Buitenhuis, C. K., Martinez Munoz, C., Verwijs-Jassen, M., Behrendt, H., Zsiros, J., and Smets, L. A. (1998). Mutational analysis of Bax and Bcl-2 in childhood acute lymphoblastic leukaemia. Int J Cancer *79*, 273-277.

Sarrazin, S., Starck, J., Gonnet, C., Doubeikovski, A., Melet, F., and Morle, F. (2000). Negative and translation termination-dependent positive control of FLI- 1 protein synthesis by conserved overlapping 5' upstream open reading frames in Fli-1 mRNA. Mol Cell Biol *20*, 2959-2969.

Sattlegger, E., Hinnebusch, A. G., and Barthelmess, I. B. (1998). *cpc-3*, the *Neurospora crassa* homologue of yeast *GCN2*, encodes a polypeptide with juxtaposed eIF2alpha kinase and histidyl-tRNA synthetase-related domains required for general amino acid control. J Biol Chem *273*, 20404-20416.

Schleiss, M. R., Degnin, C. R., and Geballe, A. P. (1991). Translational control of human cytomegalovirus gp48 expression. J Virol *65*, 6782-6789.

Schluter, G., Boinska, D., and Nieman-Seyde, S. C. (2000). Evidence for translational repression of the SOCS-1 major open reading frame by an upstream open reading frame. Biochem Biophys Res Commun *268*, 255-261.

Scolnick, E., Tompkins, R., Caskey, T., and Nirenberg, M. (1968). Release factors differing in specificity for terminator codons. Proc Natl Acad Sci USA *61*, 768-774.

Sedman, S. A., Good, P. J., and Mertz, J. E. (1989). Leader-encoded open reading frames modulate both the absolute and relative rates of synthesis of the virion proteins of simian virus 40. J Virol *63*, 3884-3893.

Sedman, S. A., and Mertz, J. E. (1988). Mechanisms of synthesis of virion proteins from the functionally bigenic late mRNAs of simian virus 40. J Virol *62*, 954-961.

Seit-Nebi, A., Frolova, L., Justesen, J., and Kisselev, L. (2001). Class-1 translation termination factors: invariant GGQ minidomain is essential for release activity and ribosome binding but not for stop codon recognition. Nucleic Acids Res *29*, 3982-3987.

Semenkovich, C. F., and Heinecke, J. W. (1997). The mystery of diabetes and atherosclerosis: time for a new plot. Diabetes *46*, 327-334.

Serin, G., Gersappe, A., Black, J. D., Aronoff, R., and Maquat, L. E. (2001). Identification and characterization of human orthologues to *Saccharomyces cerevisiae* Upf2 protein and Upf3 protein (*Caenorhabditis elegans SMG-4*). Mol Cell Biol *21*, 209-223.

Shantz, L. M., and Pegg, A. E. (1994). Overproduction of ornithine decarboxylase caused by relief of translational repression is associated with neoplastic transformation. Cancer Res *54*, 2313-2316.

Shantz, L. M., and Pegg, A. E. (1999). Translational regulation of ornithine decarboxylase and other enzymes of the polyamine pathway. Int J Biochem Cell Biol *31*, 107-122.

Shantz, L. M., Viswanath, R., and Pegg, A. E. (1994). Role of the 5'-untranslated region of mRNA in the synthesis of S-adenosylmethionine decarboxylase and its regulation by spermine. Biochem J *302*, 765-772.

Shen, W.-C., and Ebbole, D. J. (1997). Cross-pathway and pathway-specific control of amino acid biosynthesis in *Magnaporthe grisea*. Fungal Genet Biol *21*, 40-49.

Shibuya, T., Tange, T. O., Sonenberg, N., and Moore, M. J. (2004). eIF4AIII binds spliced mRNA in the exon junction complex and is essential for nonsense-mediated decay. Nat Struct Mol Biol *11*, 346-351.

Shin, B. S., Maag, D., Roll-Mecak, A., Arefin, M. S., Burley, S. K., Lorsch, J. R., and Dever, T. E. (2002). Uncoupling of initiation factor eIF5B/IF2 GTPase and translational activities by mutations that lower ribosome affinity. Cell *111*, 1015-1025.

Shirley, R. L., Ford, A. S., Richards, M. R., Albertini, M., and Culbertson, M. R. (2002). Nuclear import of Upf3p is mediated by importin-alpha/-beta and export to the cytoplasm is required for a functional nonsense-mediated mRNA decay pathway in yeast. Genetics *161*, 1465-1482.

Shirley, R. L., Lelivelt, M. J., Schenkman, L. R., Dahlseid, J. N., and Culbertson, M. R. (1998). A factor required for nonsense-mediated mRNA decay in yeast is exported from the nucleus to the cytoplasm by a nuclear export signal sequence. J Cell Sci *111 (Pt 21)*, 3129-3143.

Singh, G., and Lykke-Andersen, J. (2003). New insights into the formation of active nonsense-mediated decay complexes. Trends Biochem Sci *28*, 464-466.

Slusher, L. B., Gillman, E. C., Martin, N. C., and Hopper, A. K. (1991). mRNA leader length and initiation codon context determine alternative AUG selection for the yeast gene MOD5. Proc Natl Acad Sci USA *88*, 9789-9793.

Song, H., Mugnier, P., Das, A. K., Webb, H. M., Evans, D. R., Tuite, M. F., Hemmings, B. A., and Barford, D. (2000). The crystal structure of human eukaryotic release factor eRF1--mechanism of stop codon recognition and peptidyl-tRNA hydrolysis. Cell *100*, 311-321.

Spahn, C. M., Beckmann, R., Eswar, N., Penczek, P. A., Sali, A., Blobel, G., and Frank, J. (2001). Structure of the 80S ribosome from *Saccharomyces cerevisiae*--tRNA- ribosome and subunit-subunit interactions. Cell *107*, 373-386.

Srivastava, S., Verschoor, A., and Frank, J. (1992). Eukaryotic initiation factor 3 does not prevent association through physical blockage of the ribosomal subunit-subunit interface. J Mol Biol *226*, 301-314.

Stansfield, I., Jones, K. M., Kushnirov, V. V., Dagkesamanskaya, A. R., Poznyakovski, A. I., Paushkin, S. V., Nierras, C. R., Cox, B. S., Teravanesyan, M. D., and Tuite, M. F. (1995). The products of the *SUP45* (eRF1) and *SUP35* genes interact to mediate translation termination in *Saccharomyces cerevisiae*. EMBO J *14*, 4365-4373.

Steel, L. F., Telly, D. L., Leonard, J., Rice, B. A., Monks, B., and Sawicki, J. A. (1996). Elements in the murine *c-mos* messenger RNA 5'-untranslated region repress translation of downstream coding sequences. Cell Growth Differ *7*, 1415-1424.

Suzuki, Y., Ishihara, D., Sasaki, M., Nakagawa, H., Hata, H., Tsunoda, T., Watanabe, M., Komatsu, T., Ota, T., Isogai, T., *et al.* (2000). Statistical analysis of the 5' untranslated region of human mRNA using "Oligo-Capped" cDNA libraries. Genomics *64*, 286-297.

Takahashi, S., Araki, Y., Sakuno, T., and Katada, T. (2003). Interaction between Ski7p and Upf1p is required for nonsense-mediated 3'-to-5' mRNA decay in yeast. EMBO J *22*, 3951-3959.

Tao, J., and Frankel, A. D. (1992). Specific binding of arginine to TAR RNA. Proc Natl Acad Sci USA *89*, 2723-2726.

Tarun, S. Z., Jr., and Sachs, A. B. (1995). A common function for mRNA 5' and 3' ends in translation initiation in yeast. Genes Dev *9*, 2997-3007.

Thermann, R., Neu-Yilik, G., Deters, A., Frede, U., Wehr, K., Hagemeier, C., Hentze, M. W., and Kulozik, A. E. (1998). Binary specification of nonsense codons by splicing and cytoplasmic translation. EMBO J *17*, 3484-3494.

Thomas, A., Goumans, H., Voorma, H. O., and Benne, R. (1980). The mechanism of action of eukaryotic initiation factor 4C in protein synthesis. Eur J Biochem *107*, 39-45.

Thuriaux, P., Ramos, F., Piérard, A., Grenson, M., and Wiame, J. M. (1972). Regulation of carbamoylphosphate synthetase belonging to the arginine biosynthetic pathway of *Saccharomyces cerevisiae*. J Mol Biol *67*, 277-287.

Trachsel, H., Erni, B., Schreier, M. H., and Staehelin, T. (1977). Initiation of mammalian protein synthesis. II. The assembly of the initiation complex with purified initiation factors. J Mol Biol *116*, 755-767.

Triana-Alonso, F. J., Chakraburtty, K., and Nierhaus, K. H. (1995). The elongation factor 3 unique in higher fungi and essential for protein biosynthesis is an E site factor. J Biol Chem *270*, 20473-20478.

van Hoof, A., Frischmeyer, P. A., Dietz, H. C., and Parker, R. (2002). Exosome-mediated recognition and degradation of mRNAs lacking a termination codon. Science *295*, 2262-2264.

Varshney, U., Lee, C. P., and RajBhandary, U. L. (1991). Direct analysis of aminoacylation levels of tRNAs in vivo. Application to studying recognition of *Escherichia coli* initiator tRNA mutants by glutaminyl-tRNA synthetase. J Biol Chem *266*, 24712-24718.

Vestergaard, B., Van, L. B., Andersen, G. R., Nyborg, J., Buckingham, R. H., and Kjeldgaard, M. (2001). Bacterial polypeptide release factor RF2 is structurally distinct from eukaryotic eRF1. Mol Cell *8*, 1375-1382.

Vilela, C., Linz, B., Rodrigues-Pousada, C., and McCarthy, J. E. (1998). The yeast transcription factor genes *YAP1* and *YAP2* are subject to differential control at the levels of both translation and mRNA stability. Nucl Acids Res *26*, 1150-1159.

Vilela, C., Ramirez, C. V., Linz, B., Rodrigues-Pousada, C., and McCarthy, J. E. (1999). Post-termination ribosome interactions with the 5'UTR modulate yeast mRNA stability. EMBO J *18*, 3139-3152.

von Manteuffel, S. R., Gingras, A. C., Ming, X. F., Sonenberg, N., and Thomas, G. (1996). 4E-BP1 phosphorylation is mediated by the FRAP-p70s6k pathway and is independent of mitogen-activated protein kinase. Proc Natl Acad Sci USA *93*, 4076-4080.

Vries, R. G., Flynn, A., Patel, J. C., Wang, X., Denton, R. M., and Proud, C. G. (1997). Heat shock increases the association of binding protein-1 with initiation factor 4E. J Biol Chem *272*, 32779-32784.

Wang, C., Le, S. Y., Ali, N., and Siddiqui, A. (1995). An RNA pseudoknot is an essential structural element of the internal ribosome entry site located within the hepatitis C virus 5' noncoding region. RNA *1*, 526-537.

Wang, L., and Wessler, S. R. (1998). Inefficient reinitiation is responsible for upstream open reading frame- mediated translational repression of the maize R gene. Plant Cell *10*, 1733-1746.

Wang, Z., Fang, P., and Sachs, M. S. (1998). The evolutionarily conserved eukaryotic arginine attenuator peptide regulates the movement of ribosomes that have translated it. Mol Cell Biol *18*, 7528-7536.

Wang, Z., Gaba, A., and Sachs, M. S. (1999). A highly conserved mechanism of regulated ribosome stalling mediated by fungal arginine attenuator peptides that appears independent of the charging status of arginyl-tRNAs. J Biol Chem *274*, 37565-37574.

Wang, Z., and Kiledjian, M. (2001). Functional link between the mammalian exosome and mRNA decapping. Cell *107*, 751-762.

Wang, Z., and Sachs, M. S. (1997a). Arginine-specific regulation mediated by the *Neurospora crassa arg-2* upstream open reading frame in a homologous, cell-free *in vitro* translation system. J Biol Chem *272*, 255-261.

Wang, Z., and Sachs, M. S. (1997b). Ribosome stalling is responsible for arginine-specific translational attenuation in *Neurospora crassa*. Mol Cell Biol *17*, 4904-4913.

Wanke, C., Eckert, S., Albrecht, G., van Hartingsveldt, W., Punt, P. J., van den Hondel, C. A., and Braus, G. H. (1997). The *Aspergillus niger GCN4* homologue, *cpcA*, is transcriptionally regulated and encodes an unusual leucine zipper. Mol Microbiol *23*, 23-33.

Weissenbach, J., Martin, R., and Dirheimer, G. (1975). The primary structure of tRNAIIArg from brewers' yeast. 1. Complete digestions with pancreatic and T1 ribonucleases. Eur J Biochem *56*, 521-526.

Welch, E. M., and Jacobson, A. (1999). An internal open reading frame triggers nonsense-mediated decay of the yeast SPT10 mRNA. EMBO J *18*, 6134-6145.

Wells, S. E., Hillner, P. E., Vale, R. D., and Sachs, A. B. (1998). Circularization of mRNA by eukaryotic translation initiation factors. Mol Cell *2*, 135-140.

Weng, Y., Czaplinski, K., and Peltz, S. W. (1996a). Genetic and biochemical characterization of mutations in the ATPase and helicase regions of the Upf1 protein. Mol Cell Biol *16*, 5477-5490.

Weng, Y., Czaplinski, K., and Peltz, S. W. (1996b). Identification and characterization of mutations in the *UPF1* gene that affect nonsense suppression and the formation of the Upf protein complex but not mRNA turnover. Mol Cell Biol *16*, 5491-5506.

Weng, Y., Czaplinski, K., and Peltz, S. W. (1998). ATP is a cofactor of the Upf1 protein that modulates its translation termination and RNA binding activities. RNA *4*, 205-214.

Wera, S., Fernandez, A., Lamb, N. J., Turowski, P., Hemmings-Mieszczak, M., Mayer-Jaekel, R. E., and Hemmings, B. A. (1995). Deregulation of translational control of the

65-kDa regulatory subunit (PR65 alpha) of protein phosphatase 2A leads to multinucleated cells. J Biol Chem *270*, 21374-21381.

Werner, M., Feller, A., Messenguy, F., and Piérard, A. (1987). The leader peptide of yeast *CPA1* is essential for the translational repression of its expression. Cell *49*, 805-813.

Wiegand, H. L., Lu, S., and Cullen, B. R. (2003). Exon junction complexes mediate the enhancing effect of splicing on mRNA expression. Proc Natl Acad Sci USA *100*, 11327-11332.

Williams, M. A., and Lamb, R. A. (1989). Effect of mutations and deletions in a bicistronic mRNA on the synthesis of influenza B virus NB and NA glycoproteins. J Virol *63*, 28-35.

Wilson, P. G., and Culbertson, M. R. (1988). SUF12 suppressor protein of yeast. A fusion protein related to the EF-1 family of elongation factors. J Mol Biol *199*, 559-573.

Wintermeyer, W., Savelsbergh, A., Semenkov, Y. P., Katunin, V. I., and Rodnina, M. V. (2001). Mechanism of elongation factor G function in tRNA translocation on the ribosome. Cold Spring Harb Symp Quant Biol *66*, 449-458.

Wollerton, M. C., Gooding, C., Wagner, E. J., Garcia-Blanco, M. A., and Smith, C. W. (2004). Autoregulation of polypyrimidine tract binding protein by alternative splicing leading to nonsense-mediated decay. Mol Cell *13*, 91-100.

Wright, M. B., Howell, E. A., and Gaber, R. F. (1996). Amino acid substitutions in membrane-spanning domains of Hol1, a member of the major facilitator superfamily of transporters, confer nonselective cation uptake in *Saccharomyces cerevisiae*. J Bacteriol *178*, 7197-7205.

Wu, J., and Miller, B. L. (1997). *Aspergillus* asexual reproduction and sexual reproduction are differentially affected by transcriptional and translational mechanisms regulating *stunted* gene expression. Mol Cell Biol *17*, 6191-6201.

Yarus, M. (1989). Specificity of arginine binding by the Tetrahymena intron. Biochem *28*, 980-988.

Yoon, H., and Donahue, T. F. (1992). The *sui1* suppressor locus in *Saccharomyces cerevisiae* encodes a translation factor that functions during $tRNA_i^{Met}$ recognition of the start codon. Mol Cell Biol *12*, 248-260.

Yun, D. F., and Sherman, F. (1995). Initiation of translation can occur only in a restricted region of the *CYC1* mRNA of *Saccharomyces cerevisiae*. Mol Cell Biol *15*, 1021-1033.

Zavialov, A. V., Mora, L., Buckingham, R. H., and Ehrenberg, M. (2002). Release of peptide promoted by the GGQ motif of class 1 release factors regulates the GTPase activity of RF3. Mol Cell *10*, 789-798.

Zhang, J., Sun, X., Qian, Y., LaDuca, J. P., and Maquat, L. E. (1998a). At least one intron is required for the nonsense-mediated decay of triosephosphate isomerase mRNA: a possible link between nuclear splicing and cytoplasmic translation. Mol Cell Biol *18*, 5272-5283.

Zhang, J., Sun, X., Qian, Y., and Maquat, L. E. (1998b). Intron function in the nonsense-mediated decay of beta-globin mRNA: indications that pre-mRNA splicing in the nucleus can influence mRNA translation in the cytoplasm. RNA *4*, 801-815.

Zhang, S., Ruiz-Echevarria, M. J., Quan, Y., and Peltz, S. W. (1995). Identification and characterization of a sequence motif involved in nonsense-mediated mRNA decay. Mol Cell Biol *15*, 2231-2244.

Zhang, S., Welch, E. M., Hogan, K., Brown, A. H., Peltz, S. W., and Jacobson, A. (1997). Polysome-associated mRNAs are substrates for the nonsense-mediated mRNA decay pathway in *Saccharomyces cerevisiae*. RNA *3*, 234-244.

Zhouravleva, G., Frolova, L., Le Goff, X., Le Geullec, R., Inge-Vechtomov, S., Kisselev, L., and Philippe, M. (1995). Termination of translation in eukaryotes is governed by two interacting polypeptide chain release factors, eRF1 and eRF3. EMBO J *14*, 4065-4072.

Zhu, S., Sobolev, A. Y., and Wek, R. C. (1996). Histidyl-tRNA synthetase-related sequences in GCN2 protein kinase regulate *in vitro* phosphorylation of eIF-2. J Biol Chem *271*, 24989-24994.

Zimmer, A., Zimmer, A. M., and Reynolds, K. (1994). Tissue specific expression of the retinoic acid receptor-beta 2: regulation by short open reading frames in the 5'-noncoding region. J Cell Biol *127*, 1111-1119.

BIOGRAPHICAL SKETCH

Anthony Gaba was born in Simi Valley, California on August 26, 1973. In 1997 he received his B.S. in Biochemistry and Cell Biology from the University of California, San Diego, where he received molecular biology training in the laboratory of Dr. Martin Yanofsky. In 1999 he received his M.S. in Biochemistry and Molecular Biology from the Oregon Graduate Institute under the supervision of Dr. Matthew S. Sachs. Anthony continued his research in the laboratory of Dr. Sachs and earned his Ph.D. in 2005 from Oregon Health and Science University. Following the successful defense of his dissertation, Anthony will begin postdoctoral research in the laboratory of Dr. Michael Karin at the University of California, San Diego Medical School where he will study posttranscriptional control mechanisms in macrophages.

Publications

Wang, Z., Gaba, A., and Sachs, M. S. (1999). A highly conserved mechanism of regulated ribosome stalling by fungal attenuator peptides that appears independent of the charging status of arginyl tRNAs. *J. Biol. Chem.* **274**, 37565-37574.

Gaba, A., Wang, Z., Krishnamoorthy, T., Hinnebusch, A. G., and Sachs, M. S. (2001). Physical evidence for distinct mechanisms of translational control by upstream open reading frames. *EMBO J.* **20**, 6453-6463.

Sachs, M. S., Wang, Z., Gaba, A., Fang, P., Belk, J., Ganesan, R., Amrani, N., and Jacobson, A. (2002). Toeprint analysis of the positioning of translational apparatus components at initiation and termination codons of fungal mRNAs. *Methods* **26**, 105-114.

Gaba, A., Jacobson, A., and Sachs, M. S. (2005). Ribosome occupancy of the *CPA1* upstream open reading frame termination codon modulates nonsense-mediated mRNA decay. (In preparation).