ANALYSES OF THE FUNGAL ARGININE ATTENUATOR PEPTIDE'S ROLE IN THE REGULATION OF RIBOSOME STALLING

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A dissertation submitted to the faculty of the
OGI School of Science & Engineering
Oregon Health & Science University
in partial fulfillment of the requirements
for the degree
Doctor of Philosophy
in
Biochemistry and Molecular Biology

August 2003

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DEDICATION

Dedicated to my wife, Yao, for her love and patience.

ACKNOWLEDGMENTS

I wish to express my deep gratitude to my thesis advisor, Dr. Matthew S. Sachs. Matthew has given me numerous inspirations, instructions, suggestions and encouragements during my Ph.D. years, which were essential for the completion of this thesis. The scientific training I received in the Sachs lab will greatly benefit my career as an independent researcher. I also want to thank the members of my dissertation committee, Drs. Adam Geballe, Gebretateos Woldegiorgis and Peter Zuber. Special thanks to Dr. Adam Geballe; Adam has provided lots of suggestions and advice for my research.

I also owe a lot to many current and former members of the Sachs lab, especially Dr. Zhong Wang, Cheng Wu, Christina Spevak, Tony Gaba, Kanan Lathia, Richard Feinberg, and Baoyu Lin.

Many thanks to the people at the Northwest Translational Control Group for their suggestions and critiques. Special thanks to Dr. Alan Sachs and Dr. Uttam RajBhandary for providing technical assistance and valuable input for my research. I also thank Dr. James Cregg and people in his lab, Dr. Gebre Woldegiorgis and people in his lab, Dr. Michael Gold and people in his lab, Dr. Peter Zuber and people in his lab, and Dr. Michiko Nakano and people in her lab, for their encouragements and technical support. Thanks to Drs. Jim Whittaker and Mei Whittaker for supervising me in my first quarter at OGI. Heartfelt thanks to Dr. Joann Sanders-Loehr and Dr. Thomas Loehr for their help. Special thanks to Nancy Christie and Terrie Hadfield for all the help they have provided me during these years.

Finally, I want to thank my dearest wife, Yao Zhang, for her love, understanding and tremendous support.

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ABSTRACT

Analyses of the Fungal Arginine Attenuator Peptide's Role in the Regulation of Ribosome Stalling

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at Oregon Health & Science University
August 2003

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The arginine attenuator peptides (AAP), specified by an evolutionarily conserved upstream open reading frame (uORF) in mRNAs specifying small subunit of carbamoyl phosphate synthetase in fungi, can control the movement of ribosomes in response to arginine. Previous studies with cell-free translation systems from *Neurospora crassa* and *Saccharomyces cerevisiae* have indicated that ribosomes synthesizing the *N. crassa arg-2* and the homologous *S. cerevisiae CPA1* uORF-encoded AAPs stall at the uORF termination codon when the concentration of Arg is high, blocking ribosomes from scanning to the downstream initiation codon. These AAPs also cause Arg-regulated stalling of ribosomes involved in elongation when fused at the N-terminus of a luciferase (LUC) reporter.

The research work for this dissertation is focused on elucidating the detailed mechanism of the AAP-mediated translational control *in vitro* with cell-free systems, which includes analyzing *cis*-acting requirements for *arg-2* uORF function, examining effects of existing *trans*-acting mutations that cause translation defects *in vivo* on *arg-2*

regulation, and investigating the regulated ribosome movement by a nascent AAP domain within a larger polypeptide.

The results indicate that: (i) The conserved AAP sequence, but not the mRNA sequence, appears responsible for regulation. The highly evolutionarily conserved core of the peptide functions within the ribosome to cause stalling. Translational events at a potential stall site, such as an encounter with a stop codon or a rare codon, can influence the extent of stalling at that site. (ii) Seven tested *N. crassa* super-suppressor strains (*ssu-1*, *-2*, *-3*, *-4*, *- 5*, *-9*, and *-10*) produce amber suppressor-tRNA, but these mutant strains appear unaltered in arginine regulation. (iii) The AAP domain can transiently stall ribosomes when placed either at the N-terminus or within a polypeptide. The half-life of ribosomes stalled by the AAP during elongation increased when the Arg concentration increased. Ribosomes appear to resume translation after release from the stall.

This work demonstrates that a regulatory uORF-encoded peptide mediates translational control as a nascent peptide within the ribosome.

CHAPTER 1
INTRODUCTION

A large fraction of the biological macromolecules in organisms are proteins, most biological reactions are catalyzed by proteins, and proteins provide major life-dependent functions. Consequently, the synthesis of proteins is one of the most complex and energy-consuming cellular processes. The process of translating the mRNA into protein can be divided into three phases: initiation, elongation and termination. In the past few years, we have gained further insights into the molecular details of translation through *in vitro* biochemical studies and through high-resolution three-dimensional structures for the ribosome and translation factors. In this chapter are summarized the molecular mechanism of eukaryotic translation and the eukaryotic translational control mechanisms, especially those mechanisms involving upstream open reading frames (uORF).

1.1 MOLECULAR MECHANISM OF EUKARYOTIC TRANSLATION

Eukaryotic mRNA contains several *cis*-acting elements that play important roles both in the translation and translational control (Figure 1.1). Unlike the mRNA in prokaryotes, the 5' and 3' ends of the functional eukaryotic mRNA are modified. Almost all cellular mRNA in eukaryotes carries an m^7GpppN cap structure (where N is any nucleotide) at their 5' end, and a poly(A) tail at their 3' end. The cap is required for efficient translation (Kozak, 1999; Hershey, 2000; Sachs, 2000) and maintaining mRNA stability (Decker and Parker, 1994; Jacobson and Peltz, 1996); it also plays a significant role in nuclear export (Lewis and Izaurralde, 1997; Lewis and Tollervey, 2000). The initial length of the poly(A) tail is about 200 to 250 nt in mammals and about 60 to 90 nt in yeast (Jacobson, 1996). After export of the mRNA from nucleus to the cytoplasm, the

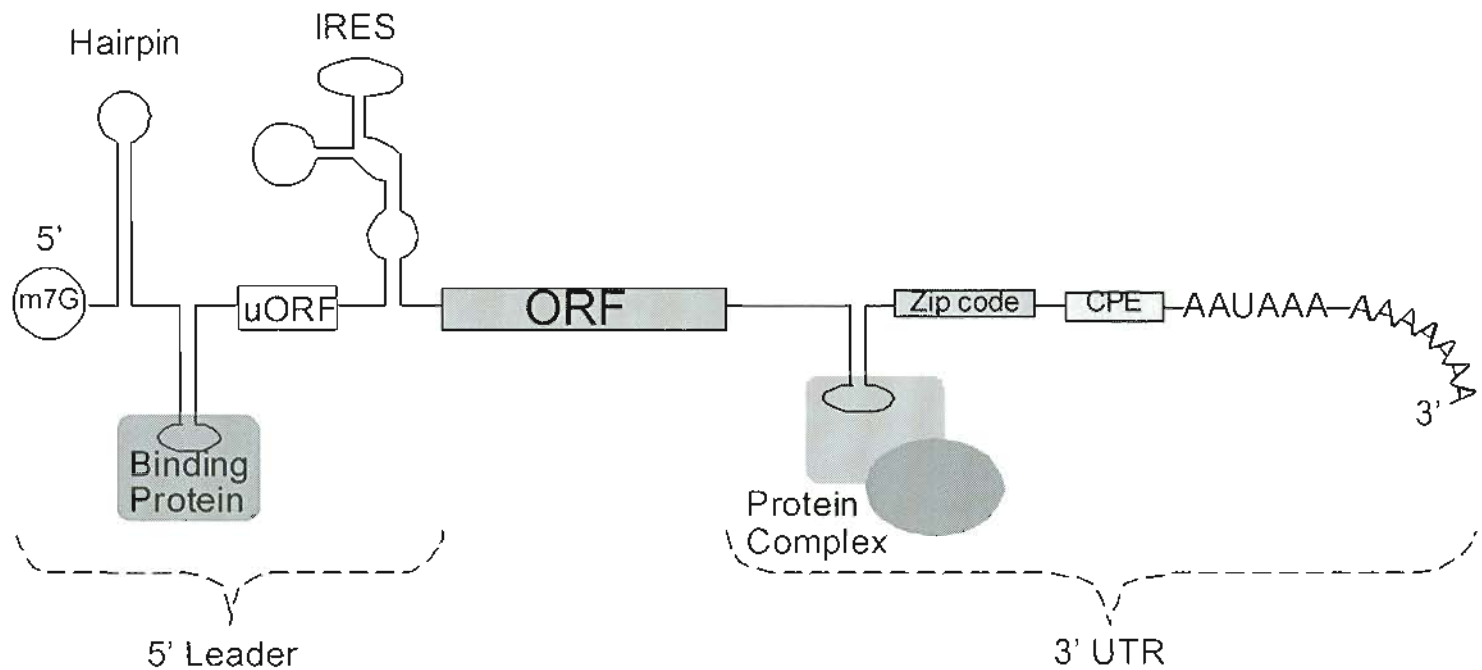

Figure 1.1. Cis-acting elements on mRNA that affect gene expression. Abbreviations (from 5' to 3'): UTR, untranslated region; m7G, 7-methyl-guanosine cap; hairpin,, hairpin-like secondary structures; uORF, upstream open reading frame; IRES, internal ribosome entry site; CPE, cytoplasmic polyadenylation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

poly(A) tail is gradually shortened to about 10-60 nt (Brawerman, 1981; Sachs and Davis, 1990). The poly(A) tail influences translation initiation and mRNA stability as does the cap (Sachs, 2000; Wilusz et al., 2001). Moreover, the 5' cap structure and the 3' poly(A) tail together can act synergistically to enhance translation (Gallie, 1991; Iizuka et al., 1994; Tarun and Sachs, 1995; Preiss and Hentze, 1998). The regulatory functions of the *cis*-acting elements in eukaryotic translation are discussed in Section 1.2.2.

1.1.1 Initiation

Initiation starts with the formation of an eIF2/GTP/Met-tRNA$_i$ ternary complex and the binding of this ternary complex to 40S ribosomal subunit to form a 43S preinitiation complex. The preinitiation complex then binds to mRNA and moves along the mRNA until encountering an initiation codon. Upon base-pairing between the anticodon of Met-tRNA$_i^{Met}$ and the start codon (typically an AUG triplet), the initiation factors dissociate from the preinitiation complex. The initiation phase concludes when the 60S ribosomal subunit joins with the 40S subunit to form the 80S elongating ribosome. At least ten initiation factors (eIFs) are involved in this process (reviewed in Pestova et al., 2001). Initiation process is illustrated in Figure 1.2 and discussed further below.

1.1.1.1 Formation of the Free 40S Ribosomal Subunits. The 40S small ribosomal subunit must be in its free form before initiation can proceed. However, at the physiological magnesium concentration (1~2 mM), the 80S ribosome is the predominant species, thus, there must exist mechanisms by which the 80S ribosome dissociates into its two subunits to allow the formation of the 43S preinitiation complex. Although the mechanism is poorly understood, it is generally thought that three initiation factors (eIF1A, eIF3 and eIF6) promote dissociation (Hershey, 2000). One plausible explanation for these initiation factors to function in this process is that they serve as anti-association factors by binding to ribosomal subunits (eIF1A and eIF3 to 40S, eIF6 to 60S), and preventing them from associating with each other by creating steric hindrance (Russell and Spremulli, 1979; Goumans et al., 1980; Raychaudhuri et al., 1984). eIF1A is also shown to inhibit the dimerization of the 40S subunits (Goumans et al., 1980; Kainuma and Hershey, 2001). However, electron microscopy studies produced controversial

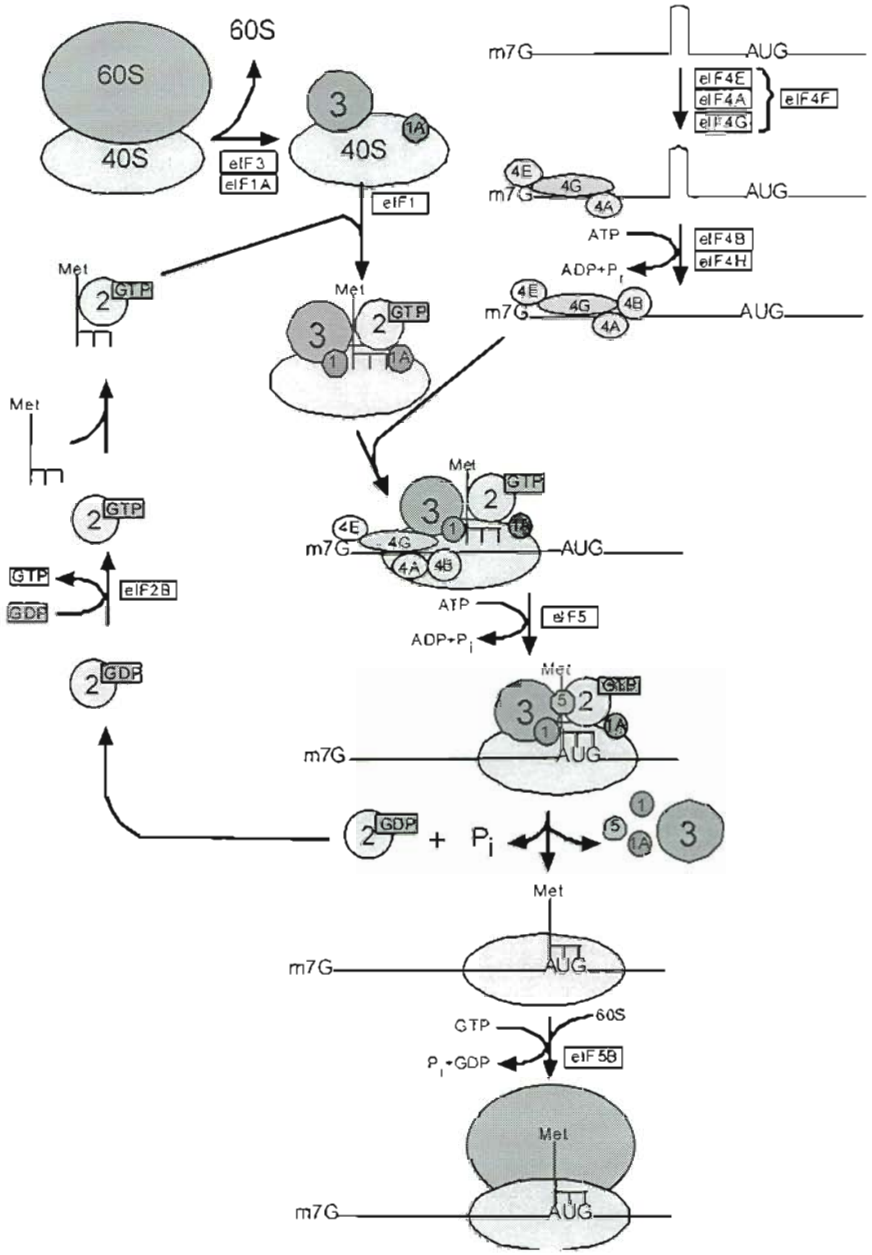

Figure 1.2. Initiation pathway in eukaryotes. Initiation factors are shown as gray circles. See text for detail. [Adapted from Hershey, 2000]

results for the stereochemical consequences of the eIF3 binding to the 40S subunit (Bommer et al., 1991; Srivastava et al., 1992) and the recent studies indicated that eIF1A, eIF3 and eIF6 may perform functions other than those originally proposed. These new studies show that eIF1A and eIF3 are involved in the formation of the preinitiation complex (see below) but not in the formation of the free 40S subunit. While eIF6 may be involved in biogenesis or assembly of the 60S ribosomal subunit, it does not participate in the 80S ribosome dissociation (Basu et al., 2001).

*1.1.1.2 **Formation of the 43S Preinitiation Complex.*** In eukaryotes, a specific tRNA derivative, methionyl-$tRNA_i$, is used to initiate translation. Met-$tRNA_i$ binds to eIF2•GTP to form a Met-$tRNA_i$•eIF2•GTP ternary complex. eIF2 discriminates this initiator tRNA from the pool of elongator tRNA by recognizing the methionyl residue and the A-U pair at the end of this tRNA's acceptor stem (Hershey, 2000). eIF2 is a heterotrimer consisting of α, β and γ subunits. Biochemical and genetic studies indicated that the α subunit is mainly involved in the translational regulation (discussed later). The β subunit plays a role in the binding of Met-$tRNA_i$ to eIF2 and interacts with mRNA and eIF5 (Hinnebusch, 2000). The γ subunit is primarily responsible for the binding of GTP and initiator tRNA, it functions as a GTPase (Dorris et al., 1995; Naranda et al., 1995; Erickson and Hannig, 1996; Huang et al., 1997). After one round of the initiation, eIF2 dissociates from the ribosome in an inactive eIF2•GDP form. It must be converted back to its active eIF2•GTP form for next round of initiation by eIF2B (Hershey, 2000; Hinnebusch, 2000).

The ternary complex binds to the 40S subunit to form the 43S preinitiation complex. eIF1A and eIF3 are involved in this process. eIF3 stabilizes the preinitiation complex by preventing 60s subunit from displacing the ternary complex, and eIF1A stimulates the association of ternary complex with 40S subunits in the absence of the 60S subunit (Merrick et al., 1973; Chaudhuri et al., 1997; Chaudhuri et al., 1999; Battiste et al., 2000). With the assistance of the other factors, eIF1A may occupy the ribosome A-site to promote the initiator tRNA binding to the P-site (Roll-Mecak et al., 2001).

Since the binding of Met•$tRNA_i$ to the 40S subunit is a critical step in the process of translation, the regulation at this step, especially the regulation of the activity of eIF2α,

becomes the focal point for controlling the rate of overall protein synthesis (discussed in section 1.2.1.1).

*1.1.1.3 **Association of the Preinitiation Complex With mRNA.*** The preinitiation complex binds to the mRNA predominantly via a cap-dependent mechanism, which results in the recognition of the initiation codon via the mechanism described by the "scanning model" (discussed in section 1.1.1.4). The 5'-cap on the mRNA is specifically recognized and bound by eIF4E, which functions as a subunit in the eIF4F heterotrimer consisting of eIF4E, eIF4A and eIF4G. The formation of the eIF4F increases the cap-binding affinity of eIF4E by at least 10 fold (Haghighat and Sonenberg, 1997; Ptushkina et al., 1998). This could explain why the cap-binding by eIF4E as a subunit in eIF4F is favored over the binding by the apo-eIF4E in translation (Sachs and Varani, 2000).

eIF4G serves as a scaffolding protein and can be roughly divided into three functional domains. The N-terminal third contains binding domains for binding poly(A)-binding protein (PABP) and eIF4E (Lamphear et al., 1995; Mader et al., 1995; Imataka et al., 1998) The central region contains an mRNA recognition motif and binding sites for eIF3 and eIF4A (Lamphear et al., 1995; Marcotrigiano et al., 2001). The C-terminal third contains another binding site for eIF4A (Korneeva et al., 2001) and a binding domain for Mnk-1/Mnk-2, which are the kinases believed to be responsible for phosphorylating eIF4E (discussed later).

Any secondary structure near the 5'-end of the mRNA reduces the accessibility of the cap structure. eIF4A, a bidirectional, ATP-dependent DEAD-Box RNA helicase, unwinds such structures in the RNA (Rozen et al., 1990). The helicase activity of eIF4A is increased when it is in the eIF4F complex (Rozen et al., 1990). It is further increased in the presence of eIF4B and eIF4H (Rogers et al., 2001 and references therein).

After the removal of the mRNA secondary structure by eIF4A, the 43S preinitiation complex binds to mRNA *via* the interaction between eIF4G (in eIF4F) and eIF3 (on the 40S subunit). The poly(A) tail also plays a role in recruiting the eIF4G to the mRNA via interactions between eIF4G and the poly(A) binding protein PAB1p. This interaction not only increased translation efficiency but also resulted in the circularization of the translated mRNA (Sachs, 2000).

The preinitiation complex may also bind to the mRNA *via* a mechanism described by the internal initiation model, in which the binding of the preinitiation complex to the mRNA is cap-independent and mediated by the internal ribosomal entry site (IRES). IRES-mediated translation initiation is discussed in Section 1.1.1.6.

1.1.1.4 ***Recognition of the Initiation Codon and Junction of the 60S Ribosome Subunit.*** The initiation codons on the vast majority of mRNAs are recognized via the mechanism described by the scanning model (Kozak, 1989). After the 43S preinitiation complex binds to the mRNA, the 40S ribosome moves downstream to seek the initiation codon. mRNA secondary structures are thought to be removed by eIF4A. Scanning consumes ATP (Kozak, 1980). Perhaps ATP is only consumed for eIF4A to perform its helicase function; the movement of ribosomes per se may just be a linear diffusion without consuming ATP (Pestova and Hellen, 2000). The 43s preinitiation complex continues scanning until it recognizes a start codon on the mRNA. The nucleotide sequence flanking the start codon is critical for its recognition (discussed in section 1.2.2.1). Once the AUG codon is recognized, the preinitiation complex binds stably, mainly through the anticodon-codon interaction between the Met-tRNA$_i$ and the mRNA. The correct recognition requires eIF1, eIF1A, eIF2 and eIF5 (Pestova et al., 1998; Asano et al., 2000; Donahue, 2000; Hershey, 2000; Pestova and Hellen, 2000). Following initiation codon recognition, eIF5 activates eIF2γ GTPase activity; the GTP bound to eIF2 is hydrolyzed to GDP, triggering the dissociation of the eIF2•GDP and other initiation factors from 40S subunit. Accompanying the dissociation of the initiation factors, eIF5B•GTP hydrolyzes its bound GTP. eIF5B is thought to occupy the ribosomal A site together with eIF1A, as do their prokaryotic homologues IF2/IF1 do (Roll-Mecak et al., 2001). The hydrolysis triggers eIF5B•GDP dissociation from the 40S subunit and the subsequent joining of 60S ribosomal subunit to form the 80S ribosome. It may also induce the ribosome to change its conformation and become elongation-competent (Pestova et al., 2000; Pestova, 2000; Roll-Mecak et al., 2001).

1.1.1.5 ***Ribosome Shunting.*** An alternative mechanism by which ribosomes reach the initiation codon is through ribosome shunting. Shunting was first found to be used in the translation initiation by cauliflower mosaic virus (CMV) (Fütterer et al., 1990; Fütterer et al., 1993), and later in the translation of rice tungro bacilliform virus (RTBV)

(Fütterer et al., 1996), Sendai virus (Curran and Kolakofsky, 1988; Latorre et al., 1998), adenovirus (Yueh and Schneider, 1996), human papillomavirus (Remm et al., 1999), and the cellular mRNA that encodes heat shock protein 70 (Yueh and Schneider, 2000). With shunting, the preinitiation complex still binds to mRNA in a 5' cap-dependent manner and scans downstream until it is blocked by a stable hairpin structure. The ribosome then 'jumps' over this hairpin to a landing site and resumes the scanning until it recognizes a downstream AUG codon. Several mechanisms have been proposed to explain shunting in the translation of the CMV 35S mRNA (Wiklund et al., 2001). The 35S mRNA 600 nt leader contains several upstream open reading frames. The complete translation of the 5' proximal uORF is a prerequisite for ribosome shunting over the hairpin that is immediately downstream of this uORF, indicating that shunting may be a special form of reinitiation (Ryabova and Hohn, 2000).

1.1.1.6 ***Internal Initiation.*** Although cap-dependent initiation is predominant, ribosomes are also able to initiate by internal initiation model in certain cases (reviewed in Hellen and Sarnow, 2001). With internal initiation, the preinitiation complex is recruited via an internal ribosome entry site (IRES) located in the 5' leader of the mRNA, not via the cap.

Internal initiation was first discovered to be used for the translation of picornaviral mRNAs (Jang et al., 1988; Pelletier and Sonenberg, 1988), and subsequently for the translation of many other mRNAs encoding viral proteins, growth factors, transcription factors, oncogenes, transporters/receptors, translation factors, activators of apoptosis, dendritically localized proteins, as well as other proteins (Hellen and Sarnow, 2001; Vagner et al., 2001). An IRES database is available at http://www.rangueil.inserm.fr/ IRES database. However, the experimental assay for assigning translation of the mRNA as IRES-mediated process must be designed carefully and the results from these assays must be interpreted cautiously (Kozak, 2001), so the number of authentic IRES elements is likely lower than the literature suggests.

An IRES, typically with a length of 400~500 nucleotides, is located in the 5'-leader region of the mRNA. There are no obvious similarities in terms of sequence, size, or structure that are identified between different IRES elements except for those on viral mRNAs from the related families. However, the integrity of secondary structure, or some

conserved motifs in certain IRES elements, is generally required to maintain IRES function. This implicates that higher-level RNA folding as a major determinant for IRES function. Three different mechanisms have been found to be used in the initiation of viral mRNAs containing IRESs, which include eIF-dependent initiation on the mRNA of encephalomyocarditis virus (EMCV), eIF-independent initiation on the mRNA of hepatitis C virus (HCV), and eIF-independent initiation and initiator tRNA-independent translation on the mRNA of insect cricket paralysis-like viruses (CrPV).

In EMCV, the initiation AUG codon is located at the 3' border of the IRES (Kaminski et al., 1990; Kaminski et al., 1994). The 43S preinitiation complex could bind directly to the initiation codon with the assistance of eIF2, eIF3, eIF4B, eIF4G and eIF4A. eIF4E and PABP are not required (Kaminski et al., 1990; Kaminski et al., 1994; Pestova et al., 1996; Pestova et al., 1996; Lomakin et al., 2000). Although PABP is not required, the poly(A) tail enhances IRES-mediated initiation in this case (Bergamini et al., 2000; Michel et al., 2000; Svitkin et al., 2001). Individual IRES may also require specific IRES *trans*-acting factors (ITAFs) for differential IRES regulation (Hellen and Sarnow, 2001).

The IRESs in the 5' leaders of HCV-related viral transcripts are approximately 360 nt and share similarities in their structures. *In vitro*, the 40S ribosomal subunit binds stably to HCV IRES without any eIFs present, and even without the presence of Met-$tRNA_i$•eIF2•GTP ternary complex. Such binding places the initiation codon in the immediate proximity of the ribosomal P site (Pestova et al., 1998).

In CrPV mRNA, an ORF encoding the structural protein precursor is separated by an intercistronic region from an upstream ORF encoding the nonstructural protein precursor. The translation of the downstream ORF is mediated by an IRES. This IRES recruits 40S ribosome in the absence of eIFs and the ternary complex. The binding of the 40s ribosome places the novel initiation codon GCU at the ribosomal A-site (not the P-site). After the assembly of the 80s ribosome, the first translocation occurs without formation of a peptide bond (Wilson et al., 2000). It is hypothesized that the ribosome P site is occupied by IRES pseudoknot, and that translocation is assisted by the interaction between the IRES and other upstream elements (Wilson et al., 2000).

Evidence has accumulated for the IRES-mediated initiation of translation of some cellular mRNAs, most of which are differentially expressed during the cell cycle or under

stress situations (reviewed in Sachs, 2000; Hellen and Sarnow, 2001; Pyronnet and Sonenberg, 2001). The mechanisms used in these cases of IRES-related initiation are less well understood. mRNA-rRNA interaction may play a role (Chappell et al., 2000) and some specific ITAFs may be involved in modulating the activity of these IRES elements (Hellen and Sarnow, 2001).

1.1.2 Elongation

Our understanding of translation elongation is mostly based on studies of this process in prokaryotes, particularly on the structural and functional studies of the prokaryotic ribosome, as well as of elongation factors EF1A, EF1B, EF2 (reviewed in Ramakrishnan, 2002). The elongation process in eukaryotic translation is thought to be analogous. Thus eukaryotic elongation factors eEF1A and eEF2 are clearly descendants of their prokaryotic counterparts (Merrick, 2000). The ribosome 'core' is also well conserved in all organisms (Spahn et al., 2001; Doudna and Rath, 2002).

Despite of similarities between eukaryotes and prokaryotes, significant differences exist. Prokaryotic elongation factor EF1B and its eukaryotic counterpart eEF1B are not well conserved; an additional elongation factor eEF3 is present in yeasts and fungi, but not in mammals (Skogerson and Wakatama, 1976). The structure of the eukaryotic ribosome is more complex than prokaryotic ribosome. It contains an additional rRNA molecule and 20-30 more proteins (Spahn et al., 2001; Doudna and Rath, 2002). The complexity of the eukaryotic ribosomes and the differences in elongation factors suggest that elongation in eukaryotes is different and may involve more complex regulations.

Nevertheless, the eukaryotic elongation process as currently understood is based on studies in prokaryotes (reviewed in Merrick, 2000; Ramakrishnan, 2002). In elongation, eEF1A•GTP binds aminoacylated tRNA to form a ternary complex. eEF1A discriminates the mischarged tRNA from correctly charged tRNA (LaRiviere et al., 2001). The ternary complex of eEF1A•GTP•aa-tRNA enters ribosomal A-site, correct codon-anticodon interaction induces conformational changes in the ribosome, which in turn triggers GTP hydrolysis by eEF1A (Pape et al., 1998). eEF1A•GDP leaves the ribosome and recycles back to eEF1A•GTP through the action of eEF1B. The cognate ternary

complex at the A-site induces this conformation change (Pape et al., 1999); ribosomes may also play a major role in monitoring the selection of the cognate tRNA (Ogle et al., 2001).

When eEF1A•GDP leaves, the tRNA is free to move its CCA end into the peptidyl transferase center on 60s subunit. The tRNA at the P-site is then deacylated and the peptide chain attached to P-site tRNA is transferred to the A-site tRNA catalyzing a new peptide bond. Following peptide bond formation, eEF2•GTP binds to the ribosome and translocation of the tRNAs and the mRNA occurs. The shape of eEF2•GTP mimics the ternary complex eEF1A•GTP•aminoacylated-tRNA (Nissen et al., 1995), and it enters the same site as the ternary complex (Agrawal et al., 1998; Wilson and Noller, 1998). Peptidyl-tRNA is forced out of the A-site and brought to the P-site, while the deacylated-tRNA at the P-site is brought to the E-site (Moazed and Noller, 1989). The whole process is driven by the energy released from hydrolysis of GTP by eEF2 (Rodnina et al., 1997). The peptidyl-tRNA possibly drags the codon of the mRNA from A-site into P-site so that the mRNA moves exactly 3 nucleotides during the translocation, maintaining the reading frame for translation. When eEF2•GDP leaves the ribosome, the next round of elongation begins.

1.1.3 Termination

The termination process starts when the stop codon enters the ribosomal A-site. Eukaryotic release factor eRF1 recognizes the stop codon at the A-site and binds to the ribosome. Binding triggers hydrolysis of the peptidyl-tRNA and the release of the nascent peptide. This in turn activates the GTPase activity of eEF3 on the ribosome to hydrolyze the GTP bound to eEF3; hydrolysis causes the release of eRF1 and eEF3 from the ribosome.

Although some crucial questions concerning the mechanism of termination used by eukaryotes remain unanswered, substantial progress has been made in the study of prokaryotic translation termination. Instead of a single release factor eRF1 that recognizes three stop codons, there are two release factors (RF1and RF2) that recognize different stop codons. Although eRF1, RF1 and RF2 all resemble tRNA, eRF1 and RF2 do not share strong similarity in structure (Song et al., 2000; Vestergaard et al., 2001).

However, a common GGQ motif is found in all eukaryotic eRF1 and prokaryotic RF1/2 (Frolova et al., 1999). Biochemical and genetic studies, along with structural analyses, indicate that the GGQ motif resembles the CCA-end at aminoacylated tRNA and mediates a nucleophilic attack on the ester bond of the peptidyl-tRNA molecule in the P site (Frolova et al., 1999; Song et al., 2000; Seit-Nebi et al., 2001). A tripeptide sequence has been functionally identified to be responsible for the specificity of codon recognition by RF1 and RF2 respectively, but there is no support for this specificity-determinant from crystal structure analysis, perhaps reflecting differences in the structures of release factors in solution and in crystals (Ito et al., 2000). Electrostatic interaction also contributes to the specific recognition by RF1/2 (Uno et al., 2002). What is involved in the specific recognition by eRF1 has not been identified.

A recent study elucidated the roles played by RF3 in translation termination (Zavialov et al., 2001). RF3 has much higher affinity for GDP than GTP, so no free RF3•GTP may exist in vivo. Instead, RF3 could bind to the ribosome in the form of RF3•GDP and the RF1/RF2-ribosome complex serves as nucleotide exchange factor for RF3•GDP. Upon hydrolysis of the ester bond by RF1/2, the conformation change would allow RF3•GDP to convert to RF3•GTP, causing a conformational change in RF3, which would now have high affinity for the ribosome, RF1/2 in turn is expelled from the ribosome. Finally GTP is hydrolyzed by RF3 and RF3•GDP is released from the ribosome free for another cycle.

In addition to the activities of release factors and other components of the translation machinery, the sequence surrounding termination codon also has an effect on termination efficiency (Bonetti et al., 1995; McCaughan et al., 1995; Major et al., 1996).

1.2 TRANSLATIONAL CONTROL OF EUKARYOTIC GENE EXPRESSION

Controlling the proper level and proper timing of protein synthesis is critical for cell growth, proliferation, and development. Translational control is a major post-transcriptional regulatory mechanism. Translational control is defined as "a change in the rate (efficiency) of translation of one or more mRNA per unit time" (Mathews et al., 2000). Compared to other regulatory mechanisms, translational control is direct, rapid

and flexible; most of time it is reversible. It provides a means to control protein synthesis in the absence of transcriptional control (Mathews et al., 2000).

The control of translation can be roughly divided into two categories: global control of translation and mRNA-specific control of the translation. Global control is achieved through regulating the activities of components in the translational machinery, such as initiation factors, elongation factors and ribosomal proteins. mRNA-specific control occurs when *cis*-acting elements in the mRNA (Figure 1.1) provide regulatory functions.

Translation can be regulated at the levels of initiation, elongation and termination. However, regulatory events appear predominantly to control initiation because initiation is typically the rate-limiting step for translation (Mathews et al., 2000). The control of translation initiation can be achieved either globally through the control of the activities of the initiation factors, or specifically through the control of the translation initiation efficiency of the open reading frames on particular mRNA.

1.2.1 Global Control of Translation

The activities of the components of the translation machinery affect the overall rates of protein synthesis. The global rate of translation is tightly regulated in response to exogenous stimuli, energy levels inside the cell, environmental changes, viral infections and other influences. The control of the global translation rate is mainly achieved through the regulation of the activities of key components of the translation machinery. Many of these components are phosphoproteins (Hershey, 1989; Mathews et al., 2000), including initiation factors eIF2, eIF2B, eIF3, eIF4E, eIF4B, eIF4G, eIF5 and eIF5B, elongation factors eEF1A and eEF2; ribosomal protein S6. Consequently, the control of phosphorylation of these proteins is the major translational control mechanism used by eukaryotic cells (Mathews et al., 2000).

Apart from control through modulating phosphorylation, other control mechanisms are also used to control the activities of translation factors, such as the regulatory mechanisms that are used by viruses or apoptotic cells to control the availability and integrity of eIF4G. Reviewed below are the well-established regulatory

mechanisms that control the functions of eIF2, eIF4E, eIF4E binding proteins (eIF4E-BP), eIF4G, eEF2 and ribosomal protein S6.

1.2.1.1 eIF2. eIF2 in the form of eIF2•GTP is responsible for selecting the initiator tRNA and delivering it to the 40s ribosomal subunit. eIF2•GDP leaves the ribosome after translation is initiated and must be recycled back to eIF2•GTP to participate in another round of initiation. The recycling rate determines the rate of translation initiation. This makes eIF2 the central control element for translation.

The replacement of the GDP bound to eIF2 with GTP is catalyzed by the guanine nucleotide exchange factor eIF2B. The rate of this replacement can be controlled through regulating eIF2 phosphorylation. The phosphorylation of eIF2 converts eIF2•GDP from an effective substrate for eIF2B into a strong competitive inhibitor for eIF2B. Because eIF2 generally is in molar excess over eIF2B, phosphorylation of only a fraction of eIF2 exerts strong inhibitory effects on eIF2B and thus on translation (Hinnebusch, 2000).

The phosphorylation site on eIF2 is identified as ser51 of the eIF2α subunit. Four eIF2α kinases have been identified: the GCN2 protein kinase in all eukaryotes, the double-stranded RNA-dependent eIF2α kinase (PKR) and the endoplasmic reticulum (ER) resident kinase (PERK) in mammals, and the heme-regulated eIF2α kinase (HRI) in vertebrates. Each is activated by different factors. This fact is reflected in their structures: they all share a conserved kinase domain but each has a unique regulatory domain (Dever, 2002).

In yeast, GCN2 can be activated by uncharged tRNA and purine nucleotide upon amino acid starvation (Hinnebusch, 2000). It can also be activated upon glucose starvation (Yang et al., 2000) or upon sodium toxicity (Goossens et al., 2001). The activation of GCN2 upon amino acid starvation in turn activates expression of GCN4, a transcription activator of amino acid biosynthesis in yeast. The translational control mechanism used to control GCN4 is discussed in section 1.3.1.1.

PKR kinase is activated by dsRNA and plays an essential role in the interferon-mediated cellular antiviral response. The phosphorylation of eIF2α by PKR leads to the shut-off of translation, which blocks virus replication and induces apoptosis of infected cells (Kaufman, 2000).

PERK kinase is activated by the presence of unfolded protein inside the ER. Phosphorylation of eIF2α by PERK leads to a halt in protein synthesis and thus relieves the ER stress (Ron, 2000). Recent studies have also implicated PERK in playing an important role in maintaining cellular homeostasis (Harding et al., 2001; Scheuner et al., 2001).

HRI kinase is activated in response to iron-deficiency and its activity is down-regulated by the level of heme inside cell (Chen, 2000).

1.2.1.2 eIF4E. eIF4E, a component in the eIF4F complex, binds to the mRNA 5'-cap and facilitates the recruitment of 43s preinitiation complex to the mRNA (see section 1.1.1.3). The activity of eIF4E is regulated by eIF4E binding protein (eIF4E-BP, discussed in section 1.2.1.3) or by phosphorylation (Gingras et al., 1999; Raught et al., 2000). The phosphorylation of eIF4E mostly correlates with increases in the translation rate. Extracellular stimuli, such as hormones, growth factors, cytokines, and mitogens increase eIF4E phosphorylation and increase global translation rate, while environmental stresses such as heat shock or virus infection decrease both eIF4E phosphorylation and the translation rate (reviewed in Gingras et al., 1999). Phosphorylation of eIF4E is reported to enhance its cap binding ability and this is thought to be the mechanism by which it increases translation initiation (Minich et al., 1994). The major phosphorylation site of mammalian eIF4E is Ser209 (Flynn and Proud, 1995; Whalen et al., 1996). Structural analyses indicate that phosphorylation of Ser209 may play a positive role in enhancing eIF4E's binding to the cap (Marcotrigiano et al., 1997). Mnk1 has been identified as the candidate kinase that phosphorylates eIF4E at Ser209 (Fukunaga and Hunter, 1997; Waskiewicz et al., 1997; Waskiewicz et al., 1999). However, the phosphorylation of eIF4E at Ser209 is not required for cap-dependent translation in vitro and in vivo (McKendrick et al., 2001; Morley and Naegele, 2002), but has other roles in cell growth control (Lachance et al., 2002).

1.2.1.3 eIF4E-BP. eIF4E binding proteins (4E-BPs), a family of three small peptides, negatively regulate the availability of eIF4E. 4E-BPs competes with eIF4G for eIF4E binding by acting as molecular mimics of eIF4E binding site of eIF4G (Marcotrigiano et al., 1999). This competitive binding interferes the formation of the eIF4F complex responsible for forming the 43S preinitiation complex. The binding of

4E-BP to eIF4E is reversible and the activities of 4E-BPs are regulated by their phosphorylation. Hyperphosphorylation of 4E-BP decreases 4E-BP binding to eIF4E, while hypophosphorylation of 4E-BP increases binding (reviewed in Gingras et al., 1999; Raught et al., 2000). Numerous extracellular stimuli, including hormones, growth factors, mitogens, and cytokines, induce the hyperphosphorylation of 4E-BP1. In contrast, nutrient deprivation, environmental stress, and certain viral infections can induce the dephosphorylation of 4E-BP (Gingras et al., 1999; Raught et al., 2000). The phosphorylation status of 4E-BP also controls its proapoptotic function (Li et al., 2002 and reference therein). Six phosphorylation sites have been identified in 4E-BP1 (numbering according to the human polypeptides): Thr37, Thr46, Ser65, Thr70, Ser83, and Ser112 (Fadden et al., 1997; Heesom et al., 1998). A stepwise, hierarchical scenario of 4E-BP phosphorylation has been proposed: Thr37 and Thr 46 are first phosphorylated by kinase FRAP/MTOR. The kinase P13K/Akt, a downstream effecter of phosphoinositide 3'-OH kinase, then phosphorylates Thr70 and Ser 65 (Gingras et al., 1999; Gingras et al., 2001).

1.2.1.4 eIF4G. eIF4G is phosphorylated in response to various stimuli (reviewed in Gingras et al., 1999; Raught et al., 2000). The phosphorylation sites on eIF4G are mapped (Raught et al., 2000), but the intracellular signaling pathway and the function of that governs phosphorylation of eIF4G are not well understood. eIF4G is also cleaved upon during viral infection (Gingras et al., 1999) and apoptosis (Clemens et al., 1998; Marissen and Lloyd, 1998). eIF4G cleavage generates two fragments: the N-terminal fragment contains the eIF4E and PABP binding domains, the C-terminal fragment contains sites for the interaction with eIF4A, eIF3, and Mnk1. Cap-dependent translation is largely inhibited after cleavage of eIF4G. eIF4G cleavage induces the internal initiation of viral mRNA translation and inhibits the cap-dependent translation of host proteins during the replication of several picornaviruses including foot and mouth disease virus, human rhinovirus and poliovirus (Foeger et al., 2002 and references therein). eIF4G cleavage during apoptosis may also trigger the IRES-mediated translation of death proteins (Henis-Korenblit et al., 2002 and reference therein).

1.2.1.5 eEF2. eEF2 can be phosphorylated at its N-terminal GTP-binding region, and this phosphorylation inhibits its binding to the ribosome, thereby reducing the

rate of translation elongation (reviewed in Proud, 2000). The eEF2 kinase is a Ca^{2+}/calmodulin-dependent kinase (Ryazanov et al., 1988), which is in turn can be phosphorylated by other kinases in response to different signals. These other kinases include the Ca^{2+}/calmodulin-dependent, cAMP-induced protein kinase (Redpath and Proud, 1993; Redpath and Proud, 1993; Proud and Denton, 1997; Hovland et al., 1999; Diggle et al., 2001), the stress-activated protein kinase SAPK4/p38δ (Knebel et al., 2001), $p90^{RSK1}$ in the MEK/Erk pathway and p70 S6 kinase in the mTOR (mammalian target of rapamycin) pathway (Wang et al., 2001).

*1.2.1.6 **Ribosomal Protein S6.*** S6, one of the ribosomal proteins in the 40S subunit, is located at the interface between the two ribosomal subunits and has direct contact with the large subunit 28S rRNA (Nygard and Nika, 1982; Nygard and Nilsson, 1990). It may also have contacts with some translation factors as shown by cross-linking experiments (Nygard and Nilsson, 1990). The phosphorylation of S6, which is catalyzed by p70 S6K, correlates with the increased translation of TOP (Terminal Oligo Pyrimidine) mRNAs, which encode components of the translation machinery (Fumagalli, 2000; Volarevic and Thomas, 2001). p70 S6K is activated in response to various stimuli, such as growth factors (Grammer et al., 1996), oxidative stress (Wang and Proud, 1997), and amino acid (Hara et al., 1998). The fact that S6 may be involved in the regulation of ribosome biogenesis thus reflects its important role in cell proliferation.

1.2.2 mRNA-Specific Control of Translation

Individual mRNA contains structural elements that can play crucial roles in controlling their own translation (Figure 1.1). These elements are located in both 5' leader and 3' untranslated regions (UTR). The structural features in the 5' leader include 5'-cap, RNA secondary structure, RNA-protein binding sites, upstream AUG codons (uAUGs) and upstream open reading frames (uORFs), and internal ribosome entry sites (IRESs). These *cis*-acting elements mainly play roles in regulating the translational efficiency of the mRNA. On the other hand, the *cis*-acting elements in the 3'UTR can exert regulatory roles in translation through controlling the cytoplasmic polyadenylation of mRNA, the temporal and spatial pattern of mRNA translation, and the stability of mRNA. The regulatory roles played by these *cis*-acting elements are summarized below.

1.2.2.1 The Regulatory Roles Played by Cis-Acting Elements in 5' Leader.

1.2.2.1.1 The Length of the 5' Leader. The length of the 5' leader typically ranges from 20 nt to 200 nt (Kozak, 1987; Mignone et al., 2002). Shortening the length between the cap and the first AUG codon to less than 20 nt may impair the recognition of this AUG codon and promote leaking scanning (Kozak, 1991).

1.2.2.1.2 The Cap Structure. The 5' m^7G cap structure is present on all known eukaryotic mRNAs and viral mRNAs except mRNAs from picornaviruses and retroviruses. Recognition and binding of the cap by eIF4E is the critical point in cap-dependent translation initiation (discussed above). The cap structure also plays a central role in maintaining mRNA stability (Wilusz et al., 2001). The decapped mRNA becomes susceptible to the 5' to 3' exonucleolytic degradation by the cytoplasmic exonuclease (Beelman et al., 1996; LaGrandeur and Parker, 1998), while the binding of eIF4E to the cap could block the access of the decapping enzyme (Vilela et al., 2000).

1.2.2.1.3 The Context of the Initiation Codon. The nucleotide sequence flanking the start codon affects the efficiency with which it is recognized by the scanning ribosome (Kozak, 1986; Kozak, 1987). The optimal initiation context has been identified as (A/G)CCaugG (Kozak, 1986; Kozak, 1987; Kozak, 1989; Kozak, 1991). Although some variations in this consensus context are found among different eukaryotic groups, the preference of a purine at the –3 position is still maintained (Cavener and Ray, 1991).

1.2.2.1.4 The Secondary Structure. The secondary structure in the 5' leader can exert both positive and negative effects on translation initiation, depending on the thermodynamic stability and the position of the structure (Kozak, 1991). A small amount of less-stable secondary structures ($\Delta G = -19$ kcal/mol) near the AUG codon enhances its recognition by the scanning ribosome, probably because these structures slow down scanning (Kozak, 1990). However, secondary structure between the cap and the AUG codon usually impairs translation initiation (Kozak, 1991). The secondary structure renders its strong inhibiting effect on initiation when it is close to the cap (less than 12 nt), or when it is very stable ($\Delta G = -50$ or -60 kcal/mol). eIF4A is thought to be responsible for unwinding secondary structure, and the inhibitory effect of strong secondary structures is alleviated by increasing eIF4A level in vitro (Svitkin et al., 2001).

Importantly, strong secondary structures that can't be unwound by the preinitiation complex are penetrable by the 80S ribosome during elongation (Kozak, 1989).

1.2.2.1.5 The Protein Binding Site. Specific RNA secondary structures also serve as binding sites for regulatory proteins. Such binding could block the preinitiation complex from loading or scanning, inhibiting translation initiation. The best studied examples are the "iron-responsive elements" (IREs)in mRNAs encoding ferritin (Rouault et al., 1988), erythroid 5-amnolevulinate synthetase (Dandekar et al., 1991), mitochondrial aconitase (Gray et al., 1996; Kim et al., 1996), succinate dehydrogenase b of Drosophila melanogaster (Kohler et al., 1995; Gray et al., 1996), and the intestinal iron exporters ferriportin (Donovan et al., 2000) and IREG1 (McKie et al., 2000). When the level of intracellular iron is low, a specific trans-acting cytoplasmic binding protein "IRE-binding protein" (IRE-BP) binds to the IRE and blocks the ribosome from scanning.

1.2.2.1.6 The Alternative Initiation Codons. There is accumulated evidence that several isoforms of a protein can be generated via alternative initiation at upstream AUG (uAUG) codons or even non-AUG codons in the 5' leader region. The regulatory mechanisms for alternative initiation are not well understood. In addition to cis-acting elements within the transcript, some trans-acting factors also may participate in this process.

Most proteins isoforms generated by the alternative initiation have roles in cell growth and differentiation. Some examples are C/EBP α, C/EBP β (Ossipow et al., 1993), eIF4GI (Byrd et al., 2002), JunD (Yazgan and Pfarr, 2001; Short and Pfarr, 2002), glucocorticoid receptor α (Yudt and Cidlowski, 2002), fibroblast growth factor-2 (reviewed in Okada-Ban et al., 2000), osteogenic growth peptide (Bab et al., 1999), transcription regulatory factor Egr3 (O'Donovan and Baraban, 1999) and p55PIK, one of the regulatory subunits of phosphoinositide (phosphatidylinositol) 3-kinase (Xia and Serrero, 1999). In most cases, the isoforms have different activities. For example, the transcription activity of full-length JunD can be suppressed after the binding of Menin tumor suppressor, but the truncated form of JunD cannot bind Menin and thus transcription activation by this isoform is not affected by the level of Menin (Agarwal et al., 1999). The Jun-N-terminal kinases (JNKs) can also bind and activate full length JunD more efficiently than truncated JunD, although the JNK binding domain and three

JNK phosphorylation target sites exist in both isoforms (Yazgan and Pfarr, 2001; Short and Pfarr, 2002).

1.2.2.2 ***Regulatory Roles Played by the 3'UTR.*** The translational control mechanisms mediated by the 3' UTR of the mRNA are best understood through studies of translation events that occur during oogenesis and early embryogenesis in Drosophila and Xenopus. These studies indicate that, unlike cis-acting elements in the 5' UTR, elements in 3' UTR generally require trans-acting factors (mostly proteins) for function. Translational control mediated by 3' UTRs is tightly coupled to cytoplasmic polyadenylation; localization and stability of mRNAs. The control mediated by 3' UTR can be critical for cell-cycle progression and establishing polarity with embryo (for reviews see Decker and Parker, 1995; Johnstone and Lasko, 2001; Macdonald, 2001; Kloc et al., 2002).

The translational activity of the mRNA may be tightly associated with its polyadenylation, as shown by studies of translational control in *Xenopus* oogenesis (for review see Mendez and Richter, 2001). In immature oocytes, the translation of Mos, cyclin A1, B1 and B2 and several other mRNAs are repressed and are only activated upon maturation. In the repressed state, the poly(A) tails on these transcripts are short (20~40 nt); they are extended to approximately 150 nt when translation is activated. As revealed by studies on *c-mos*, repression and activation are mediated by the *cis*-acting cytoplasmic polyadenylation element (CPE) located within the 3' UTR of (Wickens et al., 2000).

CPE, which has the general consensus sequence UUUUUAU (Mendez and Richter, 2001), is bound by the CPE binding protein (CPEB). CPEB itself is bound by another protein known as Maskin, which in turn binds to eIF4E. Such binding inhibits the binding of eIF4E to eIF4G, thus blocking the formation of the eIF4F complex and repressing initiation (de Moor and Richter, 1999; Stebbins-Boaz et al., 1999).

CPE-mediated translation activation is triggered by the phosphorylation of CPEB at Ser174 by Eg2 kinase (Mendez et al., 2000). Phosphorylated CPEB recruits "cleavage and polyadenylation specificity factor" (CPSF) to its specific binding sequence, the hexanucleotide AAUAAA, which is 20~30 nt downstream of CPE. CPSF in turn recruits poly(A) polymerase (PAP) to elongate the poly(A) tail (Mendez et al., 2000). The

elongation of the poly(A) tail leads to translational activation of the mRNA. The possible mechanism for activation is that PABP is recruited to the longer poly(A) tail, and this poly(A)-bound PABP binds to eIF4G, competing with Maskin for eIF4E and allowing the formation of eIF4F.

The regulation of spatial expression patterns mediated by 3' UTRs is elucidated through studies of mRNAs encoding proteins expressed during early *Drosophila* embryogenesis, such as *Oscar*, *nanos* and *gurden* mRNAs (for review see Johnstone and Lasko, 2001; Kloc et al., 2002). The Oscar protein is localized and expressed at the embryo's posterior pole. The translation of *Oscar* mRNA is repressed during its transport to posterior pole. The interaction of Bruno protein with the *cis*-acting elements in the *Oscar* 3' UTR known as Bruno response elements (BREs) is required for repression (Kim-Ha et al., 1995; Webster et al., 1997). Additional *trans*-acting factors may also be required for repression (Johnstone and Lasko, 2001).

Like *Oscar* mRNA, *nanos* mRNA is also only translated at the posterior pole, but it is not concentrated at posterior pole (96% of the *nanos* mRNA is not at the posterior pole (Bergsten and Gavis, 1999)). The translation repression of unlocalized *nanos* mRNA requires a translational control element (TCE) in the 3' UTR, and the *trans*-acting factor, Smaug, which interacts with the TCE (Gavis et al., 1996; Dahanukar et al., 1999; Smibert et al., 1999). Additional *cis*-acting elements in the 3' UTR and other *trans*-acting factors are also required for repression (Crucs et al., 2000). The repression of *nanos* mRNA translation likely occurs during elongation (Clark et al., 2000; Markesich et al., 2000).

The repression of *gurken* mRNA at the dorsoventral axis of *Drosophila* also requires the same *cis*-acting element BRE and those *trans*-acting factors required for the repression of *Oscar* mRNA (Kim-Ha et al., 1995; Cooperstock and Lipshitz, 2001). This suggests that the translational regulation of these two mRNAs is coordinated.

Cis-acting elements in 3' UTRs are also involved in the control of mRNA stability (for review, see Wilusz et al., 2001). These control mechanisms are not discussed here.

1.3 TRANSLATIONAL CONTROL BY uORFS

The presence of uORFs in 5' leader regions is a widespread feature of eukaryotic mRNAs, and there is ample evidence that uORFs can mediate translational control (reviewed in Lovett and Rogers, 1996; Geballe and Sachs, 2000; Morris and Geballe, 2000; Tenson and Ehrenberg, 2002). More than 3% of mRNAs in *S. cerevisiae* are estimated to contain one or more uORFs(Vilela et al., 1998). A recent computational analysis indicated that 15% of examined human mRNAs (2312 in total) contain uORFs. The occurrence rate is 32% in the subset of mRNAs encoding transcription factors, growth factors and their receptors, proto-oncogenes, and other regulatory proteins that are poorly translated under normal conditions (Davuluri et al., 2000). These data are reasonably consistent with an early survey (Kozak, 1987). However, despite the increasing availability of sequence data and advances in computer analysis, the reliability of these data must be questioned because of the problems in precisely defining the 5' leaders of mRNAs from cDNA data, such as accounting for the existence of multiple transcripts resulting from alternative transcription initiation or splicing (Kozak, 1996; Kozak, 1999; Kozak, 2000; Kozak, 2001).

Table 1.1 lists those mRNAs that contain uORFs known to control translation. Generally, the translation of uORFs represses the translation of the downstream gene (Morris and Geballe, 2000). In the presence of a uORF, the ribosome can reach the downstream initiation codon *via* leaking scanning or reinitiation. In leaking scanning, a population of the ribosomes may neglect and pass the uORF initiation codon and initiate translation at the downstream initiation codon. The nucleotide sequence and the RNA secondary structures flanking the uORF initiation codon help determine the efficiency of the uORF translation (Kozak, 1999). In reinitiation, ribosomes start translation at a uORF initiation codon; after terminating uORF translation, ribosomes reinitiate translation at the downstream ORF. Recently, an *in vitro* assay was developed to distinguish between leaking scanning and reinitiation (Gaba et al., 2001; Sachs et al., 2002).

The translation of the uORF can also control mRNA stability. In this case, the presence of a uORF mimics the presence of a premature stop codon in the mRNA. The

Table 1.1

Genes with uORFs Known to Be 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*	(Schleiss et al., 1991; Degnin et al., 1993; Alderete et al., 1999)
	influenza NB/NA	(Williams and Lamb, 1989)
	reovirus S1	(Fajardo and Shatkin, 1990; Belli and Samuel, 1993)
	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 and Mertz, 1988; Sedman et al., 1989)
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	(Lincoln et al., 1998; Calkhoven et al., 2000)
	C/EBPß	(Lincoln et al., 1998; Calkhoven et al., 2000)
	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., 1999; Child et al., 1999)
	huntingtin	(Lee et al., 2002)
	lck	(Marth et al., 1988)
	major vault protein	(Holzmann et al., 2001)
	mdm-2	(Brown et al., 1999)
	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*	(Zimmer et al., 1994; Reynolds et al., 1996)
	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)

[4]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.

translation of uORFs can thus cause the mRNA to be degraded by the nonsense mediated decay (NMD) pathway (reviewed in Jacobson and Peltz, 2000; Maquat, 2000).

The detailed molecular mechanisms by which uORFs regulated translation are still not well understood. uORFs can have effects that are either independent or dependent on their coding sequence. Some well-characterized examples of genes whose translation is regulated by sequence independent or dependent mechanism are summarized below.

1.3.1 Examples of Genes Regulated by uORFS Independent of Their Coding Sequence

1.3.1.1 ***Yeast GCN4.*** Translational control by GCN4 uORFs represents the best-studied example of their capacity to regulate expression via reinitiation (reviewed in Hinnebusch, 1996; Hinnebusch, 1997). GCN4 mRNA encodes a general transcription activator for more than 500 genes involved in amino acid biosynthesis. The translation of GCN4 is repressed under non-stress conditions. Under stress conditions, such as amino acid starvation or glucose starvation, the translation of GCN4 is significantly enhanced.

The 5' leader of *GCN4* mRNA is 590 nt long and contains 4 uORFs located in the region 150-360 nt upstream of *GCN4* initiation codon, each of these uORFs encodes either a dipeptide or tripeptide. A *GCN4* transcript lacking the start codons for all 4 uORFs is translated at high efficiency under both non-starvation and starvation conditions. Not every uORF is required for the translational control of *GCN4*; uORF1 and uORF4 are sufficient to retain virtually wild-type regulation. Each of these uORFs has a different role in regulation. It is not the peptide sequences encoded by these uORFs, but the nucleotide sequences surrounding the uORF stop codons that are critical for their function. uORF1 serves as a reinitiation 'promoter'. Essentially all the ribosomes initiate translation at uORF1; after translating uORF1, 50% of the ribosomes reinitiate at a downstream initiation codon. The AU-rich sequence at the third codon and immediately 3' of uORF1 is required for uORF1 to promote reinitiation. In contrast, uORF4 serves as an inhibitor to reinitiation. uORF4 inhibits 99% of the ribosomes that have translated it from reinitiating at downstream. The GC rich sequence following the uORF4 termination codon is necessary for its inhibitory activity. Replacing the corresponding

AU rich region of uORF1 with the GC rich region of uORF4 converts uORF1 into an efficient reinitiation barrier. The evidence that the translation of downstream ORF is *via* reinitiation but not *via* leaking scanning has been provided by *in vivo* genetic studies, as well as by *in vitro* primer extension inhibition assay (Gaba et al., 2001). The molecular mechanism for uORF1/4-mediated control is not understood yet; it has been proposed that the different strengths of base pairing between the mRNA and the rRNA result in these different effects (Hinnebusch, 1997).

These *cis*-acting elements, as well as the phosphorylation status of eIF2α, function together to determine the translation efficiency of *GCN4*. The phosphorylation of eIF2α affects the recycling rate of eIF2•GTP (discussed in section 1.2.1.1). Under non-starvation conditions, ribosomes translate uORF1 and resume scanning on the mRNA. Plentiful Met-tRNA$_i$•eIF2•GTP allows scanning ribosomes to acquire this ternary complex and to be able to reinitiate efficiently at downstream uORFs. The translation of uORF4 (or uORF3) disallows reinitiation at the downstream *GCN4* start codon and thus the translation of *GCN4* is repressed. Upon amino acid starvation or glucose starvation, eIF2α is phosphorylated by the GCN2 kinase, which is activated by uncharged tRNA or purine nucleotides. This phosphorylation impairs the recycling of eIF2•GTP and in turn reduces the availability of the ternary complex. Therefore, the scanning ribosomes that have translated uORF1 are less likely to acquire the ternary complex before reaching uORF3 or uORF4 initiation codons, and have to bypass them. Their continuous scanning gains time for them to reacquire the ternary complex to become capable of reinitiating translation at *GCN4*. Consistent with this model, extending the distance between uORF1 and uORF4 reduces initiation at *GCN4* under starvation conditions.

1.3.1.2 ***Other Genes with GCN4-Like Translational Control.*** *Neurospora crassa cpc-1* serves same function as *S. cerevisiae GCN4* (Paluh et al., 1988; Ebbole et al., 1991; Luo et al., 1995). The 720-nt leader of the *cpc-1* transcript contains two uORFs, which encode peptides of 2 and 41 residues respectively. The two uORFs presumably function to regulate the expression of *cpc-1* as uORF1 and uORF4 in GCN4 transcript do in response to stress conditions.

Mammalian activating transcription factor 4 (*ATF4)* contains 2 evolutionarily conserved uORFs in its 5' leader (Harding et al., 2000). The second uORF overlaps with *ATF4* coding region. Translation of *ATF4* is activated upon eIF2α phosphorylation by PERK in response to unfolded proteins in the ER (see section 1.2.1.1). Under this stress condition, the translation of *ATF4* increases by three-fold. The two uORFs repress the translation of a reporter by 500-fold under non-stress condition and are required for derepressing translation under stress. Eliminating either uORF start codon causes high level translation of the reporter and abolishes positive regulation under stress condition.

1.3.1.3 ***Mammalian CD36.*** Mammalian CD36 is a scavenger receptor involved in angiogenesis, atherosclerosis, inflammation, and lipid metabolism (reviewed in Febbraio et al., 2001). The CD36 transcript contains 3 uORFs in its 200 nt leader, and the expression of CD36 correlates with the glucose level. The elimination of uORF1 abolishes glucose-repressive translation, while elimination of uORF3 does not. The reporter gene analyses study indicate that ribosomes translate CD36 via reinitiation after translating uORF1, and that reinitiation efficiency increases with increases in glucose (Griffin et al., 2001).

1.3.1.4 ***Cauliflower Mosaic Virus (Camv) 35S ORF VII.*** The 612-nt 5' leader of CAMV 35S ORF VII contains nine small uORFs with the coding capacities of 2 to 35 codons. It can fold into an elongated hairpin structure comprising three main stems. Studies indicate that the translation of CAMV 35S ORF VII occurs via ribosome shunting (discussed in section 1.1.1.5). Efficient ribosome shunting requires cis-acting elements in 5' leader, which include 5' cap, 5' proximal uORF1, stem section 1, as well as a proper distance (5-10 nt) between uORF1 and stem section 1. When the four-codon uORF1 is replaced with a six-codon uORF whose translation inhibits polypeptide and ribosome release at termination codon, the shunting is also inhibited. This observation has led to a model that ribosome shunting is a special form of reinitiation (reviewed in Hohn et al., 2001). Reinitiation is enhanced by a viral trans-acting factor (Hohn et al., 2001; Park et al., 2001).

1.3.1.5 ***Maize Lc.*** Lc, a member of the maize (Zea mays) R gene family, encodes a basic helix-loop-helix transcriptional activator in the anthocyanin biosynthetic pathway (Ludwig et al., 1989). The expression of R genes is repressed under normal

growth conditions; the overexpression of R genes lacking the 5' leader has been associated with developmental defects (Lloyd et al., 1992). The 235-nucleotide 5' leader of Lc mRNA contains a 38-codon uORF located 60 nucleotides downstream of the 5' end. The initiation codon for this uORF is in an ideal sequence context (Damiani and Wessler, 1993). An earlier study indicated that elimination of the uORF AUG codon and the other two in-frame upstream AUGs in the uORF by point mutations increased the translation of downstream reporter by 20 to 30 folds (Damiani and Wessler, 1993). A later study directly proved that the translation of the uORF is required for the repression; increasing translation of the uORF enhances repression. The data indicated that ribosomes translate the downstream ORF via reinitiation, and that repression results from inefficient reinitiation of ribosomes at downstream ORF after they translate the uORF. The uORF peptide sequence is not critical for repressing reinitiation; instead, the intercistronic sequence downstream of the uORF is important for the repression (Wang and Wessler, 1998). In addition to the inhibitory effect exerted by uORF, recent studies show that the hairpin structure in 5' leader inhibits ribosome loading at the 5' end of the Lc mRNA (Wang and Wessler, 2001).

1.3.2 Examples of Genes Regulated by Coding-Sequence-Dependent uORFs

The synthesis of the nascent peptide encoded by a uORF can regulate the movement of the ribosome that has translated it (reviewed in Lovett and Rogers, 1996; Tenson and Ehrenberg, 2002). A model has been proposed for explaining this form of the translational control (Geballe and Morris, 1994) and has been corroborated by the later studies. In this model, ribosomes translate the downstream ORF *via* leaky scanning. The nascent peptide encoded by the uORF interacts with the translational machinery either inside the exit channel or at the peptidyl transferase center to cause ribosomes to stall on the transcript. The stalled ribosomes block the access of translating scanning ribosome to the initiation codon of downstream ORF and thus reduce its translation. Although ribosome stalling is the general cause for this kind of translational control, the nascent peptides encoded by different uORFs may achieve this by different molecular mechanisms. Three best-studied examples of this form of translational control are summarized below.

1.3.2.1 Mammalian S-Adenosylmethionine Decarboxylase (AdoMetDC). Mammalian S-adenosylmethionine decarboxylase (AdoMetDC) is a key enzyme in the polyamine biosynthesis pathway. The transcript of AdoMetDC has a 330-nucleotide 5' leader, which is highly conserved between human and bovine. A uORF encoding the peptide MAGDIS is present at 14-nt from the 5' end of the transcript. This uORF has a key role both in cell-specific regulation and feedback control by polyamine levels (Hill and Morris, 1992; Hill and Morris, 1993; Ruan et al., 1994; Ruan et al., 1996).

The translation of *AdoMetDC* is suppressed in resting normal T cells and in T-cell lines with normal cellular levels of polyamines, but not in nonlymphoid cell lines; the association of a single ribosome with the *AdoMetDC* transcript is observed upon suppression (Hill and Morris, 1992). This cell-specific translational control requires the presence of the uORF, and the coding sequence of the uORF (Hill and Morris, 1993; Ruan et al., 1994). Mutagenesis study indicate that aspartic acid at the fourth codon and isoleucine or valine at the fifth codon of the uORF are required for uORF to function both in yeast and in mammalian cells (Mize et al., 1998).

The regulation of *AdoMetDC* by polyamines can be reconstituted *in vitro* in wheat germ extracts (Raney et al., 2000). Primer extension inhibition assays confirm that the translation of uORF causes ribosome stalling at its termination codon with a high level of spermidine, the half-life of the ribosome stall correlates with the concentration of polyamines (Law et al., 2001). A recent study further indicates that the high level of polyamine actually impedes the hydrolysis of the peptidyl-tRNA upon translation termination, thus slowing down the release of the ribosome from the mRNA (Raney et al., 2002).

1.3.2.2 Human Cytomegalovirus UL4. UL4 encodes a structural virion glycoprotein. The UL4 ORF is contained in three viral transcripts with an identical 3' end but different 5' leaders. Three uORFs were found to be present in the 5' leader of the most abundant transcript (Chang et al., 1989). The 22-codon uORF2 causes translational repression of the UL4 ORF. uORF2 is conserved among several clinical strains of CMV; uORF2 only acts as a cis-element to repress the translation of the downstream UL4 ORF (Degnin et al., 1993). The repression requires the peptide coding sequence. The C-

terminal coding sequence of uORF2 and the termination codon are critical for control (Schleiss et al., 1991; Degnin et al., 1993).

The initiation codon of uORF2 is not in good initiation context and most ribosomes scan past it to initiate at a downstream ORF (Cao and Geballe, 1994; Cao and Geballe, 1995). With the primer extension inhibition assay, it has been shown that a coding sequence-dependent ribosomal stall occurs at uORF2 termination codon *in vitro* and *in vivo*. This stall blocks the access of ribosomes to the downstream ORF causing translation repression of UL4 (Cao and Geballe, 1996).

A more detailed mechanism by which the synthesis of the nascent peptide encoded by uORF2 stalls ribosome has been delineated in later studies. The synthesis of the uORF2-encoded peptide impairs the hydrolysis of peptidyl-tRNA when the termination codon is in the A-site (Cao and Geballe, 1996). Ribosomal release at the termination codon can occur without the hydrolysis of the peptidyl tRNA (Cao and Geballe, 1998). Translational repression by uORF2 appears to be critical for establishing the level and timing of UL4 expression, but the uORF is not essential for viral replication (Alderete et al., 1999; Alderete et al., 2001).

*1.3.2.3 **Small Subunit of Fungal Arginine-Specific Carbamoyl Phosphate Synthetase.*** Fungi have two carbamoyl phosphate synthetases (CPS). CPS-P is involved in the pyrimidine biosynthetic pathway and is feedback inhibited by pyrimidines. CPS-A is involved in the arginine biosynthetic pathway. CPS-A is a heterodimer, located in the cytosol in S. cerevisiae and in mitochondria in filamentous fungi (Davis, 1986).

The small subunit of CPS-A, a glutamine amidotransferase, is responsible for transferring the glutamine amide nitrogen to large subunit, which catalyzes the synthesis of carbamoyl phosphate from ammonia, ATP and HCO_3. The expression of the small subunit is regulated at both transcriptional and translational level. At the transcriptional level, it is subject to the general control of amino acid biosynthesis. At the translational level, it is the only enzyme in the arginine biosynthetic pathway that is subject to the negative regulation by arginine.

In vivo, the expression of the CPS-A small subunit is increased by ten-fold upon amino acid starvation, and is repressed four-to-ten-fold by arginine (Davis, 1986; Davis and Weiss, 1988; Sachs, 1998). As discussed below, translational control is mediated by

an evolutionarily conserved uORF (Figure 1.3). This uORF is found in *S. cerevisiae CPA* in (Nyunoya and Lusty, 1984) and in *N. crassa* (Orbach and Sachs, 1991), *Magnaporthe grisea* (Shen and Ebbole, 1997), *Trichoderma virens* (Baek and Kenerley, 1998), *Aspergillus nidulans* (GenBank AJ223085), *Botrytis cinerea* and *Cochliobolus heterostrophus* (Dr. Scott E. Baker, per. comm.) *arg-2*. Among these uORFs, the functions of the yeast *CPA1* and *Neurospora arg-2* uORFs have been the best studied.

Yeast *CPA1* was among the first eukaryotic genes in which a uORF was identified. The 250-nucleotide 5' leader of the *CPA1* transcript contains a 25-codon uORF (Nyunoya and Lusty, 1984; Werner et al., 1985). The presence of this uORF is essential for regulation of *CPA1* expression. The elimination of the uORF initiation codon by *in vitro* or *in vivo* mutagenesis abolished arginine-specific repression. Studies of constitutive mutants led at the identification of mutations in the uORF that cause loss of repression by arginine; these include the introduction of premature stop codons either at 8^{th} glutamine codon or at 20^{th} tryptophan codon, the C11T mutation and the D13N mutation. Regulation appeared to occur at the translation level since none of the above mutations were reported to change the level of mRNAs (Werner et al., 1987). Additional mutagenesis studies revealed other *cis*-acting requirements for the function of *CPA1* uORF: (i) the 5' or the 3' sequences surrounding the uORF are not required for the regulation but could influence the efficiency of repression by arginine; (ii) the uORF could act in *cis* to repress other genes in high Arg, either present as a uORF in front of *GCN4* or in-frame fused to *lacZ*; (iii) the peptide sequence encoded by the uORF is necessary for the regulation (Delbecq et al., 1994); (iv) the active domain of the peptide encoded by the uORF spans from codon 6 to codon 23 (Delbecq et al., 2000). Most recently, the uORF is implicated in the nonsense mediated decay (NMD) pathway. It appears that the presence of the uORF is required for the decay of the *CPA1* mRNA by RNA surveillance proteins (Messenguy et al., 2002).

Neurospora arg-2 contains a 24-codon uORF in the 5' leader of its transcript. The genomic sequence of *arg-2* provides some evidence that the expression of *arg-2* is subject to cross-pathway control by *cp-c1*, because two *cpc-1* binding sites are present upstream of transcription starting site of *arg-2* and two are present in each of the two

Figure 1.3. Evolutionarily conserved AAP sequence. Abbreviations: *N.c., Neurospora crassa; M.g., Magnaporthe grisea; T.c., Trichoderma virens; A.n., Aspergillus nidulans; B.c., Botrytis cinerea; C.h., Cochliobolus heterostrophus; S.c.. Saccharomyces cerevisiae.*

introns within the uORF coding region (Orbach and Sachs, 1991). The expression of *arg-2* is developmentally regulated; it is transcribed at its highest level during spore germination and early exponential growth (Sachs and Yanofsky, 1991). *arg-2* repression by arginine occurs at multiple levels. Long exposure of *Neurospora* to high Arg reduces both the level of *arg-2* transcript and the translation of *arg-2*. Short exposure of *Neurospora* to high Arg reduces the association of *arg-2* transcript with polysomes, and results in a decrease in the rate of *arg-2* protein synthesis but not in the level of *arg-2* transcript. This suggests that translational control functions as an immediate response to changes in arginine availability. Analyses of the expression of *arg-2-lacZ* reporter genes show that the expression of the transcript with the wild-type *arg-2* 5' leader is repressed two-fold by a high level of arginine; the elimination of the uORF start codon, as well as other mutations that result in the translation of a truncated uORF, abolish this arginine-specific repression *in vivo*. In all of cases, arginine did not affect the level of *arg-2-lacZ* mRNA but reduced the amount of the transcripts associated with polysomes. These results indicate the presence of the uORF in *arg-2* transcript is essential and important for negative regulation of *arg-2* by arginine at the translational level (Luo et al., 1995; Luo and Sachs, 1996).

Genetic studies identified a D12N mutation in *arg-2* uORF, which like the D13N mutation in *CPA1* uORF (Werner et al., 1987), abolished the arginine-specific regulation (Freitag et al., 1996). Unlike the case with *CPA1*, additional biochemical analyses showed conclusively that this mutation eliminated translational control.

To better address the mechanism of arginine-specific translational control by the *N. crassa arg-2* uORF, a homologous *Neurospora* cell-free *in vitro* translation system was developed. Using synthetic mRNA containing wild type and mutated *arg-2* 5'-leader regions upstream of the firefly luciferase (LUC) coding region, it was established that Arg-specific regulation by *arg-2* uORF was fully reconstituted in this cell-free system (Wang and Sachs, 1997). Analyses with a primer extension inhibition assay (toeprint assay) in the *Neurospora* cell-free system demonstrated that ribosomal stalling at the uORF termination codon in the presence of high Arg is responsible for arginine-specific translational attenuation. The results from *in vivo* and *in vitro* analyses are highly consistent (Wang and Sachs, 1997). Analyses of additional mutations in the 5' leader of

arg-2 in the cell-free system indicated that the uORF itself is sufficient for the Arg-specific regulation; neither the intercistronic region nor the species of the stop codon used are required for the regulation. The stop codon itself is not required; direct in-frame fusion of the uORF coding region to the downstream LUC coding region also confers Arg-specific translational attenuation. In this case, the ribosome stalls after the uORF-coding region. That the translation of the *arg-2* uORF stalls both terminating and elongating ribosome shows that the nascent peptide encoded by *arg-2* uORF regulates the movement of translating ribosomes through a novel mechanism. Based on the function of the peptide encoded by the uORF in the 5' leader of fungal CPS-A transcript, it has been named as Arginine Attenuation Peptide (AAP) (Wang et al., 1998). The *N crassa* and *S. cerevisiae* AAPs were shown to function in *N. crassa*, *S. cerevisiae* and wheat germ extracts; their function do not to rely on the charging status of arginyl-tRNAs (Wang et al., 1999). Finally, direct biochemical evidence has been obtained that the translation of the downstream ORF in the presence of CPS-A uORF is *via* regulation of a leaky-scanning mechanism (Gaba et al., 2001).

To further study the detailed molecular mechanism by which the AAP functions, my thesis research has focused on analyzing *cis*-acting requirement for *arg-2* uORF function (Chapter 2), examining effects of existing *trans*-acting mutations that cause translation defects *in vivo* on *arg-2* regulation (Chapter 3), and investigating the regulated ribosome movement by the nascent AAP domain within a polypeptide (Chapter 4).

CHAPTER 2

EVOLUTIONARILY CONSERVED FEATURES OF THE ARGININE ATTENUATOR PEPTIDE PROVIDE THE NECESSARY REQUIREMENTS FOR ITS FUNCTION IN TRANSLATIONAL REGULATION*

2.1 INTRODUCTION

The fungi contain two carbamoyl phosphate synthetases (Davis, 1986). CPS-P produces carbamoyl phosphate for the synthesis of pyrimidines and CPS-A produces carbamoyl phosphate for the synthesis of Arg. The *Neurospora crassa arg-2* and *Saccharomyces cerevisiae CPA1* genes encoding the CPS-A small subunit are subject to a unique form of Arg-specific negative translational regulation that requires a conserved *cis*-acting peptide coding region present as an upstream open reading frame (uORF) in the 5'-leader of each transcript (Werner et al., 1987; Delbecq et al., 1994; Luo et al., 1995; Freitag et al., 1996; Luo and Sachs, 1996). Translational regulation by eukaryotic uORFs is becoming an increasingly well-documented form of genetic control (Geballe and Sachs, 2000; Sarrazin et al., 2000; Schluter et al., 2000) but there is little understanding of the mechanistic basis for uORF function in most cases.

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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.

The roles of the *arg-2* and *CPA1* uORFs in translational control have been investigated using cell-free translation extracts from *N. crassa* and *S. cerevisiae* in which Arg-specific regulation mediated by the AAP is reconstituted. When either uORF is placed in the 5'-leader of capped and polyadenylated synthetic RNA transcripts encoding firefly luciferase (LUC), synthesis of the LUC is reduced when extracts contain a high concentration of Arg (Wang et al., 1999 and references therein). The positions of ribosomes at rate-limiting steps in translation have been assessed using a primer-extension inhibition (toeprint) assay; Arg causes ribosomes to stall at the *arg-2* and *CPA1* uORF termination codons in each extract (Wang and Sachs, 1997; Wang et al., 1999). As a consequence of stalling, fewer ribosomes initiate translation at the downstream initiation codon; the stalled ribosomes appear to block ribosomes engaged in scanning (Wang and Sachs, 1997; Kozak, 1999). While ribosomes normally stall at these uORF termination codons, when the uORF coding regions are fused directly to LUC, ribosomes also stall during elongation in the region immediately downstream of these coding regions (Wang et al., 1998; Wang et al., 1999). The regulatory effects of Arg appear independent of the extent of arginyl-tRNA charging because the tRNAs appear maximally charged at concentrations of Arg substantially below those that exert regulatory effects (Wang et al., 1999).

The product of the *arg-2* and *CPA1* uORFs has been named the Arg attenuator peptide (AAP) because of its function in translational regulation (Wang et al., 1998; Wang et al., 1999). Similar peptides are encoded by uORFs in the transcripts of the corresponding genes of three other euascomycetes, *Magnaporthe grisea*, *Trichoderma virens* and *Aspergillus nidulans* (Figure 2.1). Comparisons of these uORF-encoded peptides indicate that the central amino acid sequence and the overall length are highly conserved (Figure 2.1). The function of the *M. grisea*, *T. virens* and *A. nidulans* uORFs has not been tested. Naturally occurring polymorphisms observed in the second uORF of the cytomegalovirus (CMV) UL4 transcript, whose peptide sequence is important for it to function to stall ribosomes, can result in the loss of ability to control translation (Alderete et al., 1999). Because of this, and because the *Schizosaccharomyces pombe* carbamoyl phosphate synthetase small subunit gene (SPBC56F2.09C) apparently lacks a uORF, it is important to determine whether these other fungal uORFs function in regulation.

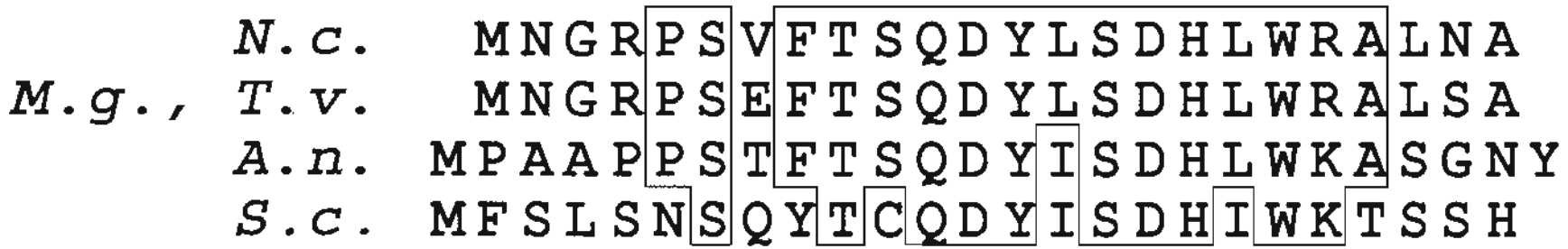

Figure 2.1. Comparisons of the AAPs from *N. crassa* (*N.c.*; Orbach et al., 1990), *Magnaporthe grisea* (*M.g.*; Shen and Ebbole, 1997), *Trichoderma virens* (*T.v.*; Baek and Kenerley, 1998), *Aspergillus nidulans* (*A.n.*; Genbank AJ224085) and S. *cerevisiae* (*S.c.*; Werner et al., 1987). Residues conserved in at least three AAPs are boxed.

Mutations in the *N. crassa* and *S. cerevisiae* AAPs that alter amino acid residues in the conserved central region eliminate regulation (Werner et al., 1987; Werner et al., 1990; Freitag et al., 1996; Luo and Sachs, 1996; Wang and Sachs, 1997; Wang and Sachs, 1997; Wang et al., 1999). But, while a primary role for the AAP coding sequence is indicated, it has not been established whether the sequence of the RNA also has a regulatory role outside of its capacity to encode the AAP. Such a regulatory role needs to be examined because RNA sequences can bind to Arg directly (Puglisi et al., 1992; Geiger et al., 1996; Yarus, 1998). Furthermore, as in the transcriptional attenuation of bacterial amino acid biosynthetic operons, specific sequences within the leader peptide's coding region might form structural elements at the level of nucleic acid critical for regulation (Yanofsky, 2000). Also, it is unclear whether other features of the AAP coding region – such as its length, irrespective of coding sequence – are important for regulation.

We examined the role of the fungal uORFs that confer Arg-specific regulation by investigating the importance of the uORF-encoded peptide sequence and the RNA sequence encoding the peptide to more fully understand the requirements for these uORFs' regulatory function. These data indicate that the most highly conserved features of these uORF-encoded peptides are both necessary and sufficient to confer Arg-specific regulation and that the nascent peptide moiety itself, and not structural features of the RNA encoding it, is responsible for regulation.

2.2 EXPERIMENTAL PROCEDURES

2.2.1 DNA Templates and RNA Synthesis

Plasmids were designed to produce capped and polyadenylated synthetic RNA encoding firefly LUC with the wild-type or mutant AAP sequences in the RNA 5'-leader region (Figure 2.2). Most plasmids (Table 2.1) contained mutations introduced by PCR with mutagenic primers (Freitag et al., 1996; Luo and Sachs, 1996) or by megaprimer PCR (Sarkar and Sommer, 1990). Others were constructed by fragment-insertion or fragment-replacement using synthetic oligonucleotides (Table 2.2). Oligonucleotides

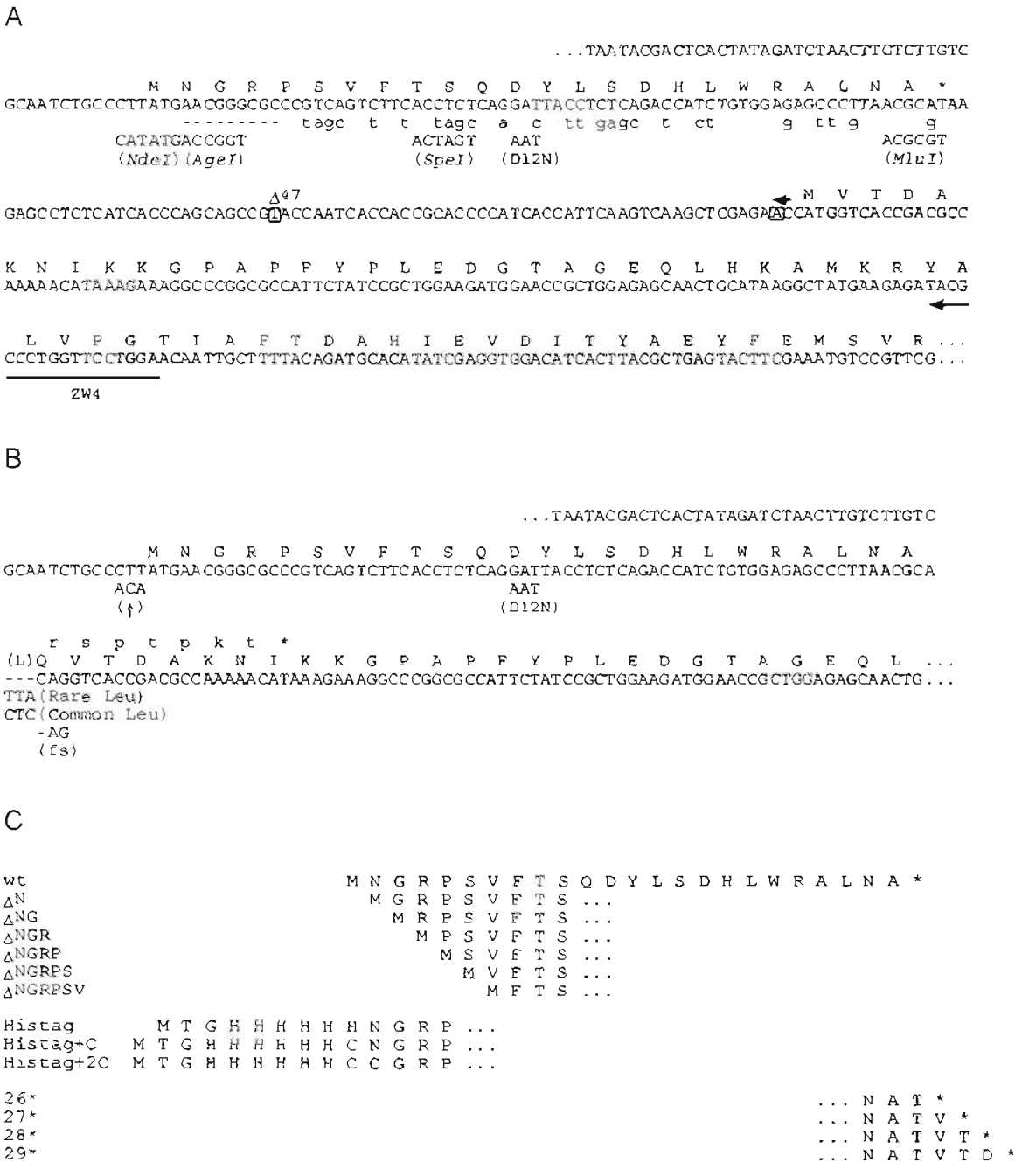

Figure 2.2. The 5' leader regions and AAP sequences of arg-2-LUC genes used in this study. (A) Sequences of wild-type and mutant templates in which the AAP is encoded as a uORF. The sequence shown begins with the T7 RNA polymerase-binding site and ends within the LUC coding region. The amino acid sequences of the arg-2 AAP and the N-terminus of LUC are indicated. Mutations that result in new restriction enzyme sites are shown below the wild-type sequence, as is the D12N mutation, which eliminates regulation. The multiple silent mutations that change the RNA sequence but not the peptide coding sequence in a shortened AAP are indicated in lower case below the wild-type sequence; short hyphens indicate deleted nucleotide sequence in this construct. 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. (B) Sequences of templates containing wild-type and mutant AAP-LUC fusion genes. The sequence shown begins with the T7 RNA polymerase-binding site and ends within the LUC coding region; the amino acid sequence of the N-terminus of the AAP-LUC fusion polypeptide is indicated. Point mutations are shown below the wild-type sequence. The (↑) mutation improves the initiation context for translation and the D12N mutation eliminates Arg-specific regulation. Common (CTC) or rare (TTA) Leu codons were inserted at codon-25 of the polypeptide as indicated (the wild-type peptide lacks a Leu codon at this position as indicated by the dashes). The 1-nt deletion within codon-25 that causes a frameshift (indicated fs) results in a predicted reading frame for a 31-residue peptide; the sequence of the frameshifted region of this polypeptide is indicated in lower case letters above the sequence of the wild-type peptide. (C) Peptide sequences of additional mutant AAPs used in this study. Sequences between ellipses match the wild-type sequence. Plasmids and oligonucleotides are described in Table 2.1, Table 2.2 and Table 2.3.

Table 2.1

Firefly LUC Constructs Made by PCR or Megaprimer PCR (MP)

	5'leader structure	Method	Forward primer	Backward primer	PCR Template/ligation vector	Restriction sites for ligation
pR301	*Age*I, *Spe*I, *Mlu*I	MP	FP24	FP25, FP26-1	pR104[1]/pR101[2]	*Bgl*II/*Nco*I
pR302	C-extension+1	PCR	FP64	FP07	pR301/pR301	*Mlu*I/*Nco*I
pR303	C-extension+2	PCR	FP65	FP07	pR301/pR301	*Mlu*I/*Nco*I
pR304	C-extension+3	PCR	FP66	FP07	pR301/pR301	*Mlu*I/*Nco*I
pR305	C-extension+4	PCR	FP67	FP07	pR301/pR301	*Mlu*I/*Nco*I
pR306	C-extension+5	PCR	FP68	FP07	pR301/pR301	*Mlu*I/*Nco*I
pR401	*Nde*I	MP	FP19	FP32	pR101/pR101	*Bgl*II/*Nco*I
pR402	N-deletion (ΔN)	PCR	FP38	FP07	PR104/pR401	*Nde*I/*Nco*I
pR403	N-deletion (ΔNG)	PCR	FP39	FP07	PR104/pR401	*Nde*I/*Nco*I
pR404	N-deletion (ΔNGR)	PCR	FP40	FP07	PR104/pR401	*Nde*I/*Nco*I
pR405	N-deletion (ΔNGRP)	PCR	FP43	FP07	pR404/pR401	*Nde*I/*Nco*I
pR406	N-deletion (ΔNGRPS)	PCR	FP44	FP07	pR404/pR401	*Nde*I/*Nco*I
pR407	N-deletion (ΔNGRPSV)	PCR	FP45	FP07	pR404/pR401	*Nde*I/*Nco*I
pSH101	D12N, His 6	MP	FP19	FP20	pRH101/pR101	*Bgl*II/*Nco*I
pRHC101	Wild type, His 6+Cys	MP	FP24	FP35	pRH101/pR101	*Bgl*II/*Nco*I

pSHC101	D12N, His 6+Cys	MP	FP24	FP35	pSH101/pR101	*Bgl*II/*Nco*I
pRHC102	Wild type, His 6+2Cys	MP	FP24	FP36	pRH101/pR101	*Bgl*II/*Nco*I
pSHC102	D12N, His 6+2Cys	MP	FP24	FP36	pSH101/pR101	*Bgl*II/*Nco*I
pRLN103	AAP-LUC fusion, 25th Rare Leu	PCR	FP01	FP58-1	pR107[3]/pR101	*Bgl*II/*BstE*II
pRLN104	D12N, AAP-LUC fusion, 25th Rare Leu	PCR	FP01	FP58-1	pSF104[4]/pR101	*Bgl*II/*BstE*II
pRLN203	AAP-LUC fusion, 25th Common Leu	PCR	FP01	FP58-2	pR107/pR101	*Bgl*II/*BstE*II
pRLN204	D12N, AAP-LUC fusion, 25th Common Leu	PCR	FP01	FP58-2	pSF104/pR101	*Bgl*II/*BstE*II
pR1031	*Nde*I in Δ47[5]	PCR	FP32	FP07	pR103/pR101	*NdeI*/*Nco*I
PRER001	PR301	PCR	FP37	FP07	PR301/pR401	*NdeI*/*Nco*I
pRER102	D12N + silent mutations	MP	FP19	FP69	pRER101/pR401	*NdeI*/*Mlu*I
pRRAT102	ΔAUG in rat uORF	PCR	FP73	FP07	pRAT101/pR101	*Bgl*II/*Nco*I

[1] pR104 is made with pLATER®EX1 mutagenesis system (Promega) (see text) to convert the AAP 2nd and 3rd codons into an *AgeI* site. The mutagenic oligonucleotide used was FP11.
[2] pR101 contains wild type AAP sequence in the 5' leader of luciferase (Wang and Sachs, 1997).
[3] pR107 is a wild type AAP-LUC fusion construct (Wang et al., 1999).
[4] pSF104 is a D12N AAP-LUC fusion construct (Wang et al., 1999).
[5] pR103 is a 47-nt deletion in the intercistronic region of pR101 (Wang et al., 1999).

Table 2.2

Firefly LUC Constructs Made by Oligonucleotide Insertion

Construct	5'-leader structure	Plus strand	Minus strand	Ligation Vector	Restriction site(s) for ligation
pRH101	Wild type His6	FP102-1	FP102-2	pR104[1]	*Age*I
pRER101	Wild type silent mutations	FP104-1	FP104-2	pRER001	*Nde*I/*Mlu*I
pRAN101	Wild type *A. nidulans* AAP	FP105-1	FP105-2	pR401	*Nde*I/*Nco*I
pRMG101	Wild type *M. grisea* AAP	FP106-1	FP106-2	pR401	*Nde*I/*Nco*I
PRRAT101	Wild type rat uORF	FP109-1	FP109-2	pR401	*Nde*I/*Nco*I

[1]pR104 was constructed using the Altered Sites® II *in vitro* Mutagenesis Systems (Promega). To place the wild type 5' leader *arg-2* sequence into the pALTER®-EX1 vector, the vector was cut with restriction enzyme *Hpa*I to produce a blunt end, oligo FP8 containing a *Bgl*II site was ligated here; then the fragment between *Bgl*II and *Nco*I in this vector was replaced with fragment between the same two sites in plasmid pR101 (Wang and Sachs, 1997).

used for plasmid constructions are listed in Table 2.3. Additional plasmids used were described previously (Wang and Sachs, 1997; Wang and Sachs, 1997; Wang et al., 1998).

Plasmid DNA templates were purified by equilibrium centrifugation (Wang and Sachs, 1997) or by using the Qiagen Plasmid Midi Prep kit; templates were linearized with *Eco*RI. Capped, polyadenylated RNA was synthesized with T7 RNA polymerase from linearized plasmid DNA templates and the yield of RNA was quantified as described (Wang and Sachs, 1997).

2.2.2 Cell-Free Translation of RNA and Primer Extension Inhibition (Toeprint) Assays

The reaction conditions for *in vitro* translation using *N. crassa* extracts were as described (Wang and Sachs, 1997; Wang et al., 1998). Cell-free translation extracts were prepared from *N. crassa* as described (Wang and Sachs, 1997). In some experiments, this preparation procedure was modified by changing the method of grinding the cells (Otero et al., 1999): following harvesting of mycelial pads and rinsing with buffer A, the mycelial pads were frozen in liquid nitrogen, placed in a pre-chilled (-70°C) mortar, and then powdered with a pestle in the presence of liquid nitrogen until a fine paste was obtained. The paste was then transferred into a 50 ml polysulfone Oak Ridge centrifuge tube (Nalgene) and thawed on ice before centrifugation. This modification improved the activity of extracts.

The primer extension inhibition (toeprint) assays were accomplished as described using primer ZW4 (Wang and Sachs, 1997); 8 µl of sample instead of 4 µl was loaded onto each gel lane. The gels were dried and exposed to screens of a Molecular Dynamics PhosphorImager for approximately 24 hours. All toeprint data shown were representative of multiple experiments.

2.3 RESULTS

2.3.1 The Conserved Fungal AAPs Function in Arg-Specific Regulation

The functions of the *M. grisea*, *A. nidulans* and rat uORFs in Arg-specific regulation were tested in parallel with the *N. crassa* uORF in the *N. crassa* cell-free

Table 2.3

Oligonucleotides Used

Oligo Name	Sequence (5' to 3')
FP1	CCGCA AGGAA TGGTG CAT
FP7	TGTTT TGGCG TCGG TGA
FP8	GGAAG ATCTTC C
FP11	CTGCC CTTAT GACCG GTCGC CCGTC AGTC
FP19	GAGCA CCGCC GCCGC AAG
FP20	TGGTC TGAGA GGTAA TTCTG AGAGG
FP21	TTTCT TTATG TTTTT GGCGT CGGTG
FP22	CCGTT GAGCA CCGCC GCC
FP23	GGTCA TTGTG GCAGA TTGCG ACAAG AC
FP24	GCCGT TGAGC ACCGC CGCCG CAAGG AATGG
FP25	GGTAA TCCTG ACTAG TGAAG ACTGA CGGGC
FP26-1	AGCCT CTTAC GCGTT AAG
FP32	GGTCA TATGG GCAGA TTG
FP35	GACTG ACGGG CGACC GGTGC AATGG TGATG GTGAT GGTG
FP36	GACTG ACGGG CGACC GCAGC AATGG TGATG GTGAT GGTG
FP37	GGAAT TCCAT ATGAC CGGTC GCCCG TCAGT C
FP38	GGAAT TCCAT ATGGG TCGCC CGTCA GTCTT C

FP39	GGAAT TCCAT ATGCG CCCGT CAGTC TTCAC C
FP40	GGAAT TCCAT ATGCC GTCAG TCTTC ACCTC T
FP43	GGAAT TCCAT ATGCA GTCTT CACCT CTCAG G
FP44	GGAAT TCCAT ATGGT CTTCA CCTCT CAGGA T
FP45	GGAAT TCCAT ATGTT CACCT CTCAG GATTA C
FP58-1	CACGG TGACC TGTAA TGCGT TAAGG GCTCT CCA
FP58-2	CACGG TGACC TGGAG TGCGT TAAGG GCTCT CCA
FP62	GTCTT CACTA GTCAG AATTA CCTC
FP64	GGCGG CACGC GTTGT AAGAG CCTCT CATCA CCC
FP65	GGCGG CACGC GTTGG TCTAA GAGCC TCTCA TCACC C
FP66	GGCGG CACGC GTTGG TCACC TAAGA GCCTC TCATC ACCC
FP67	GGCGG CACGC GTTGG TCACC GACTA AGAGC CTCTC ATCAC CC
FP68	GGCGG CACGC GTTGG TCACC GACGC CTAAG AGCCT CTCAT CACCC
FP69	CGCTC AAATA GTTTT GGCT
FP73	TCACT ATAGA TCTAA CTTGT CTTGT CGCAA TCTGC CCATC TGAGA TATT
OPF102-1	CCGGT CACCA TCACC ATCAC CATA
OPF102-2	CCGGT ATGGT GATGG TGATG GTGA
OPF104-1	TATGC CTAGC GTTTT TACTA GCCAA GACTA TTTGA GCGAT CACTT GTGGA GGGCT TTGAA
OPF104-2	CGCGT CAAAG CCCTC CACAA GTGAT CGCTC AAATA GTCTT GGCTA GTAAA AACGC TAGGCA
OPF105-1	TATGC CAGCG GCACC TTCCA CCTTC ACTTC TCAGG ATTAC ATCTC GGACC ACCTC TGGAA
	AGCTT CCGGA AACTA TTGAG AGCCT CTCAT CACCC AGCAG CCGC

OPF105-2	CATGG CGGCT GCTGG GTGAT GAGAG GCTCT CAATA GTTTC CGGAA GCTTT CCAGA GGTGG TCCGA GATGT AATCC TGAGA AGTGA AGGTG GAAGG TGCCG CTGGC A
OPF106-1	TATGA ACGGT CGCCC GTCAG AATTC ACTTC TCAGG ACTAT CTCTC AGACC ATCTG TGGAG GGCCC TGTCC GCTTA AGAGC CTCTC ATCAC CCAGC AGCCG C
OPF106-2	CATGG CGGCT GCTGG GTGAT GAGAG GCTCT TAAGC GGACA GGGCC CTCCA CAGAT GGTCT GAGAG ATAGT CCTGA GAAGT GAATT CTGAC GGGCG ACCGT TCA
OPF109-1	TATGA GATAT TTGTG ATTTA ATTTT AGTCA CAAAA CATCT TCAAA C
OPF109-2	CATGG TTTGA AGATG TTTTG TGACT AAAAT TAAAT CACAA ATATC TCA

translation system. The uORF in the rat carbamoyl phosphate synthetase gene (Nyunoya et al., 1985) encodes the peptide (MYRL*), which differs from the fungal uORF-encoded peptides. Equal amounts of each RNA sample were translated in reaction mixtures containing low (10 μM) or high Arg (500 μM); LUC activity and the positions of ribosomes on each RNA were assayed (Figure 2.3).

For each of the fungal uORFs, a high concentration of Arg caused decreased synthesis of LUC and caused ribosomes to stall at the uORF termination codon (Figure 2.3, compare lanes 2 and 1, 5 and 4, 8 and 7; relative LUC activities are indicated at the top of each pair of lanes). Thus, each of the fungal uORFs acted similarly to cause Arg-specific regulation in the *N. crassa* cell-free system. In reaction mixtures containing high Arg that were programmed with RNA containing the *N. crassa* and *M. grisea* uORFs, which showed stronger Arg-specific regulation than reaction mixtures programmed with RNA containing the *A. nidulans* uORF, additional toeprint signals were observed 21-30 nt upstream of the toeprint corresponding to the termination codon. These signals are considered likely to correspond to ribosomes stalled behind the ribosomes stalled at the termination codon (Wang and Sachs, 1997). High Arg also caused a reduction in the signal corresponding to ribosomes at the *N. crassa* and *M. grisea* uORF initiation codons. This is presumably because the toeprint assay preferentially detects ribosomes that are closest to the primer-binding site when multiple ribosomes are present on an mRNA (Wang and Sachs, 1997).

Comparison of toeprint signals from reaction mixtures programmed with RNA containing the rat uORF and RNA in which the rat uORF initiation codon was eliminated revealed ribosomes at the rat uORF's initiation codon and termination codon (Figure 2.3, compare lanes 10 and 11 with 13 and 14). Thus, the rat uORF appeared to be translated. However, in contrast to the Arg-specific regulation of LUC synthesis and of ribosome stalling conferred by each of the fungal uORFs, the rat uORF did not confer Arg-specific regulation (Figure 2.3, compare lanes 11 and 10).

2.3.2 Effects of Deleting the N-Terminus of the AAP

While the overall length of the AAP and its central region are evolutionarily conserved, the sequence of the AAP's N-terminus is not conserved Figure 2.1). Possibly,

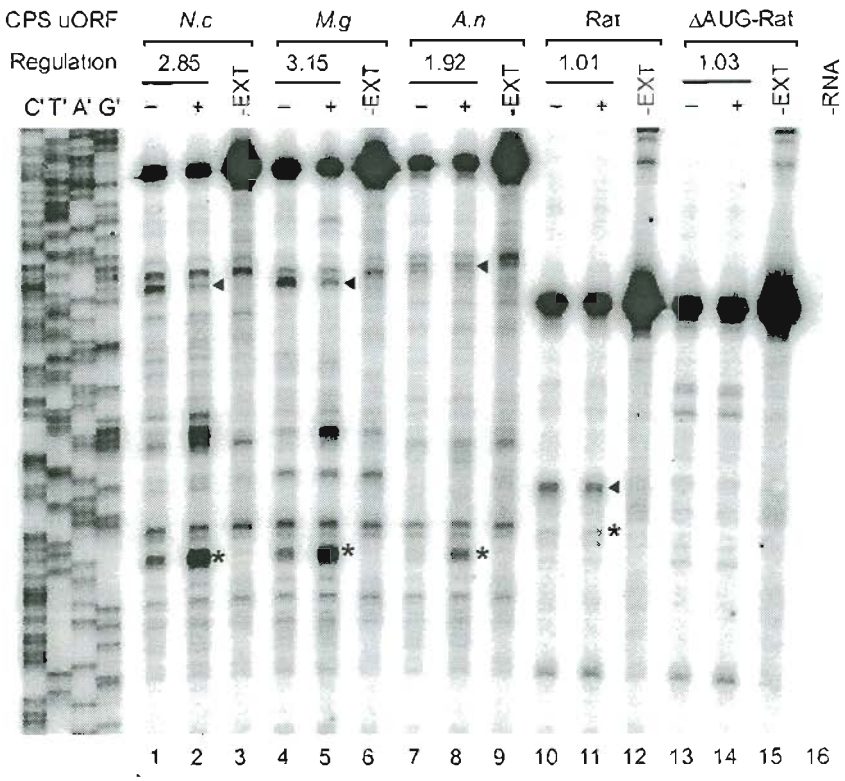

Figure 2.3. *In vitro* translation in *N. crassa* extracts of mRNAs containing uORFs. The transcripts examined are indicated at the top; the ratio of LUC activity produced after 30 min in reaction mixtures containing 10 μM Arg versus 500 μM Arg are also indicated at the top. Equal amounts of synthetic RNA transcripts (120 ng) were translated in 20 μl reaction mixtures at 25°C. Reaction mixtures contained 10 μM (-) or 500 μM (+) of Arg and 10 μM each of the other 19 amino acids. After 20 min of translation, 3 μl of the translation mixtures were toeprinted with primer ZW4 and analyzed next to dideoxynucleotide sequencing of the wt-Δ47 construct (pR1031, Table 2.1; (Wang and Sachs, 1997)). The nucleotide complementary to the dideoxynucleotide added to each sequencing reaction is indicated above the corresponding lane so that the sequence of the template can be directly deduced; the 5'-to-3' sequence reads from top to bottom. The products obtained from primer extension of each RNA (120 ng) in the absence of translation reaction mixture (-EXT, lanes 3, 6, 9, 12, 15) and from translation reaction mixture not programmed with RNA (-RNA, lane 16) are shown for comparison. The arrowheads indicate the position of the premature transcription termination products corresponding to ribosomes bound at the uORF initiation codon. The asterisks indicate the positions of premature transcription termination products corresponding to ribosomes at the uORF termination codon.

the length of the N-terminus is important, even if the sequence is not, since shortening the C-terminus eliminates function (Wang and Sachs, 1997 and below). To test this, a series of deletions were constructed to shorten the AAP at its N-terminus. First, a unique *Nde*I restriction site was introduced into the *N. crassa arg-2* sequence in the region that included the AAP initiation codon. Introduction of this site improved the initiation context of the uORF but did not change its predicted coding sequence (Figure 2.2A). Next, a series of deletions were constructed using PCR and mutagenic primers (Table 2.1). Regulation exerted by the uORF containing the *Nde*I site, and uORFs containing deletions of amino acid residues 2, 2-3, 2-4, 2-5, 2-6 or 2-7 were compared to regulation mediated by the wild-type uORF and by a uORF in which Asp-12 was mutated to Asn (D12N). The latter mutation abolishes regulation *in vivo* (Freitag et al., 1996) and *in vitro* (Wang and Sachs, 1997; Wang and Sachs, 1997; Wang et al., 1998; Wang et al., 1999).

Equal amounts of capped and polyadenylated synthetic RNAs representing each construct were translated in *N. crassa* extracts (Figure 2.4). Relative to a construct containing the uORF in the wild-type initiation context, regulation by Arg was increased by introduction of the *Nde*I site, which improves the initiation context of the uORF (Figure 2.4). Increased initiation at the uORF start codon was evident from the increased toeprint signal that corresponded to ribosomes at the uORF initiation codon in the *Nde*I construct compared to the wild-type construct in extracts containing low Arg (Figure 2.4, compare lane 5 with lane 1). This is consistent with the previously observed increases in regulation occurring when the uORF initiation context is improved by other mutations (Wang and Sachs, 1997).

Deletion of codon 2 (ΔN), codons 2 and 3 (ΔNG), or codons 2-4 (ΔNGR) did not substantially affect regulation based on LUC synthesis or ribosome stalling at the uORF termination codon (Figure 2.4). In contrast, deletion of codons 2-5 (ΔNGRP) reduced regulation based on these criteria, and deletion of codons 2-6 (ΔNGRPS) or codons 2-7 (ΔNGRPSV) eliminated regulation, as did the D12N mutation (Figure 2.4). Since codon-5 of the *N. crassa* AAP defines the beginning of the region in which all of the AAP sequences obtained to date are conserved (Figure 2.1) it appears that the regulatory function of the AAP is maintained with deletion of the N-terminus up to but not including

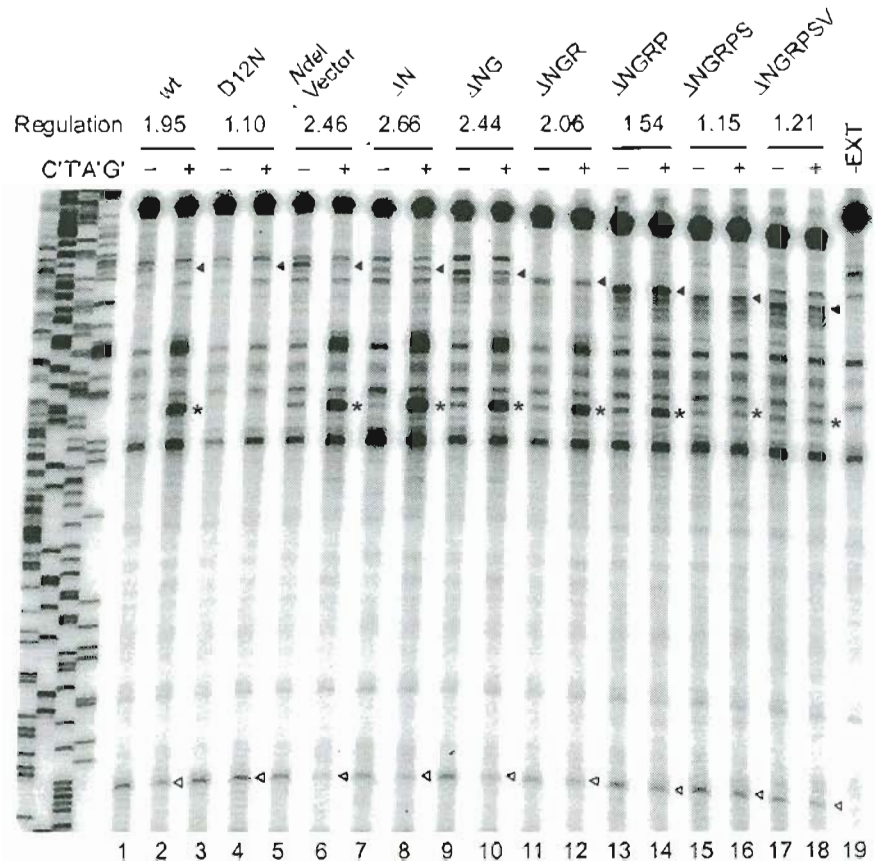

Figure 2.4. Effects of deletion of the amino acid residues at the uORF N-terminus on Arg-specific regulation. The constructs examined (Figure 2.2C) are indicated at the top; LUC activity measurements and reaction conditions were as described in Figure 2.3. Dideoxynucleotide sequencing reactions for the template encoding the wild-type AAP with the *NdeI* site (pR401, Table 2.1) are on the left. The products of a reaction obtained from primer extension of pure *NdeI*-vector RNA (120 ng) in the absence of translation reaction mixture (-EXT, lane19) are shown for comparison. Arrowheads and asterisks are as for Figure 2.3; open arrowheads indicate ribosomes at the LUC initiation codon.

the conserved region. Therefore, the results indicate that the nonconserved N-terminal segment of the AAP is dispensable for regulation.

The N-terminal region of the AAP could also be extended (Figure 2.2C) without abolishing its regulatory function (data not shown). All of the extended AAPs tested retained regulation based on LUC and toeprint assays; in contrast, similarly extended AAPs which also contained the D12N mutation showed no regulatory capacity.

2.3.3 The RNA Sequence Is Not Important for Regulation Beyond Its Capacity to Encode a Functional AAP

Is the AAP alone required for regulation, or does the RNA that encodes it also play an additional regulatory role? To test this possibility, we examined the role of the AAP-encoding RNA sequence in its entirety by effecting a radical change in the RNA sequence specifying the functional 20-residue AAP in which residues 2-4 were deleted (ΔNGR). The coding region for this shortened AAP was chemically synthesized with 26 of 63 possible base substitutions incorporated, all of them silent (Figure 2.2A). At least one nucleotide was changed in every codon, except for the Trp-codon, which cannot tolerate change, and the Asn codon near the C-terminus, which formed part of the *Mlu*I site used for cloning. A 27^{th} mutation was introduced in a second construct, in addition to the 26 silent mutations, which changed the conserved Asp to Asn; this latter mutation is predicted to eliminate regulation. We reasoned that, if the RNA sequence were not important beyond its capacity to encode the AAP, then the coding region in which over 40% of the nucleotides have been silently substituted should confer Arg-regulation, while the construct containing these silent mutations and one additional nucleotide substitution to change the important Asp residue to Asn should not.

Analyses of reporter gene activity by LUC assay and toeprint assays (Figure 2.5) indicated that the RNA sequence was not important for regulation beyond its capacity to encode the AAP. Regulation was apparent for RNA containing only silent mutations in the AAP coding sequence (Figure 2.5, compare lanes 1 and 2 with lanes 6 and 7). A single additional nucleotide substitution that changed Asp to Asn eliminated regulation (Figure 2.5, lanes 4 and 5). Therefore, the RNA sequence does not appear to have a regulatory role other than to encode a functional AAP.

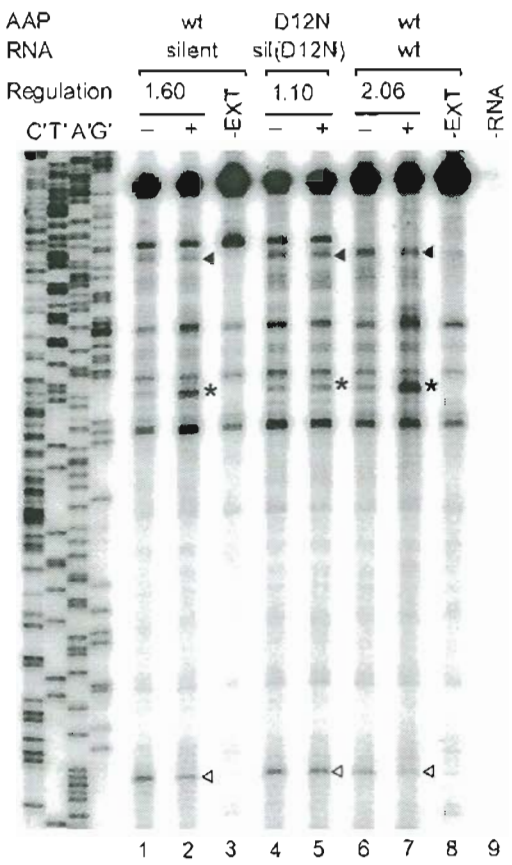

Figure 2.5. Effects of silent mutations in the RNA sequence encoding a shortened, functional AAP (ΔNGR) on Arg-specific regulation. The constructs examined (wt silent, pRER101; D12N silent, pRER102; wt, pR404) are indicated at the top; LUC activity measurements and reaction conditions were as described in Figure 2.3. Dideoxynucleotide sequencing reactions for the template containing the wild-type AAP with silent mutations (pRER101) are on the left. The products obtained from primer extension of wild-type RNA containing silent mutations (120 ng) and wild-type RNA (120 ng) in the absence of translation reaction mixture (-EXT, lane 3 and lane 8 respectively), and from translation reaction mixture not programmed with RNA (-RNA, lane 9) are shown for comparison. The arrowheads indicate the position of the premature transcription termination products corresponding to ribosomes bound at the AAP initiation codon. Arrowheads, asterisks and open arrowheads are as for Figure 2.4.

Introduction of the silent mutations into the RNA sequence slightly reduced regulation and the strength of the toeprint signal corresponding to ribosomes at the uORF termination codon (Figure 2.5). One possible reason for this, consistent with the LUC activity and primer extension data, is that these substitutions caused changes in the structure of the mRNA that reduced initiation at the AAP start codon.

2.3.4 Lengthening but Not Shortening the uORF-Encoded AAP's C-Terminus Permits Regulation

The synthesis of a polypeptide containing the wild-type *arg-2* AAP coding sequence fused directly to LUC is subject to Arg-specific regulation; ribosomes stall at sites immediately distal to the 24-codon AAP located within the LUC coding region (experiments described below; Wang et al., 1998; Wang et al., 1999). This suggested the possibility that the carboxyl-terminus of the uORF could be extended without causing loss of regulatory function. To test this possibility, a series of constructs were made to extend the uORF-encoded *N. crassa* AAP at its C-terminus; the stop codon (normally codon-25) was moved to positions 26, 27, 28, 29 or 30. As the basis for these constructs, a plasmid was used in which several unique restriction sites (*Age*I, *Spe*I, *Mlu*I) were introduced into the AAP coding region to facilitate construction; the introduction of the *Age*I site changed codon-2 from Asn to Thr and the other mutations were silent mutations (Figure 2.2A and C). Toeprint signals corresponding to ribosomes stalled at the uORF termination codon and to additional ribosomes stalled 21-30 nt upstream of ribosomes at the termination codon were similar in RNAs specifying the wild type uORF or the mutant uORF containing *Age*I, *Spe*I, and *Mlu*I sites (Figure 2.6, compare lanes 13 and 14 with lanes 7 and 8). A slight increase in regulation was observed with the mutant uORF relative to the wild-type uORF; increased regulation was associated with an increased toeprint signal corresponding to ribosomes at the uORF initiation codon (Figure 2.6, compare lanes 13 and 14 with lanes 7 and 8), consistent with observations on the effect of introducing the *Nde*I site into the uORF coding region (Figure 2.4) described above.

Additional controls were analyzed in parallel with the extended-uORF constructs (Figure 2.6). Elimination of the uORF initiation codon (ΔAUG) eliminated regulation and all translation-specific toeprint signals in the uORF coding region (Figure 2.6, lanes 1

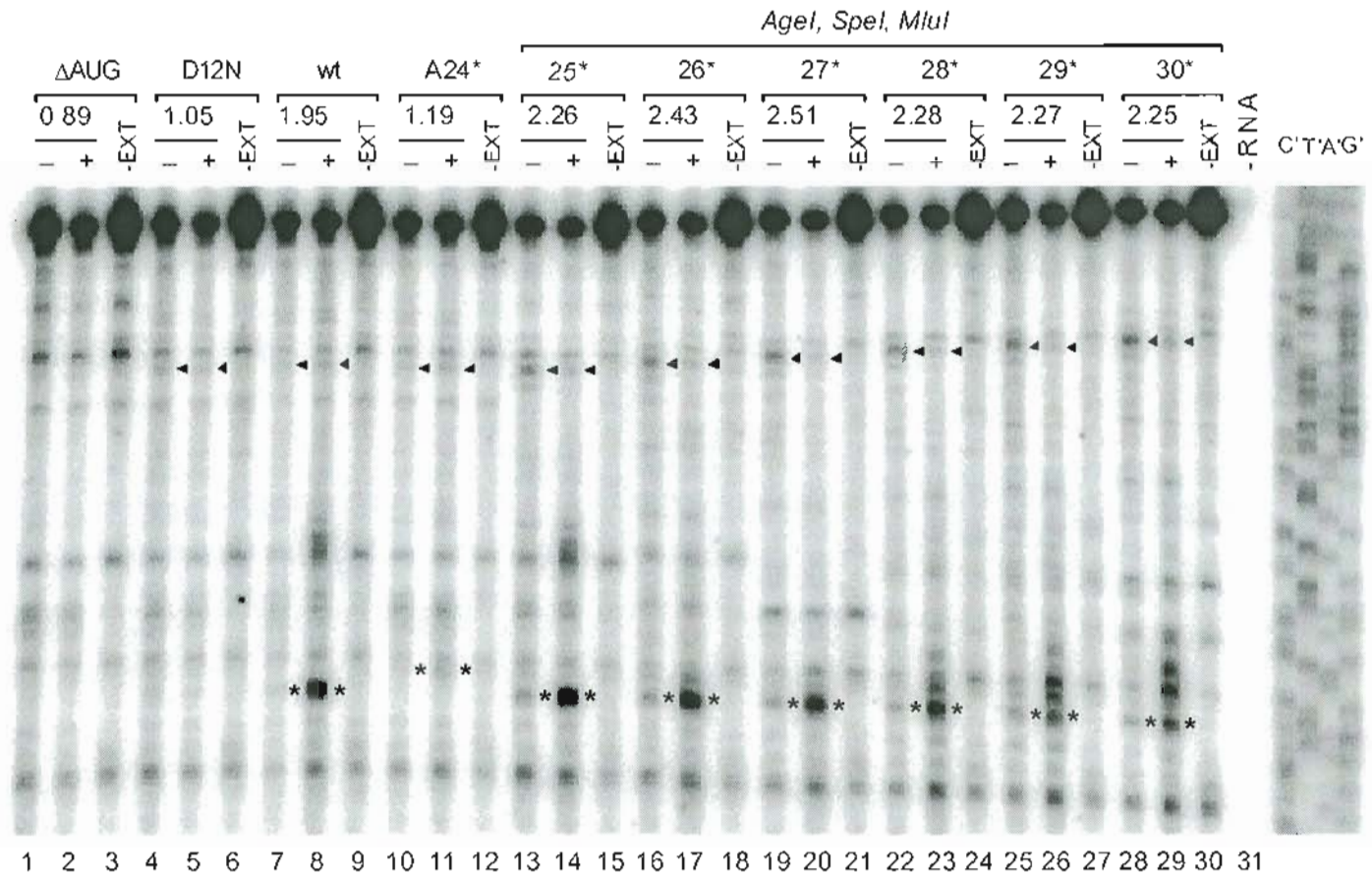

Figure 2.6. Effects of addition of the amino acid residues at the AAP C-terminus on Arg-specific regulation. The transcripts examined (Figure 2.2C) are indicated at the top; LUC activity measurements and reaction conditions were as described in Figure 2.3. Dideoxynucleotide sequencing reactions for the template encoding an AAP with the termination codon at codon-30 are on the right. The products obtained from primer extension of each pure RNA (120 ng) in the absence of translation reaction mixture (-EXT, lane 3, 6, 9 12, 15, 18, 21, 24,27,30) and from translation reaction mixture not programmed with RNA (-RNA, lane 31) are shown for comparison. Arrowheads and asterisks are as for Figure 2.3.

and 2). The D12N mutation eliminated regulation and all Arg-specific translational effects on toeprint signals (Figure 2.6, lanes 4 and 5). Shortening the AAP coding region by changing Ala-24 to a stop codon (A24*) eliminated regulation and shifted the toeprint corresponding to the termination codon by one codon, as expected (Figure 2.6, lanes 10 and 11). The results of these control experiments were all consistent with previous observations (Wang and Sachs, 1997).

LUC activity and toeprint analyses of the positions of ribosomes on the extended uORFs revealed a number of striking features. Moving the termination codon from codon-25 to codon-26, -27, -28, -29 or -30 slightly increased regulation. The positions of primer extension products corresponding to the uORF termination codon (asterisks) and to the initiation codon (arrowheads) appeared in positions exactly as predicted from the sizes of the extended uORFs. Arg increased the signals corresponding to ribosomes at uORF termination codons and decreased the signal corresponding to ribosomes at initiation codons. Arg also increased the stalling of ribosomes at codon-25 and subsequent sense codons in the C-terminally extended uORFs independent of its effects at the uORFs' termination codons, indicating that stalling during elongation occurred as well as stalling during termination.

It was of interest to determine whether the coding region could be extended to the point at which increased stalling at the termination codon in response to Arg was lost. In an AAP-LUC fusion construct, elongating ribosomes stall in a window corresponding to approximately six codons in the presence of high Arg (Figure 2.7, compare lanes 2 and 1). A single nucleotide deletion at codon 25 causes a frameshift so that the AAP reading frame has a termination codon at codon-32 (Figure 2.2, B). Primer-extension analyses of this frameshift construct indicated that, while Arg-regulated stalling of ribosomes occurred between codons 25 and 30, Arg did not affect stalling of ribosomes at codon-32. An indication of the high precision of the toeprint assay for mapping ribosomes is also evident from this experiment. As a consequence of the frameshift, the AAP initiation codon is one nt closer to the primer used for toeprinting, and this is observed in the positions of the toeprint signals corresponding to ribosomes initiating at the start of the AAP in wild-type and frameshifted constructs (Figure 2.7, compare arrowheads for lanes 4 and 2).

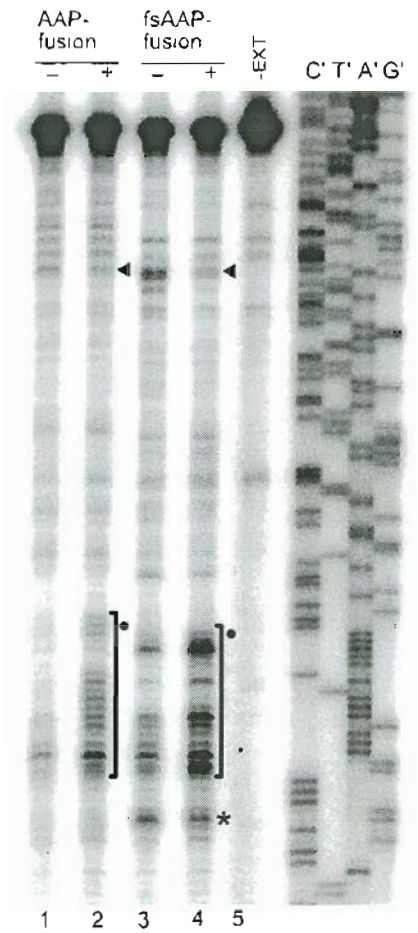

Figure 2.7. Ribosome stalling in an AAP-LUC fusion construct and a frameshifted AAP-LUC fusion construct. The transcripts examined (Figure 2.2B) are indicated at the top; transcripts were translated in reaction mixtures as described in Figure 2.3. Dideoxynucleotide sequencing reactions for the template encoding the frameshifted AAP-LUC fusion peptide (Figure 2.2B) are on the right. The products obtained from primer extension of RNA encoding the frameshifted AAP-LUC fusion peptide (120 ng) in the absence of translation reaction mixture (-EXT, lane 5) are shown for comparison. The arrowheads indicate the position of the premature transcription termination products corresponding to ribosomes bound at the AAP initiation codon. The closed circles indicate the position of premature termination products corresponding to ribosomes stalled in the presence of high Arg at the 25th codon immediately following the 24 codons of the AAP. The brackets indicate the position of premature termination products corresponding to ribosomes stalled in the presence of high Arg at the codons after the 25th codon. The asterisks indicate the position of ribosomes at the termination codon (codon-32).

An unexpected result was observed in the experiments shown in Figure 2.6. While toeprint signals are observed in the region 21-30 nt upstream of the termination codon of functional AAPs when the termination codon is at its normal position (e.g., Figure 2.3~ 2.6; see also additional experiments in (Wang and Sachs, 1997; Wang et al., 1998; Wang et al., 1999)), when the uORF was extended at its C-terminus, these strong upstream signals disappeared. The explanation for this is unclear. Were these signals to correspond to ribosomes stalled behind ribosomes stalled at termination codons, then it might have been expected that they remain in the same position relative to the termination codon in each of the carboxy-terminally extended constructs.

2.3.5 Arg-Specific Stalling during Elongation Is Better Facilitated by a Rare Codon than a Common Codon

Does the rate of translation affect the extent of ribosome stalling at a given site? Rare codons are considered to cause slowing of translation (Komar et al., 1999 and references therein). Therefore, we tested the effect on stalling of placing a rare codon versus a common codon at the same position in the mRNA (Figure 2.2B) and examined the extent of ribosomes stalling at that codon (Figure 2.8). In high Arg, a construct containing the rarest Leu codon (UUA; 1.7% usage (Edelmann and Staben, 1994)) at codon-25 of the AAP-LUC fusion gene showed greater stalling at that position than a construct containing the most common Leu codon at that position (CUC; 42% usage (Edelmann and Staben, 1994)) at that position (Figure 2.8, compare lanes 2 and 6). When less stalling was observed at this position, relatively more stalling was observed downstream, and the extent of regulation was similar in both constructs as determined by LUC assay (Figure 2.8). When constructs contained the D12N mutation, regulation by Arg was eliminated (Figure 2.8).

2.4 DISCUSSION

The results indicate that the synthesis of a core peptide of evolutionarily conserved sequence can regulate ribosome stalling on RNA in response to the concentration of Arg. The sequence of the template RNA encoding this peptide does not

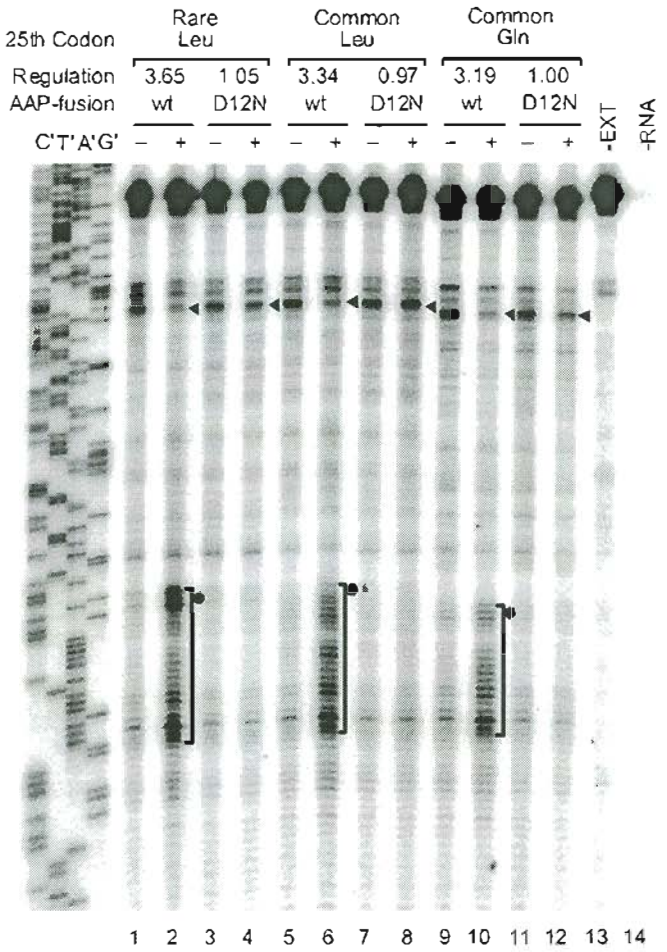

Figure 2.8. Effects of 25th rare *vs.* common Leu codon on Arg-specific regulation of AAP-LUC fusion constructs. The transcripts examined (Figure 2.2B) are indicated at the top. LUC activity measurements and reaction conditions were as described in Figure 2.3. The transcripts encoded the wild-type (wt) AAP-LUC fusion or the D12N AAP-LUC in improved initiation contexts. Dideoxynucleotide sequencing reactions for the template containing the wild-type AAP-LUC fusion with rare Leu codon at its 25th position are on the left. The products obtained from primer extension of pure AAP-LUC RNA (120 ng) in the absence of translation reaction mixture (-EXT, lane 13) and from translation reaction mixture not programmed with RNA (-RNA, lane 14) are shown for comparison. The arrowheads indicate the position of the premature transcription termination products corresponding to ribosomes bound at the AAP initiation codon. The closed circles indicate the position of premature termination products corresponding to ribosomes stalled in the presence of high Arg at the 25th codon immediately following the 24 codons of the AAP. The brackets indicate the position of premature termination products corresponding to ribosomes stalled in the presence of high Arg at the codons after the 25th codon of the peptide.

have a discernible active role in this process although it can affect the extent of stalling and the positions of stalled ribosomes. The ribosomes that have synthesized the nascent peptide moiety appear to become transiently sensitive to stalling by Arg (or a close metabolite) as they translate nearby downstream RNA sequence. The data suggest this control mechanism is also used by the fungi *M. grisea*, *T. virens* and *A. nidulans*, since the peptides encoded by uORFs in the corresponding genes of each of these fungi also function to stall ribosomes in response to Arg in the *N. crassa* cell-free system.

The rat CPS transcript contains a uORF that is different from the fungal CPS-A transcript uORFs. While the rat uORF is recognized by fungal ribosomes *in vitro* (Figure 2.3), it does not appear to function to modulate translation in response to Arg. This is consistent with its lack of sequence similarity, since even minor changes affecting conserved AAP residues eliminate its regulatory function. While the carbamoyl phosphate produced by mammalian CPS I can be used for Arg biosynthesis (Wu and Morris, 1998), and there are indications for post-transcriptional control mediated by the 5'-leader of the rat CPS mRNA (Christoffels et al., 1995), the regulatory role of this uORF, if any, remains unknown.

Currently, the most thoroughly studied of the uORFs whose encoded peptide sequences are important for translational control are the fungal uORFs encoding the AAP, uORF2 of cytomegalovirus UL4, and the uORF of mammalian S-adenosyl decarboxylase (Geballe and Sachs, 2000 and references therein). The limited introduction of silent mutations into the coding regions of the *S. cerevisiae CPA1* (Delbecq et al., 1994) and cytomegalovirus UL4 (Degnin et al., 1993) uORFs, as well as the introduction of silent mutations at all five mutable codons in the mammalian S-adenosyl decarboxylase uORF (Hill and Morris, 1993), were consistent with the lack of importance of those RNA sequences for regulation other than through their capacity to encode a specific peptide sequence. We have analyzed the regulated stalling of ribosomes mediated by AAPs encoded by natural *S. cerevisiae*, *M. grisea*, *A. nidulans*, and *N. crassa* sequences, as well as by a synthetic AAP coding region in which silent mutations alter over 40% of its nucleotide sequence (Wang and Sachs, 1997; Wang et al., 1998; Wang et al., 1999 and this work). The data indicate that it is highly unlikely that there is a role for the RNA in mediating Arg-regulated ribosome stalling outside of its capacity to encode the AAP.

Alignment of the wild-type fungal AAP coding sequences and the shortened, multiply substituted functional *N. crassa* AAP coding sequence shows no more than two consecutive nucleotides are conserved among all of the sequences, except for the Trp codon, TGG. Furthermore, we have found that this codon can be changed to a Tyr codon (TAC) and regulation maintained (C. Spevak, P. Fang and M. S. Sachs, unpublished). Thus, if the RNA sequences encoding AAPs were important outside of their coding roles, then only very limited primary conserved sequence must be important. Such a limited sequence might include an Arg codon because analyses of RNA aptamers that bind Arg indicate that an Arg codon is often part of the binding site (Yarus, 1998). But for the fungal AAPs, no Arg codons, either in the AAP reading frame or in alternative reading frames, are conserved among all of the sequences (data not shown).

In addition to providing insight into the requirements for the role of a nascent peptide in translational regulation, these results indicate that rare codons can cause conditional effects in translation that depend on other contributing factors. While very little stalling of ribosomes occurred when either a rare Leu-codon or a common Leu-codon were present at codon-25 in an AAP-LUC construct, at a low concentration of Arg, the rare codon enabled substantially more stalling than a common codon at a high concentration of Arg (Figure 2.8). These results indicate that, while a termination codon is not required for Arg-specific ribosome stalling after synthesis of the AAP, a slow step in translation such as an encounter with a stop codon or a rare codon facilitates stalling. Such a step may provide more time or a more favorable environment for the nascent AAP to exert its regulatory function.

The Arg-specific control of ribosome movement mediated by fungal AAPs so far represents a unique form of regulatory control in response to an amino acid. Related small molecules –polyamines – cause the translational regulation of mammalian S-adenosylmethionine decarboxylase via the action of a uORF whose sequence is critical for control (Ruan et al., 1996; Mize et al., 1998). It will be interesting to determine whether the control mechanisms are similar. The amino acid methionine is involved in the post-transcriptional regulation of the first enzyme committed to Arabidopsis methionine biosynthesis, cystathionine γ-synthase. A high level of methionine reduces the level of this mRNA; an evolutionarily conserved sequence within the cystathionine γ-

synthase coding region is required for regulation (Chiba et al., 1999). The mechanism remains to be determined. In mammalian systems, Arg is observed to affect the post-transcriptional control of the level of an mRNA specifying a cationic amino acid transporter; this mechanism appears to involve sequences in the 3' untranslated region of the mRNA (Aulak et al., 1999).

The stalling of ribosomes during translation is commonly thought to be mediated by secondary structures in the mRNA or by encounters with rare codons; nascent peptides have also been implicated in ribosome stalling in some instances (Crombie et al., 1992; Tsalkova et al., 1999 and references therein). Nascent peptides encoded by uORFs in eukaryotic and prokaryotic transcripts also are implicated in ribosome stalling (Lovett and Rogers, 1996; Geballe and Sachs, 2000). The data presented here provide the strongest evidence to date that a eukaryotic peptide can cause ribosomes involved in either termination or elongation to stall, and that, while mRNA structure or the presence of rare codons may influence the extent of stalling, it is the nascent peptide itself that is primarily responsible for controlling the subsequent movement of the ribosome.

CHAPTER 3

NEUROSPORA CRASSA SUPERSUPPRESSOR MUTANTS MAINTAIN ARG-SPECIFIC REGULATION AND ARE AMBER CODON-SPECIFIC*

3.1 INTRODUCTION

Neurospora crassa has ten mapped supersuppressor (*ssu*) genes (reviewed in Perkins et al., 2001). These were identified as allele-specific but not locus-specific suppressors and have been presumed to suppress premature termination mutations. *ssu-1* is the best characterized supersuppressor. It was originally isolated as an extragenic suppressor of the am^{17} mutation (amination defective), which lies within NADP-specific glutamate dehydrogenase (Seale, 1968). Direct amino acid sequence analysis of tryptic fragments of glutamate dehydrogenase produced from the *ssu-1* am^{17} strain showed that Glu-313 was replaced by Tyr (Seale et al., 1977). Codon-313 is CAG. On the basis of reversion analyses with specific mutagens, am^{17} was predicted to be a UAG (amber) mutation (Burns et al., 1984); this was recently confirmed by sequencing (Fincham et al., 2000). Analyses of mutations affecting genes involved in nitrogen regulation have identified additional *ssu-1* suppressible alleles that are presumed amber mutations (Perrine and Marzluf, 1986; Okamoto et al., 1993).

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Fang, P., Wu, C., and Sachs, M. S. (2002). Neurospora crassa supersupressor mutants are amber codon-specific. Fungal Genet. Biol. 36, 167-175.

The spectrum of suppression by eight *ssu* genes was characterized by analyzing their capacity to suppress ten different suppressible alleles (Seale, 1976). Overall they have similar spectra of action, except for *ssu-3* (Seale, 1976), and each is proposed to function as an amber-suppressor (Burns et al., 1984). However, there has been no direct test of the spectrum of action of the *Neurospora* supersuppressors on each of the nonsense codons. Furthermore, it has been presumed, but not established, that suppression is mediated by tRNA.

Cell-free translation systems are invaluable for investigating translational mechanisms (Sonenberg et al., 2000). We developed an *N. crassa* cell-free translation system to study Arg-specific translational control by the arginine attenuator peptide (AAP) that is encoded by an upstream open reading frame (uORF) in the *N. crassa arg-2* mRNA. The synthesis of luciferase (*LUC*) reporters in cell-free extracts can be measured enzymatically and by [^{35}S]Met-labeling; the positions of ribosomes on transcripts can be determined by primer extension inhibition (toeprint) assays (Wang and Sachs, 1997; Wang and Sachs, 1997; Wang et al., 1998; Wang et al., 1999; Fang et al., 2000; Gaba et al., 2001). Here we used these approaches to obtain evidence that *ssu* strains, *ssu-1*, *-2*, *-4*, *-5*, *-9*, and *-10* suppress amber (UAG) termination codons, but not ochre (UAA) or opal (UGA) terminators. The suppressor activity is clearly associated with the tRNA pools in *ssu-1*, *-2*, *-4*, *-5*, and *-10*.

3.2 MATERIALS AND METHODS

3.2.1 Strains

ssu-1(WRN33) am^{17} A (FGSC #1687) (Seale, 1968); *ssu-2(WRU35) am^{17} al A* (FGSC #1689) (Seale, 1968); *ssu-3(WRU118) am^{17} a* (FGSC #1851) (Seale, 1968); *ssu-4(WRU18) am^{17} A* (FGSC #1852) (Seale, 1968; Seale et al., 1969); *ssu-5(Y319-45) ad-3B (2-17-34) A* (FGSC #2483)(Newcombe and Griffiths, 1973); *ssu-9(WRU98) am^{17} a* (FGSC #7584) (Seale, 1976); *ssu-10(WR68) am^{17} a* (FGSC #7574) (Seale, 1968) were obtained from the Fungal Genetics Stock Center (University of Kansas Medical School).

3.2.2 DNA Templates and RNA Synthesis

Plasmids were designed to produce capped and polyadenylated synthetic RNA encoding firefly *LUC* with *AAP* sequences in the RNA 5'-leader region (Figure 3.1). For the constructs designed as in Figure 3.1A and Figure 3.1B, the *AAP* and *LUC* coding regions were always in-frame with respect to each other, and were separated either by a single sense codon (CAG or AAG) or by a single nonsense codon (UAA/UAG/UGA). Constructs (Table 3.1) contained mutations introduced by replacing a restriction-fragment with synthetic oligonucleotides (Table 3.2). The pQQ101 plasmid encoding sea pansy *LUC* (Wang et al., 1998) was used to produce RNA to use as an internal control in measuring LUC synthesis in translation extracts.

Capped, polyadenylated RNA was synthesized with T7 RNA polymerase from plasmid templates linearized with *Eco*RI and the yield of RNA was quantified as described (Fang et al., 2000).

3.2.3 Analyses of Suppression in Cell-Free Extracts

The preparation and use of *N. crassa* extracts was as described (Fang et al., 2000 and references therein). The Tricine SDS-PAGE gel system (Schägger and von Jagow, 1987) was used for examining radiolabeled polypeptides.

3.2.4 tRNA Purification

Total RNA was prepared from 100 mg samples of frozen mycelia of wild type or supersuppressor mutants as described (Luo et al., 1995). To purify tRNA, total RNA in 10 mM Tris-HCl (pH7.5), 0.2 M KCl was applied to a 1 ml DEAE-cellulose column (typically, 350 µg in 300 µl); the column was washed with 10 volumes of 10 mM Tris-HCl (pH7.5), 0.2 M KCl, and tRNA was eluted with 2 volumes of 10 mM Tris-HCl (pH7.5), 1.0 M KCl. The fractions with the highest OD_{260} were pooled, precipitated with ethanol and re-dissolved in nuclease-free water; the typical yield was 50 µg of nucleic acid.

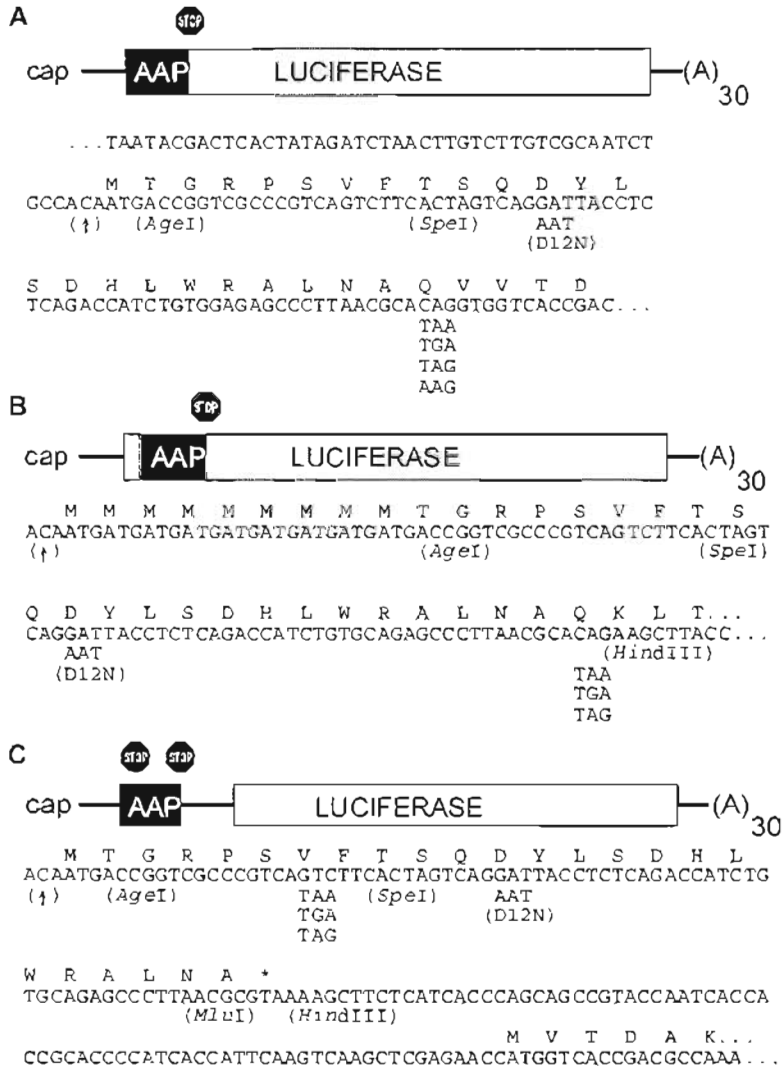

Figure 3.1. Constructs used. Diagrams of the synthetic mRNA transcripts, and the relevant 5'-regions of the templates used for RNA synthesis, are shown. (A) AAP-LUC fusion constructs containing sense- or nonsense-triplets at codon-25. The sequence shown begins with the T7 RNA polymerase-binding site, includes the 24-codon arg-2 AAP, and includes a portion of the LUC coding region. Relevant restriction enzyme sites are indicated. Mutations at codon-25 are shown, as is the D12N mutation; the latter mutation results in an AAP that is not functional in regulation. The (↑) mutation improves the initiation context for AAP-LUC translation (Wang and Sachs, 1997). (B) Met9AAP-LUC fusion constructs containing sense- or nonsense-codons between the AAP and LUC coding regions. Eight additional Met-codons are placed at the N-terminus of the AAP. The sequence shown begins with the improved initiation context at the start of the Met9AAP open reading frame; the sequence upstream of this matches the sequence in panel A. (C) V7* uORF constructs. The AAP is present as a uORF; premature nonsense codons have been introduced at codon-7 as indicated. The sequence shown begins with the improved initiation context at the start of the uORF; the sequence upstream of this matches the sequence in panel A.

Table 3.1

Constructs Used in This Study

Construct	5'-leader structure	Plus strand[a]	Minus strand[a]	Ligation Vector	Restriction site(s) for ligation
pKL101	wt-AAP-TAA-LUC	KBL1	KBL2	pR301[b]	*MluI*/*Bst*EII
pKL102	wt-AAP-TGA-LUC	KBL3	KBL4	pR301	*MluI*/*Bst*EII
pKL103	wt-AAP-TAG-LUC	KBL5	KBL6	pR301	*MluI*/*Bst*EII
pKL104	wt-AAP-CAG-LUC	KBL7	KBL8	pR301	*MluI*/*Bst*EII
pKL105	wt-AAP-AAG-LUC	KBL7	KBL10	pR301	*MluI*/*Bst*EII
pKLS101	D12N-AAP-TAA-LUC	KBL1	KBL2	pS301[b]	*MluI*/*Bst*EII
pKLS102	D12N -AAP-TGA-LUC	KBL3	KBL4	pS301	*MluI*/*Bst*EII
pKLS103	D12N -AAP-TAG-LUC	KBL5	KBL6	pS301	*MluI*/*Bst*EII
pKLS104	D12N -AAP-CAG-LUC	KBL7	KBL8	pS301	*MluI*/*Bst*EII
pKLS105	D12N -AAP-AAG-LUC	KBL9	KBL10	pS301	*MluI*/*Bst*EII
pKL202	Met9-wt-AAP-TAA-LUC	OPF111-1	OPF111-2	pKL101	*Bgl*II/*Age*I
pKL203	Met9-wt-AAP-TGA-LUC	OPF111-1	OPF111-2	pKL102	*Bgl*II/*Age*I
pKL204	Met9-wt-AAP-TAG-LUC	OPF111-1	OPF111-2	pKL103	*Bgl*II/*Age*I
pKL205	Met9-wt-AAP-CAG-LUC	OPF111-1	OPF111-2	pKL104	*Bgl*II/*Age*I
pR7011	wt-V7TAA-AAP-LUC	OPF112-1	OPF112-2	pR701[c]	*NdeI*/*Spe*I
pR7012	wt-V7TGA-AAP-LUC	OPF113-1	OPF113-2	pR701	*NdeI*/*Spe*I
pR7013	wt-V7TAG-AAP-LUC	OPF114-1	OPF114-2	pR701	*NdeI*/*Spe*I

[a]The plus-strand has the same sequence as the mRNA; the minus-strand is complementary to the mRNA
[b]pR301 and pS301 was constructed as described previously (Fang et al., 2000).
[c]pR701 was constructed using PCR to introduce a *Hin*dIII site after the uORF stop codon in pRER001(Fang et al., 2000). The primers for PCR were KL-11 (5'-GGCGG CACGC GTAAA AGCTT CTCAT CACCC AG-3') and FP07 (Wang et al., 1999). The PCR product was digested with *MIu*I and *BstE*II and ligated into vector pR301 digested with the same two enzymes.

Table 3.2. Oligonucleotides Used in This Study

Oligonucleotide	Sequence (5' to 3')
KBL1	CGCGT AAGTG
KBL2	GTGAC CACTT A
KBL3	CGCGT GAGTG
KBL4	GTGAC CACTC A
KBL5	CGCGT AGGTG
KBL6	GTGAC CACCT A
KBL7	CGCGC AGGTG
KBL8	GTGAC CACCT G
KBL9	CGCGA AGGTG
KBL10	GTGAC CACCT T
OPF111-1	GATCT AACTT GTCTT GTCGC AATCT GCCAC AATGA TGATG ATGAT GATGA TGATG ATGAA G
OPF111-2	CCGGT CTTCA TCATC ATCAT CATCA TCATC ATCAT TGTGG CAGAT TGCGA CAAGA CAAGT T
OPF112-1	TATGA CCGGC CGCCC GTCCT AATTC A
OPF112-2	CTAGT GAATT AGGAC GGGCG GCCGG TCA
OPF113-1	TATGA CCGGC CGCCC GTCCT GATTC A
OPF113-2	CTAGT GAATC AGGAC GGGCG GCCGG TCA
OPF114-1	TATGA CCGGC CGCCC GTCCT AGTTC A
OPF114-2	CTAGT GAACT AGGAC GGGCG GCCGG TCA

3.3 RESULTS

3.3.1 LUC Enzyme Assays Indicate Efficient Suppression of UAG Codons

We prepared capped and polyadenylated synthetic mRNAs that specified the 24-codon arginine attenuator peptide (AAP) followed by a sense codon (CAG or AAG) or a termination codon (UAA, UGA or UAG) and then by the firefly *LUC* coding region (Figure 3.1A). Equal amounts of each mRNA were used to program cell-free translation extracts prepared from wild-type *N. crassa* and seven *ssu* mutants. These mutants were chosen because they contained both the *ssu* mutation and an additional forcing mutation (*am*^{17} or *ad-3B (2-17-34)*) that required continued *ssu* function for growth on minimal medium. Additionally, sea pansy *LUC* mRNA was added to each extract to provide an internal control for measuring translational efficiency. The synthesis of both firefly LUC and sea pansy LUC in *N. crassa* extracts programmed with both mRNAs can be readily measured using a dual LUC assay (Wang et al., 1998). The capacity to suppress nonsense codons was determined by examining the production of enzymatically active firefly LUC (Table 3.3) from mRNAs containing a nonsense triplet at codon-25 (between the *AAP* and *LUC*) to mRNAs containing a sense codon at this position.

The efficiency of translation of mRNAs containing either the AAG- or CAG-sense codon relative to the sea pansy *LUC*-encoding mRNA internal control was similar in all of the extracts tested (data not shown). The production of firefly LUC from mRNA containing the CAG codon compared to the AAG codon was 95±6% in the first batch of extracts and 101±10% in the second batch. That mRNAs containing these commonly used sense codons were translated with similar efficiencies in all extracts validates this approach for testing the effects of premature termination mutations on *AAP-LUC* expression, since multiple batches of extracts from genotypically different strains translated control transcripts with little variability.

Extracts from wild-type cells programmed with mRNAs containing nonsense codons between the *AAP* and *LUC* produced approximately 1% of functional enzyme compared to those programmed with mRNA containing a sense codon at the corresponding position (Table 3.3). Therefore, as expected, extract prepared from the wild type did not efficiently suppress termination at nonsense codons. The reduction in

Table 3.3

Suppressor Activity as Determined by LUC Assay

Strain	Construct							
	UAG[a]		UGA		UAA		CAG	
	Activity[b]	Regulation[c]	Activity	Regulation	Activity	Regulation	Activity	Regulation
wt	0.6	0.9	1.2	2.6	0.8	1.0	100	3.2
ssu-1	21.8	3.1	2.9	3.6	1.8	1.6	100	2.0
ssu-2	21.5	6.9	3.0	8.5	1.1	2.0	100	4.0
ssu-3	0.6	1.3	2.4	5.5	1.3	1.9	100	4.2
ssu-4	33.9	2.2	4.1	3.9	1.8	1.0	100	1.7
ssu-5	17.4	2.5	3.1	2.4	1.8	1.3	100	2.0
ssu-9	3.6	2.8	1.8	3.0	1.6	1.4	100	2.2
ssu-10	19.8	3.3	3.9	4.8	1.9	1.8	100	1.8

[a]The sequence of the UAG, UGA, UAA and CAG constructs is shown in Figure 3.1A.
[b]To measure LUC activity, wild-type and *ssu N. crassa* extracts were programmed with 12 ng of the indicated RNAs and incubated for 30 min at 25°C. Reaction mixtures contained 20 μM of each of the 20 amino acids. Reactions were stopped by freezing with liquid nitrogen, and aliquots of the ice-thawed mixtures (5 μl) were used for LUC assays. LUC activity from the translation of transcripts containing each stop codon was normalized to that obtained from *AAP-LUC* transcript containing CAG at the 25th position.
[c]Regulation was calculated as the ratio of LUC activity produced after 30 min in reaction mixtures containing 20 μM Arg or 500 μM Arg and 20 μM each of the other 19 amino acids.

LUC activity was comparable to that previously observed when the *AAP* reading frame was frame-shifted with respect to the *LUC* reading frame (Wang et al., 1998).

The results obtained with extracts from *ssu* mutants were strikingly different, as demonstrated by an experiment in which an extract was prepared from each mutant and all extracts analyzed in parallel (Table 3.3). For every mutant except *ssu-3*, suppression of UAG codons was observed (Table 3.3). Analyses of two independent extract preparations from each mutant yielded similar levels of suppression for *ssu-1*, *ssu-2* and *ssu-4* and no evidence for suppression by *ssu-3* (Table 3.3 and data not shown). These results are consistent with *in vivo* studies in which the *ssu-3* suppressor is reported to act on a different spectrum of mutations than the other suppressors (Seale, 1976). *ssu-1* suppression *in vivo* is estimated at 20% (Seale et al., 1977), similar to the level we observed *in vitro* (Table 3.3 and data not shown). We observed variability in the extent of suppression in the *ssu-5*, *ssu-9*, and *ssu-10* mutant extracts. In one extract preparation, *ssu-5*, *ssu-9* and *ssu-10* each had a lower UAG-suppression value (approximately 4%) than in the other preparation (14-20%). The basis for this is not known but it does not affect the primary conclusion to be drawn from these experiments that each mutant except for *ssu-3* suppressed the UAG amber codon.

The UAA and UGA nonsense codons were not strongly suppressed in these experiments (Table 3.3). However, in wild-type extracts, when the UGA codon was present, slightly more LUC was produced than for the UAA codon (Table 3.3 and Figure 3.4). This indicated that there was slight but measurable read-through of UGA codons in wild-type extracts. In each suppressor extract, read-through of UGA codons appeared slightly elevated relative to wild type; additional slight effects on the read-through of UAA codons were observed in extracts from some but not all mutants. However, the effects observed with UGA and UAA codons were in all cases much smaller than those observed with UAG codons. That is, the *ssu* strains (except *ssu-3*) had a much greater capacity to suppress UAG amber codons than the other nonsense codons (Table 3.3).

The *AAP*-coding region enables the translational control of AAP-LUC synthesis by Arg. When a relatively high concentration of Arg is added to extracts, ribosomes stall after synthesis of the wild-type AAP, reducing synthesis of the AAP-LUC fusion polypeptide (Wang et al., 1999; Fang et al., 2000). Consistent with this, when there were

sense codons at position-25, LUC enzyme activity was negatively regulated by Arg in all extracts (Table 3.3). Therefore, regulation of AAP-LUC synthesis by Arg can be another indication for synthesis of the fusion polypeptide by read-through of the termination codon. That is, not only should the AAP-LUC polypeptide be produced if read-through occurs; its production should be regulated by Arg. As would be predicted if read-through were occurring in *ssu-1*, *ssu-2*, *ssu-4*, *ssu-5*, *ssu-9*, and *ssu-10* extracts, LUC activity was Arg-regulated (Table 3.3). LUC synthesis from mRNA containing the UGA codon, which appeared to be more suppressible than the UAA codon based on measurements of enzyme activity, was also more greatly affected by the presence of Arg than from mRNA containing the UAA codon (Table 3.3).

3.3.2 [^{35}S]Met Assays Indicate Suppression

Direct evidence that suppression *in vitro* resulted from the read-through of nonsense codons was obtained by [^{35}S]Met labeling experiments. We examined the polypeptides produced by programming extracts with mRNAs containing the *AAP* and *LUC* separated by nonsense or sense codons. To detect the short polypeptide produced by termination at stop codons following the *AAP* coding region, we added 8 Met codons at the AAP's N-terminus to create an AAP with 9 Met-codons in this region (Figure 3.1B). The short peptide containing a single Met was not readily detectable by [^{35}S]Met-labeling (data not shown).

In extracts from the wild type, the predominant radiolabeled polypeptide produced from mRNAs containing UAA, UGA or UAG nonsense codons migrated at a position consistent with the predicted size (3,520 Da) of the Met9AAP (Figure 3.2, lanes 1-6). When the codon was CAG, the predominant polypeptide corresponded in size to AAP-LUC (Figure 3.2, lanes 7 and 8). Additional polypeptides, which presumably are intermediates in LUC synthesis, are also present. These polypeptides are unlikely to be products arising from ribosomes scanning past the first initiation codon and initiating at downstream start codons because they were absent when the upstream in-frame termination codon was present. In extracts programmed with mRNA containing premature termination codons, some apparently full-length LUC was produced, since a polypeptide in the expected size range was present in these reaction mixtures but not

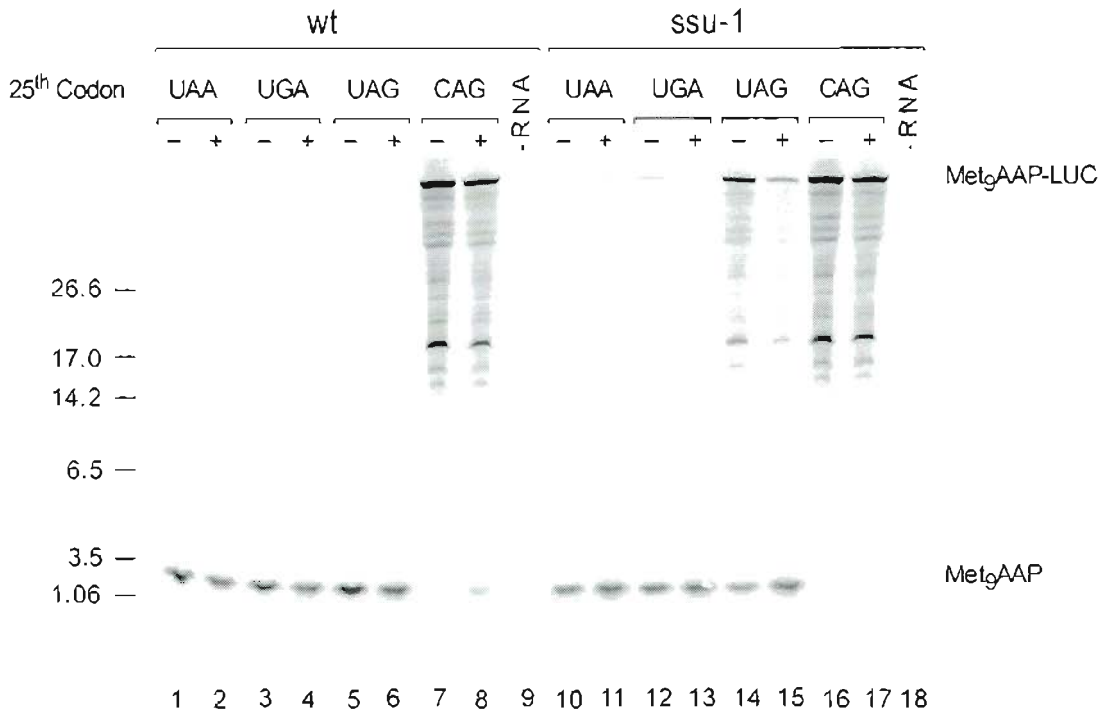

Figure 3.2. Analysis of [^{35}S]Met-labeled polypeptides produced by translation of synthetic RNA transcripts in wild type and *ssu-1* extracts. Micrococcal nuclease-treated *N. crassa* extracts (20 µl containing 40 µCi of [^{35}S]methionine (1175 Ci/mmol) were programmed with 120 ng of the indicated RNAs (Figure 3.1B) and incubated for 30 min at 25°C. Reaction mixtures contained 20 µM (-) Arg or 2000 µM (+) Arg and 20 µM each of the other 19 amino acids. Reactions were stopped by adding an equal volume of 2X SDS-PAGE loading buffer and examined by Tricine-SDS-PAGE in 16.5% polyacrylamide gels (Schägger and von Jagow, 1987). Radiolabeled translation products were visualized by phosphorimaging; the positions at which prestained molecular mass markers migrated are indicated on the left in kilodaltons. The positions of the Met9AAP and Met9AAP-LUC polypeptides are indicated on the right.

mixtures lacking mRNA template (Figure 3.2, lane 9). Phosphorimaging analysis indicated that, in all cases, the amount of this polypeptide was less than 5% of the amount produced when the codon was CAG, in reasonable agreement with the LUC enzyme measurement results obtained with these mRNAs (data not shown).

The results obtained with *ssu-1* for mRNAs containing UAA, UGA, and CAG were similar to those observed with wild type (Figure 3.2, lanes 10-13, 16 and 17). In contrast, with UAG, a substantial increase in the synthesis of full-length AAP-LUC polypeptide was observed (Figure 3.2, compare lanes 14 and 15 with lanes 5 and 6). Similar results were obtained in analyses of *ssu-2*, *-4*, *-5*, *-9*, and *–10*; *ssu-3* resembled wild type (data not shown).

In *ssu-1* and wild-type extracts programmed with the CAG-containing mRNA, high Arg caused the synthesis of the full length polypeptide to decrease and the signal corresponding to the Met9AAP to increase (Figure 3.2, lanes 7 and 8, lanes 16 and 17, and data not shown). This regulatory effect with this mRNA was also observed using extracts from the other supersuppressor strains (data not shown). A similar regulatory effect was observed for *ssu-1*, *-2*, *-4*, *-5*, *-9*, and *–10* extracts programmed with mRNA containing the suppressible UAG amber codon (Figure 3.2, lanes 14 and 15; data not shown). Quantitation of the amount of [^{35}S]Met-labeled LUC produced by suppression of the UAG codon in the *ssu-1* extract in low and high Arg (Figure 3.2, lanes 14 and 15) indicated that Arg reduced synthesis 3-fold, in good agreement with the data in Table 3.3. These results are consistent with the occurrence of read-through of the UAG codon and the model for AAP function in which high Arg causes ribosomes that have translated the AAP to stall.

Arg further reduced the relatively low amount of fusion polypeptide synthesis obtained from mRNA containing UGA at codon-25 in all extracts examined (Figure 3.2 and data not shown), consistent with the results in Table 3.3. These data indicate that, when synthesis of the AAP-LUC polypeptide is detectable above background levels, it is regulated by ribosome stalling. The results for the UAA construct in each extract, and for the UAG codon in the wild-type extract, were not always consistent with the enzyme activity data (Figure 3.2 and data not shown). In these cases, LUC synthesis was very

low, and we presume that these discrepancies arise as consequences of the inefficient translation of these transcripts.

3.3.3 Toeprint Assays Indicate Amber-Specific Suppression in Another Context

To map ribosomes on mRNA, we used constructs in which the AAP was encoded by a separate uORF (Figure 3.1C). By changing Val-7 (GUC) in the uORF-encoded reading frame for the AAP to UAG, UAA and UGA (Figure 3.1C), we established that translating ribosomes moved past the UAG at codon-7 but not UAA or UGA codons in *ssu-1* extracts. We accomplished this by mapping the positions of ribosomes using a primer extension inhibition assay. In the presence of high Arg, ribosomes stall following translation of the *AAP* coding region with UAA-25 in their A-site (Wang and Sachs, 1997). If suppression of a terminator at codon-7 occurs, a signal corresponding to ribosomes at codon-25 is expected in high Arg because the ribosomes will stall there. If suppression does not occur, no signal is expected, since translating ribosomes will not reach that site.

When synthetic mRNA containing the Val-7 codon within the wild-type *AAP* was used to program wild-type or *ssu-1* extracts, Arg-dependent stalling was observed at UAA-25 (Figure 3.3, compare lanes 2 and 1, 12 and 11). The D12N mutation in the *AAP*, which eliminates Arg-specific ribosome stalling, did so in both extracts (Figure 3.3, compare lanes 4 and 3, 14 and 13). Ribosome stalling at the wild-type uORF termination codon was associated with the reduced synthesis of LUC; enzyme synthesis was not reduced for the D12N mutation (Figure 3.3). Thus, the *ssu-1* extract and the wild-type extract responded similarly to Arg in this regulatory response.

Translation of transcripts containing UAA, UGA or UAG at codon-7 in wild-type extract did not yield toeprint signals corresponding to UAA-25 (Figure 3.3, lanes 5-10). Under these conditions, the synthesis of LUC as measured by enzyme activity was also not regulated, consistent with the toeprinting data, since ribosomes did not reach the termination codon for the full-length *AAP*, and ribosomes must reach this site for regulation to occur. The situation was markedly different for the *ssu-1* extract when UAG was placed at codon-7. An Arg-regulated stall at UAA-25 is apparent when UAG

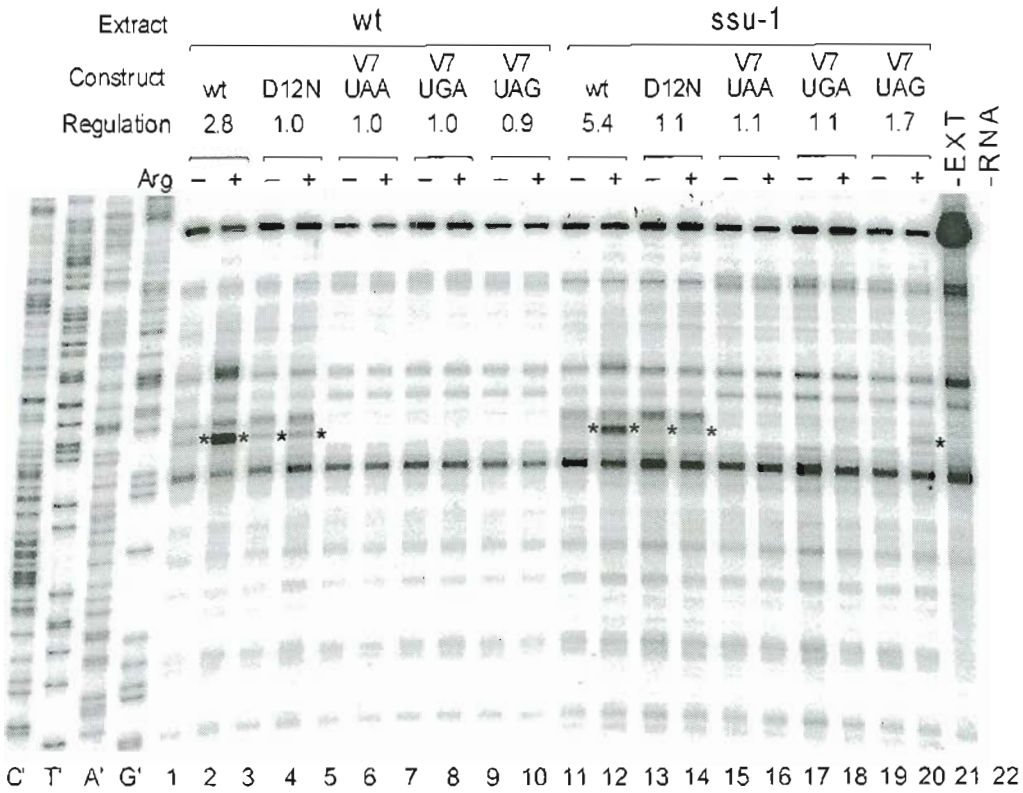

Figure 3.3. Toeprinting analysis of suppression of UAA, UGA, UAG stop codons by *ssu*-1 extracts. The transcripts examined (Figure 3.1C) are indicated at the top. Equal amounts of transcripts (120 ng) were translated in 20 µl reaction mixtures at 25°C. Reaction mixtures contained 10 µM (-) Arg or 500 µM (+) Arg and 10 µM of the other 19 amino acids. After 20 min of translation, 3 µl of each translation mixture was used for toeprinting with primer ZW4 (Wang and Sachs, 1997). Dideoxynucleotide sequencing reactions for the template containing the UAG replacement of Val-7 (GUC) are on the left. For dideoxynucleotide sequencing, the nucleotide complementary to the dideoxynucleotide added in each reaction is indicated above the corresponding lane so that the sequence of the template can be directly deduced. The sequence reads 5' to 3' from top to bottom. The products obtained from primer extension of pure V7UAG RNA (18 ng) in the absence of translation reaction mixture (-EXT, lane 21) and from translation reaction mixture not programmed with RNA (-RNA, lane 22) are shown for comparison. The asterisks indicate the positions of transcription termination products corresponding to ribosomes at the uORF termination codon. Regulation was determined by comparing LUC activity measurements in reaction mixtures containing 10 µM Arg to 500 µM Arg.

but not UAA or UGA is present at codon-7 (Figure 3.3, lanes 15-20). Furthermore, LUC synthesis in the *ssu-1* extract was Arg-regulated when the mRNA contained UAG-7, albeit less than for the wild-type uORF (Figure 3.3). Reduced regulation would be expected because suppression is not totally efficient and thus not all ribosomes that initiate translation of the uORF would reach codon-25 and stall there. These data show that ribosomes read-through the premature UAG codon to terminate at the natural uORF UAA codon in the *ssu-1* extract. In this construct, the UAG at codon-7 is followed by a codon containing only pyrimidines (UUC, Figure 3.1C); in the constructs used above, the UAG codon is followed by one containing only purines (AAG, Figure 3.1A and Figure 3.1B). Therefore, the *ssu-1* mutant can suppress amber codons in two different contexts.

In no case in which a stop codon was present at codon-7 was a toeprint signal corresponding to ribosomes observed at that site. These data indicate that ribosomes release rapidly from the mRNA at the premature termination codon, in contrast to the situation at the natural uORF termination codon.

3.3.4 Suppressor Activity Is Associated with the tRNA Fraction

We purified tRNA using DEAE-cellulose chromatography from suppressor strains and the wild type. By adding this tRNA to extracts from wild type and measuring LUC synthesis from the constructs containing UAG, UAA and UGA at codon-25, we tested whether UAG-suppressor activity resided in the tRNA fraction in each mutant (Figure 3.4). Suppression by supplementation with tRNA was measured by comparing LUC synthesis for UAG- and UGA- constructs to LUC synthesis from UAA constructs in unsupplemented and supplemented wild-type extracts.

The addition of two different amounts of tRNA from wild type *N. crassa* had no apparent effect on the synthesis of LUC from constructs containing any of the nonsense codons at position-25 as determined by measuring LUC synthesis in unsupplemented and supplemented wild-type extracts (Figure 3.4 and data not shown). In contrast, supplementation with tRNA from each supersuppressor strain increased the synthesis of LUC in extracts programmed with mRNA containing UAG but not UGA or UAA at codon-25 (Figure 3.4 and data not shown). This suppressor activity was strongest with tRNA from *ssu-1*, *-2*, *-4*, *-5*, and *-10*; *ssu-9* and *ssu-3* tRNA showed slighter suppressor

effects in this assay. These data strongly support the hypothesis that the suppressor activity resides in tRNA for *ssu-1*, *-2*, *-4*, *-5*, and *–10* and are consistent with this being the case for *ssu-9* and *ssu-3*.

3.4 DISCUSSION

The experiments described here indicate that the *N. crassa* supersuppressor strains *ssu-1*, *ssu-2*, *ssu-4*, *ssu-5*, *ssu-9*, and *ssu-10* specifically enable the suppression of UAG amber codons. The specificity of suppressor activity for UAG codons was seen with three different assays in a cell-free system: restoration of the production of active enzyme through suppression, restoration of the synthesis of full-length polypeptide through suppression, and (for *ssu-1*) measurement of the capacity of ribosomes to proceed past a termination codon through suppression. Suppressor activity resided in tRNA as determined by isolating bulk tRNA and showing that this tRNA had suppressor activity. These experiments thus indicate that *N. crassa* supersuppressor strains contain amber-specific suppressor tRNAs.

The six *N. crassa* supersuppressors for which activity was clearly seen *in vitro* are each amber-specific, a result contrasting with the spectrum of suppressors found in *Saccharomyces cerevisiae* and *Escherichia coli*, in which suppressors for each of the three nonsense codon are known (Sherman, 1982; Murgola, 1995). While, in *E. coli*, a mutated tRNA can have activity on both UAA and UAG codons, this has not been the case for *S. cerevisiae*, although both UAA and UAG suppressors can be generated by different mutations within the same tRNA. The simplest explanation for this difference in the spectra of mutant action between *N. crassa* and these other organisms is that it arises from the choice of suppressible alleles used to select *N. crassa* supersuppressors. Thus, while it is clear that amber-suppressors can be obtained in *N. crassa*, it remains an open question whether suppressors of the other nonsense codons can be obtained in this organism.

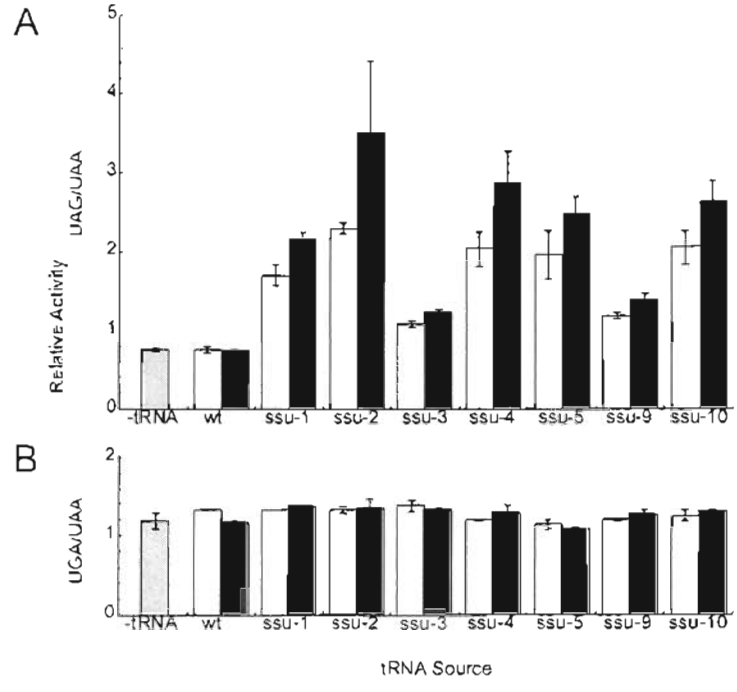

Figure 3.4. Suppression of premature stop codons in firefly *LUC* by purified tRNA. (A). Effect of purified tRNA on translation of *AAP-UAG-LUC*. 6ng RNA encoding *AAP-UAG-LUC* (from template pKL102) or 6ng mRNA encoding *AAP-UAA-LUC* (from template pKL101) were used to program protein synthesis in 10 µl reaction mixtures. Purified *N. crassa* tRNA was added at either 50ng/µl (white bars) or 100ng/µl (black bars). The source of tRNA is indicated under each group of bars. Reactions to which no tRNA was added are shown for reference (gray bar). LUC activity obtained from translation of *AAP-UAG-LUC* RNA was normalized to that from translation of *AAP-UAA-LUC* RNA. Bars show the average of the results of two independent experiments using duplicate samples in each; standard errors are also shown. (B) Effect of purified tRNA on translation of *AAP-UGA-LUC*. 6ng RNA encoding *AAP-UGA-LUC* (DNA template pKL103) was used to program translation as described above, and the results normalized to those obtained from translation of *AAP-UAA-LUC* RNA as described for panel A.

CHAPTER 4

THE INTERNAL ARGININE-ATTENUATOR-PEPTIDE DOMAIN IN A NASCENT POLYPEPTIDE REGULATES THE STALLING OF RIBOSOME THAT SYNTHESIZES IT IN RESPONSE TO ARGININE

4.1 INTRODUCTION

Nascent peptides containing specific sequences can regulate the movement of ribosomes on the mRNA that encodes them (Lovett and Rogers, 1996; Geballe and Sachs, 2000; Morris and Geballe, 2000; Gaba et al., 2001; Gong and Yanofsky, 2002; Raney et al., 2002; Tenson and Ehrenberg, 2002). The arginine attenuator peptide (AAP) functions in this manner to regulate gene expression. It is encoded by an evolutionarily conserved upstream open reading frame (uORF) in transcripts specifying the small subunit of arginine-specific carbamoyl phosphate synthetase and its role is to reduce gene expression in response to arginine.

In vivo, the wild-type *Neurospora crassa arg-2* AAP reduces the amount of the mRNA associated with polysomes when Arg is plentiful, reducing synthesis of ARG-2 polypeptide (Luo et al., 1995; Luo and Sachs, 1996). The amino acid sequence of the uORF-encoded AAP is critical for regulation. Thus, the D12N mutation in the AAP, identified by classical genetic selection, abolishes regulation (Freitag et al., 1996). The function of the AAP was established through *in vitro* studies with cell-free translation systems from *N. crassa* and *Saccharomyces cerevisiae* (Wang and Sachs, 1997; Wang et al., 1999). The assays used to determine the function of Arg-specific regulation included measuring the activity of a luciferase (LUC) reporter construct, detecting [^{35}S]Met-labeled translation products by SDS PAGE, and using a primer extension inhibition

(toeprinting) assay to map the positions of ribosomes involved in rate-limiting steps of polypeptide translation on mRNA. These *in vitro* studies revealed that ribosomes initiate translation of the downstream ORF in transcripts containing *N. crassa arg-2* or the homologous *S. cerevisiae CPA1* uORFs by scanning past these uORFs (Gaba et al., 2001). Ribosomes stall at the uORF termination codon when the concentration of Arg is high, blocking ribosomes from scanning to the downstream initiation codon, thus decreasing gene expression. Mutations that eliminate regulation *in vivo* eliminate stalling *in vitro* (Wang and Sachs, 1997), and the amino acid sequence of the AAP, not the sequence of the mRNA encoding it, is responsible for making ribosomes stall in response to Arg (Fang et al., 2000). Regulation by the AAP is not mediated by the level of aminoacylated arginyl-tRNA but appears to be directly mediated by Arg (Wang et al., 1999).

Unlike all of the other uORF-encoded peptides examined to date, which are observed to cause stalling only during termination, the AAP also causes Arg-regulated stalling of ribosomes during elongation when it is fused at the N-terminus of LUC (Wang et al., 1998; Wang et al., 1999). Ribosomes stall in the coding region immediately following the AAP.

Although the above studies strongly argue that it is the nascent AAP that interacts with the ribosome to cause stalling in the presence of high Arg, it was not directly proved that the nascent AAP is associated with the ribosome under these conditions. Also, while the toeprinting analyses show that the synthesis of the nascent AAP causes ribosome stalling during elongation, it has not been shown whether ribosomes fall-off from the mRNA or resume translation after stalling. Finally, it would be of considerable interest to know whether the AAP could function as an internal regulatory domain within a polypeptide.

Here we use reporter constructs in which it is possible to detect the AAP by [^{35}S]Met to address these important questions. Visualization of short and long polypeptides containing the AAP was accomplished by placing 9 Met-codons at the N-terminus of reporter constructs.

4.2 MATERIALS AND METHODS

4.2.1 DNA Templates and RNA Synthesis

Plasmids were designed to produce capped and polyadenylated synthetic RNA encoding Met_9-AAP-LUC fusion polypeptides (Fang et al., 2002), or 215-residue polypeptides containing wild type or D12N AAP domains near their N-termini and between residues 101-123, as shown in Figure 4.1. The plasmids (Table 4.1) contained mutations introduced by PCR with mutagenic primers (Freitag et al., 1996; Luo and Sachs, 1996) or by megaprimer PCR (Sarkar and Sommer, 1990). Oligonucleotides used for plasmid constructions are listed in Table 4.2. Additional plasmids used were described previously (Wang and Sachs, 1997; Wang and Sachs, 1997; Wang et al., 1998; Fang et al., 2000).

Plasmid DNA templates were purified by equilibrium centrifugation (Wang and Sachs, 1997); templates were linearized with *Eco*RI. Capped, polyadenylated RNA was synthesized with T7 RNA polymerase from linearized plasmid DNA templates and the yield of RNA was quantified as described (Wang and Sachs, 1997).

4.2.2 Analyses of Translation Products

The reaction conditions for *in vitro* translation using *N. crassa* extracts were as described (Fang et al., 2000, and references therein). The tricine SDS-PAGE gel system (Schägger and von Jagow, 1987) was used for analyzing [^{35}S]Met-radiolabeled polypeptides. The precipitation of peptidyl-tRNA from cell-free translations with cetyltrimethylammonium bromide (CTAB) was as described (Gilmore and Blobel, 1985).

The gels were dried and exposed to screens of a Molecular Dynamics PhosphorImager for approximately 16 hours. The ImageQuant® 5.1 application from Molecular Dynamics was used for quantitative analysis.

4.2.3 Isolation of Nascent Peptide-Ribosome Complex

To analyze [^{35}S]Met-radiolabeled ribosome-associated nascent peptides, 20 µl *in vitro* translation reaction mixture was diluted with 80 µl buffer A (30 mM HEPES-KOH,

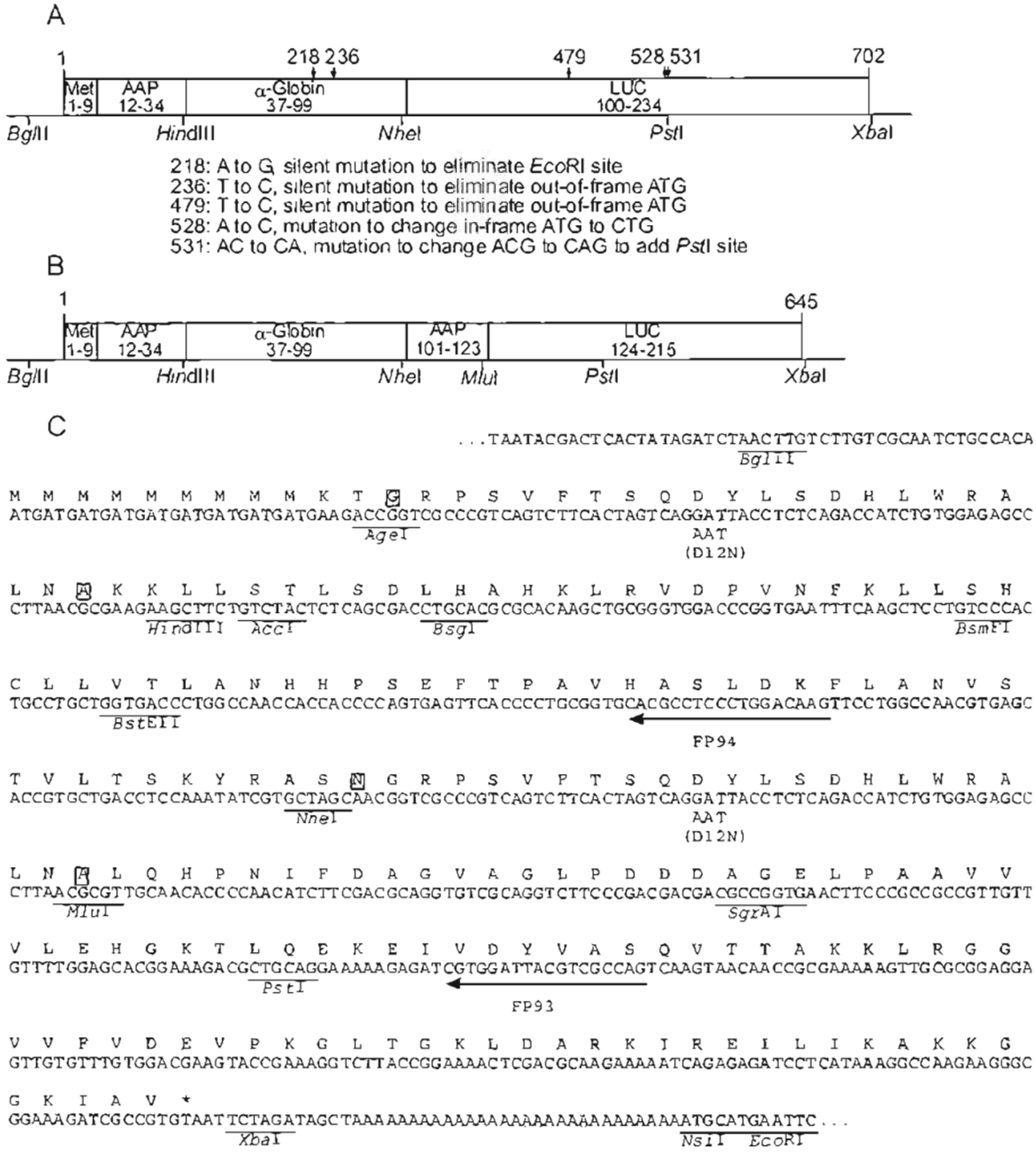

Figure 4.1. Schematic of the Met_9-AAP-Globin-LUC and Met_9-AAP-Globin-AAP-LUC mRNA. (A). The Met_9-AAP-Globin-LUC mRNA. The coding region in the mRNA is a fusion of the Met_9-AAP and domains of rabbit α-Globin (residues 80-141) and firefly LUC (residues 418-551) with two bridging codons. All ATG codons except for those at the N-terminus of the AAP have been removed (including out-of-frame ATG codons), as indicated below the diagram. The additional mutations to eliminate or introduce restriction enzyme sites are also indicated below the diagram. (B). The Met_9-AAP-Globin-AAP-LUC mRNA. The mRNA is the same as the Met_9-AAP-Globin-LUC mRNA except that a second AAP coding motif encoding codons 2-24 of the AAP replaces the codons 101-123 in the coding region of Met_9-AAP-Globin-LUC mRNA, and

the silent mutations at Leu124 (CTC to TTG) introduce a *Mlu*I site. (C). Sequences of the DNA templates for making Met_9-AAP-Globin-AAP-LUC mRNA. The sequence shown begins with the T7 RNA polymerase-binding site and ends with the *Eco*RI site after the poly(A) tail. The amino acid sequences of Met_9-AAP-Globin-LUC protein are indicated. The 'D12N' mutation (numbering based on 24-residue wild-type AAP), which eliminates regulation, is shown below the wild-type sequence. The restriction sites in this region, which only cut once in the plasmid, are underlined; the names of the corresponding restriction enzymes are indicated below the underlined sequences. The N-terminal and C-terminal amino acid residues in each AAP domain are also boxed. The sequence for which the reverse complement was synthesized and used as primer FP93 or FP94 for toeprint analysis is indicated by a horizontal arrow below the sequence.

Table 4.1

Firefly LUC Constructs Made by PCR or Megaprimer PCR (MP)

Construct	Structure	Method	Forward primer	Backward primer	PCR Template/ligation vector	Restriction sites for ligation
pTmp1	Met_9-AAP_{wt}-α - Globin - LUC	MP	FP82	FP83, FP84	pSPΔ[1]/pKL401[2]	*Hind*III/*Nhe*I
pTmp2	Met_9-AAP_{D12N} - α - Globin -LUC	MP	FP82	FP83, FP84	pSPΔ[1]/pKLS401[2]	*Hind*III/*Nhe*I
pGLR101	Met_9-AAP_{wt} - α - Globin - LUC[3]	PCR[4]	FP85, FP 87_2	FP87_1, FP88	pKL401/pTmp1	*NheI*/*EcoR*I
pGLS101	Met_9-AAP_{D12N} - α - Globin -LUC[3]	PCR[4]	FP85, FP 87_2	FP87_1, FP88	pKL401/pTmp2	*NheI*/*EcoR*I
pGLR102	Met_9-AAP_{wt}-α-Globin-LUC[5]	PCR	FP91	FP88	pGL101/pGL101	*NheI*/*EcoR*I
pGLS102	Met_9-AAP_{D12N}-α-Globin-LUC[5]	PCR	FP91	FP88	pGL101/pGLS101	*NheI*/*EcoR*I
pGLR201	Met_9-AAP_{wt} - α - Globin -AAP_{wt} - LUC	PCR	FP89	FP7[6]	pR301[7]/pGL102	*NheI*/*Mlu*I
pGLR202	Met_9-AAP_{D12N} - α - Globin - AAP_{wt} - LUC	PCR	FP89	FP7	pR301/pGLS102	*NheI*/*Mlu*I
pGLR203	Met_9-AAP_{wt} - α - Globin - AAP_{D12N} - LUC	PCR	FP89	FP7	pS301[7]/pGL102	*NheI*/*Mlu*I
pGLR204	Met_9-AAP_{D12N} - α - Globin - AAP_{D12N} - LUC	PCR	FP89	FP7	pS301/pGLS102	*NheI*/*Mlu*I

[1] pSPΔ (Drabkin et al., 1993) is a gift from Dr. Uttam RajBhandary at MIT.
[2] pKL401 is a Met_9-AAP_{wt}-LUC fusion construct, originally named as pKL205 (Fang et al., 2002); pKLS401 is a Met_9-AAP_{D12N}-LUC fusion construct.

[3] The LUC coding region in this construct encodes residue 418-551 of firefly luciferase with mutations indicated in Figure 4.1.
[4] pKL401 was amplified with primer pair of FP85, FP87_1, and primer pair of FP87_2, FP88 respectively; two PCR products were cut with *Pst*I separately and ligated together. The ligated product was then cut with *Nhe*I and *EcoR*I, and ligated into vector pTmp1 or pTemp2 cut with the same two enzymes to produce pGL101 or pGLS101.
[5] The LUC coding region in this construct encodes residue 461-551 of firefly luciferase with mutations indicated in Figure 4.1, a *Nhe*I site, 3 bridging codons, and a *Mlu*I site separate α–Globin coding region from the LUC region.

Table 4.2

Oligonucleotides Used in This Study

Oligo Name	Sequence (5' to 3')
FP82	ACCAT GAAGC TTCTG TCTAC TCTCA GC
FP83	CAGGG AGGCG TGCAC CGCAG GGGTG AACTC ACTGG GGTGG TGG
FP84	CCGGG GAGCT AGCAC GATAT TTGGA GGTCA G
FP85	GACAA GGCTA GCTGG CTACA TTCTG GAGAC
FP87_1	CTCTT TTTCC TGCAG CGTCT TTCC
FP87_2	GGAAA GACGC TGCAG GAAAA AGAG
FP88	GCTAT GACCA TGATT ACG
FP89	TCTGC CGCTA GCAAC GGTCG CCCGT CAGTC
FP91	GCTCC CGCTA GCTTG GAATC CAACG CGTTG CAACA CCCCA ACATC TTC
FP93	CTGGC GACGT AATCC ACG
FP94	CTTGT CCAGG GAGGC GTG

pH7.6, 2 mM DTT, 3 mM $Mg(OAc)_2$, 100 mM KOAc, 3.125 mg/ml cycloheximide), layered on a 600 µl sucrose cushion (15% sucrose, 10 mM Tris-HCl, pH7.5, 70 mM NH_4OAc, 4mM $Mg(OAc)_2$, 1 mM DTT, 3.125 mg/ml cycloheximide), and centrifuged at 75,000 rpm in 2ml Beckman® polyallomer Eppendorf tubes in a TLA100.3 rotor for one hour at 4°C. The supernatants were removed and the pellets washed once with 1ml cushion buffer (no sucrose), and resuspended with 20µl 2X SDS-PAGE loading buffer. Samples were analyzed by tricine SDS-PAGE.

4.2.4 Primer Extension Inhibition (Toeprinting)

The primer extension inhibition (toeprinting) assays were accomplished as described (Wang and Sachs, 1997) using primers FP93 and FP94 (Figure 4.1C and Table 4.2); 8 µl of sample instead of 4 µl was loaded onto each gel lane. The gels were dried and exposed to screens of a Molecular Dynamics PhosphorImager for approximately 24 hours. All toeprinting data shown were representative of multiple experiments.

4.3 RESULTS

4.3.1 Increasing the Amount of Arg Caused Increased Accumulation of Met_9-AAP Translated from Met_9-AAP–LUC Fusion mRNA Encoding Wild-Type AAP

Direct detection of [^{35}S]Met-labeled peptides encoded by short uORFs without immunoprecipitation or further purification has been proven unsuccessful in several cases (Cao and Geballe, 1996; Raney et al., 2000). Our attempts to detect [^{35}S]Met-labeled wild-type AAP or a [^{35}S]Met- and [^{35}S]Cys-labeled, functional His-tagged AAP (each specified by a uORF) were also unsuccessful. Since the N-terminus of the AAP can be extended without affecting function (data not shown), we constructed uORF coding regions for both wild-type and D12N *N. crassa* AAPs so that the peptides would contain an additional 8 Met codons at their N-termini (Fang et al., 2002). The Met_9-AAP is readily detectable without immunoprecipitation (Fang et al., 2002). The wild-type but not the D12N Met_9-AAP conferred Arg-specific regulation as assessed by toeprinting,

measurement of [^{35}S]Met-labeled LUC, and LUC activity assays (experiments below and Fang et al., 2002) .

To obtain further insight into the mechanism of nascent AAP-mediated ribosome movement during translation, we used wild-type and 'D12N' (the 'D12N' designation refers to the 12-th amino acid position in the naturally occurring *arg-2* uORF, not the position in subsequent constructs) Met_9-AAP-LUC fusions. We first examined the effect of the increased concentration of arginine on the synthesis of these fusion proteins by analyzing the [^{35}S]Met radiolabeled polypeptides by gel electrophoresis. Equal amounts of capped and polyadenylated mRNAs encoding Met_9-AAP-LUC fusions proteins were translated in *N. crassa* extracts containing different concentrations of Arg for 30 minutes (Figure 4.2). mRNAs encoding Met_9-AAP_{wt}-LUC and Met_9-AAP_{D12N}-LUC produced major translation products whose migration was consistent with the predicted size of 606 residues for the full length polypeptides (Figure 4.2 and data not shown). The mRNA encoding Met_9-AAP_{wt}-LUC protein also produced a short intermediate translation product in high Arg (Figure 4.2, lane 2-4) but not low Arg (Figure 4.2. lane 1) whose migration was consistent with the predicted size of 32 residues for Met_9-AAP_{wt} peptide. The accumulation of this Met_9-AAP_{wt} product increased with increased Arg, while the mRNA encoding the Met_9-AAP_{D12N}-LUC protein did not produce this small product at any concentration of arginine (Figure 4.2, lane 5-8). As the amount of the small Met_9-AAP_{wt} product increased, the amount of larger translation products decreased (Figure 4.2, compare lane 2 with lane 1, lane 3 with lane 1, lane 4 with lane 1). In contrast, the accumulation of the full length Met_9-AAP_{D12N}-LUC product was not affected by Arg (Figure 4.2, lane 5-8).

The decreased accumulation of Met_9-AAP-LUC products with increased Arg also correlated with increased Arg-regulation as measured by LUC assay (Figure 4.2, lanes 2-4). Consistent with these observations, previous toeprinting results indicated that increases in the Arg concentration in translation reactions caused more ribosome stalling when Met_1-AAP-LUC constructs were translated (Wang et al., 1999). These results are consistent with the idea that the Met_9-AAP_{wt} product is the nascent wild type AAP and that as Arg increases, more of this product is trapped inside the ribosome.

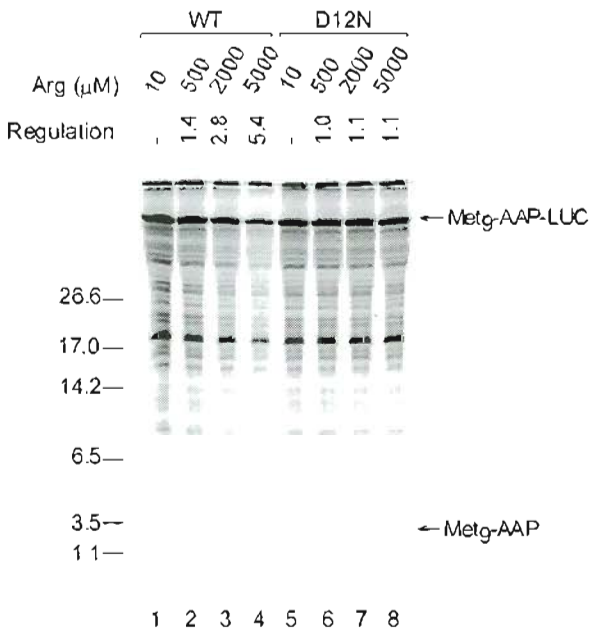

Figure 4.2. Effect of increasing the concentration of Arg on translation of Met_9-AAP-LUC transcripts in *N. crassa* cell-free extracts. Micrococcal nuclease-treated *N. crassa* extracts (10 μl containing 10 μCi of [^{35}S]methionine) were programmed with 60 ng of the indicated RNAs and incubated for 30 min at 25°C. Reaction mixtures contained 10 μM, 500 μM, 2000 μM or 5000 μM Arg and 10 μM each of the other 19 amino acids. Reactions were stopped by adding an equal volume of SDS loading buffer and examined by Tricine-SDS-PAGE in 16.5% polyacrylamide gels. Arginine regulation (indicated at the top of the gel), was calculated as the ratio of luciferase enzyme activity produced after 30 min in nonradioactive reaction mixtures containing 500 μM, 2000 μM or 5000 μM Arg versus 10 μM Arg as described (Wang and Sachs, 1997). Positions of protein products corresponding to full-length LUC or the Met_9-AAP are indicated.

4.3.2 Synthesis of the Met_9-AAP_{wt}-LUC Polypeptide Is Delayed by High Arg

The relationship between the synthesis of Met_9-AAP products and full-length polypeptides was examined through time-course studies. Equal amounts of mRNAs encoding Met_9-AAP_{wt}-LUC and Met_9-AAP_{D12N}-LUC fusions were translated in extracts containing 10 or 2000 μM Arg. Aliquots were removed at intervals and the [^{35}S]Met radiolabeled translation products were analyzed by SDS-PAGE (Figure 4.3).

The appearance of full-length Met_9-AAP_{wt}-LUC polypeptide was slowed by high Arg (Figure 4.3A). In the reaction containing 10 μM Arg, the appearance of this product was first visible at 8 min (Figure 4.3A, lane 5), as for the full length product corresponding to the Met_9-AAP_{D12N}-LUC polypeptide (Figure 4.3B, lane 5 and lane 13). However, in 2000 μM Arg, synthesis of the full length Met_9-AAP_{wt}-LUC product was delayed until 10 minutes (Figure 4.3A, lane 14). The short Met_9-AAP_{wt} product also accumulated in 2000 μM Arg (Figure 4.3A, lanes 10-16); it was first observed after 2 minutes (Figure 4.3A, lane 10), reached its highest level at 6-8 minutes, and then decreased. In contrast, Met_9-AAP_{D12N}-LUC synthesis was not affected by high Arg (Figure 4.3B) and the Met_9-AAP_{D12N} product was not observed in high Arg (Figure 4.2 and Figure 4.3B). These data suggest that AAP-mediated ribosome stalling is associated with the accumulation of the Met_9-AAP_{wt} product and the delayed synthesis of the fusion polypeptide.

4.3.3 Nascent Wild-Type Met_9-AAP Arrested during Elongation Is Largely Associated with Stalled Ribosome

Was the Met_9-AAP that accumulated in high Arg associated with ribosomes? mRNAs encoding Met_9-AAP-LUC fusions were translated in *N. crassa* extracts containing 10 or 2000 μM Arg for 10 minutes and a portion of each reaction mixture subjected to ultracentrifugation to pellet ribosomes. Both the unfractionated extract and the resuspended ribosome pellet were analyzed by SDS-PAGE In low Arg, the translation of the mRNA for the Met_9-AAP_{wt}-LUC fusion did not result in the accumulation of Met_9-AAP_{wt} products and no Met_9-AAP products were observed in

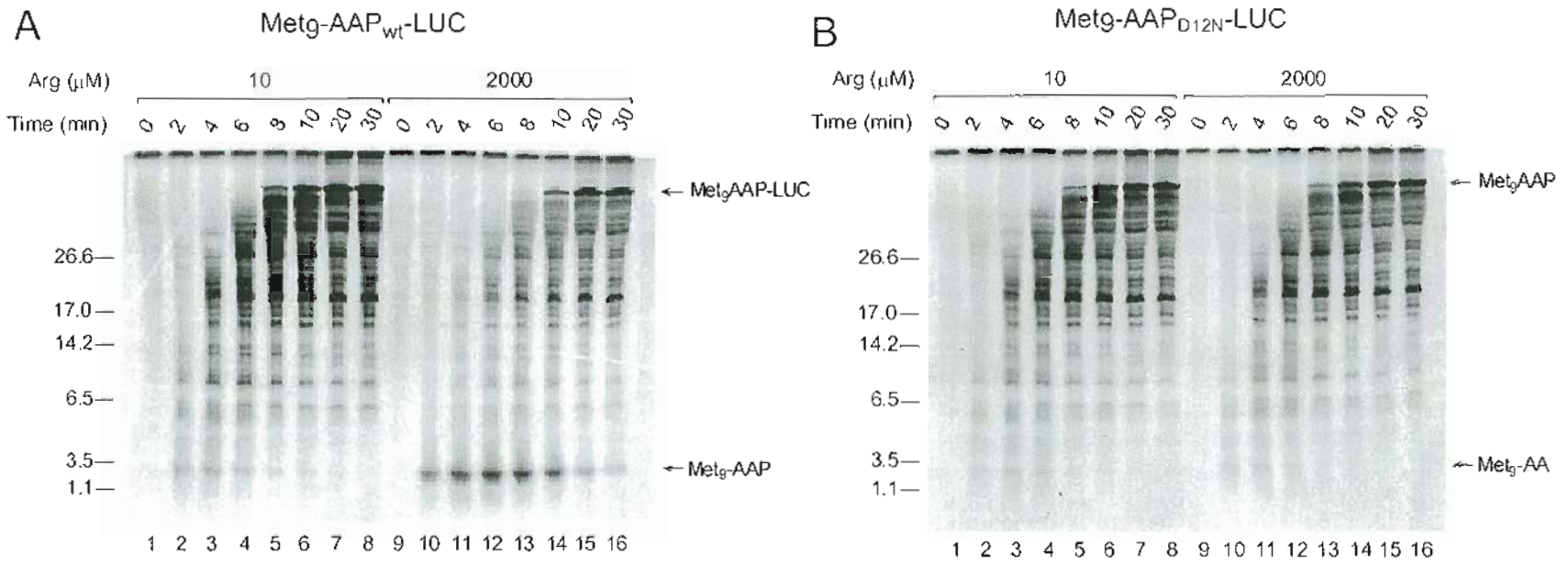

Figure 4.3. Translation timecourse for Met_9-AAP-LUC transcripts in *N. crassa* cell-free extracts. Micrococcal nuclease-treated *N. crassa* extracts (50 μl containing 50 μCi of [^{35}S]methionine) were programmed with 600 ng of wild-type AAP RNA (A) or D12N AAP RNA (B), and incubated at 25°C in the presence of either 10 μM or 2000 μM Arg and 10 μM each of the other 19 amino acids. 10 μl of reaction mixtures was removed into an equal volume of SDS loading buffer at the indicated timepoints and examined by Tricine-SDS-PAGE in 16.5% polyacrylamide gels. Positions of polypeptides corresponding to the Met_9-AAP-LUC and Met_9-AAP are indicated to the right.

ribosome pellet (Figure 4.4, lane 1 and 2). However, in high Arg, the translation of this mRNA led to accumulation of Met_9-AAP_{wt} product (Figure 4.4, lane 3) and the bulk of this product was in the ribosome pellet (Figure 4.4, lane 4). To determine that the Met_9-AAP_{wt} product in the ribosome pellet was nascent peptide associated with the peptidyl transferase center, we incubated translation extracts for 6 minutes, then treated with puromycin for 4 minutes. Puromycin would be expected to release the nascent peptides from the ribosome. The treatment with puromycin largely decreased the amount of Met_9-AAP_{wt} product in the ribosome pellet (Figure 4.4, compare lane 7 with lane 8). Treatment of extracts containing low Arg did not give unexpected side effects (Figure 4.4, lane 5 and 6). In no case did the translation of the mRNA encoding the Met_9-AAP_{D12N}-LUC fusion cause accumulation of Met_9-AAP_{D12N} products (Figure 4.4, lanes 9 and 11), nor were Met_9-AAP_{D12N} products observed in the ribosome pellet (Figure 4.4, lanes 10 and 12). These results indicated that the nascent wild-type AAP was stalled by high Arg in the peptidyl transferase center.

4.3.4 Ribosome Stall after Synthesizing an Internal AAP Domain in High Arg and Resume the Polypeptide Synthesis after Stalling

A series of constructs were designed to assess whether the AAP would function as an internal domain and to determine whether ribosomes resumed protein synthesis after stalling (Figure 4.1). The open reading frame in the mRNAs transcribed from these constructs contains four coding motifs. The N-terminal coding motif encodes Met_9-AAP, the second coding motif encodes residues 80-141 of rabbit alpha-globin, the third coding motif encodes residues 2-23 of the AAP, and the C-terminal coding motif encodes residues 418-551 of the firefly luciferase. All of in-frame or out-of-frame ATG codons except the 9 Met-codons at the N-terminus of the open reading frame were eliminated (Figure 4.5A). The constructs were designed so that each of the AAPs was either wild-type or D12N (four combinations).

The regulatory effects of each AAP domain in these Met_9-AAP-Globin-AAP-LUC reporters were tested by [^{35}S]Met pulse-chase experiments. Equal amounts of mRNA encoding each reporter were used to program *N. crassa* extracts containing 10 or 2000 μM Arg. Edeine was added after 2 min of incubation to block further initiation but

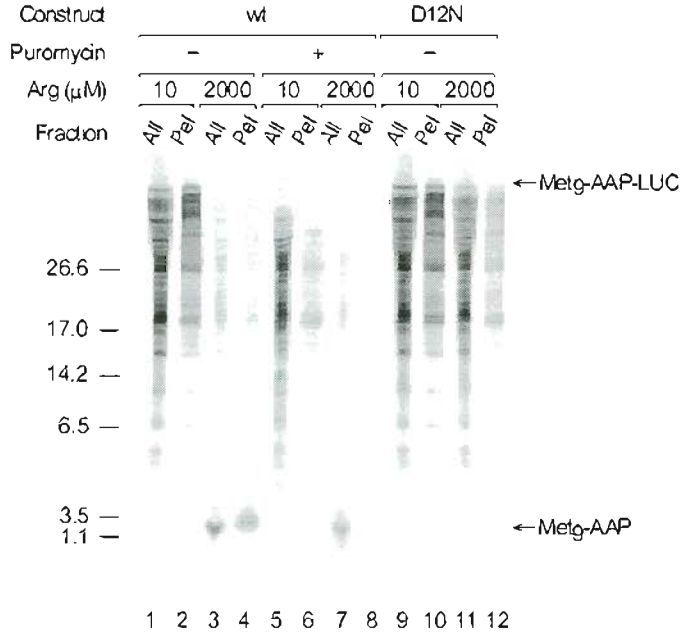

Figure 4.4. Detection of ribosome-associated Met_9-AAP. Nuclease-treated *N. crassa* extracts (40 μl containing 40 μCi of [^{35}S]Met) were programmed with 240 ng of wild-type Met_9-AAP-LUC RNA or D12N Met_9-AAP-LUC RNA, and incubated at 25°C in the presence of either 10 μM or 2000 μM Arg and 10 μM each of the other 19 amino acids. Puromycin (final concentration 2.5 mM) was added after 6 min of translation. 20 μl of reaction mixture was removed after 10 min translation and added to an equal volume of SDS-PAGE loading buffer (lanes labeled "All"). The remaining 20 μl reaction mixture was processed to pellet ribosomes and analyzed as described in experimental procedures (lanes labeled "Pel"). Positions of polypeptides corresponding to Met_9-AAP-LUC and Met_9-AAP are indicated on the right.

not elongation, and aliquots were removed at intervals for analysis by SDS-PAGE. The synthesized polypeptides were therefore first 'pulsed' with [^{35}S]Met for 2 minutes (since Met-codons are only at the N-termini), and all intermediate and full-length radiolabeled products visualized by SDS-PAGE should be derived from the translation initiated before the addition of edeine.

The translation of mRNA encoding Met_9-AAP-Globin-AAP-LUC with both N-terminal and internal D12N AAP domains yielded similar full-length products in low or high Arg (Figure 4.5, A and B, arrows), as expected since D12N AAP domains should not cause ribosome stalling in response to high Arg. The translation of mRNA encoding the reporter with a wild-type AAP domain at its N-terminus and a D12N AAP domain internally accumulated a short intermediate translation product in high Arg (Figure 4.5D, filled arrowhead), but not in low Arg (Figure 4.5C) whose migration was consistent with the predicted size of the Met_9-AAP_{wt} peptide. The synthesis of the full length product was also delayed by high Arg. The disappearance of the short product was accompanied by the accumulation of full length product (Figure 4.5D, lane 4-8).

The translation of the mRNA encoding the reporter protein with a D12N AAP domain at its N-terminus and a wild-type AAP domain internally accumulated an intermediate translation product in high Arg whose migration was consistent with the predicted size of 123 residues for Met_9-AAP-Globin-AAP peptide (Figure 4.5F, lane 4-12, open arrowhead). Less of this product was also observed in low Arg (Figure 4.5E, lane 4-7, open arrowhead). The synthesis of the full length product was delayed by high Arg. The decrease in the level of the intermediate products was accompanied by an increased level of full length products.

The regulatory functions exerted by N-terminal and internal AAP domains were further examined by the translation of mRNA encoding wild-type AAP domains both at N terminus and internally. The translation of this mRNA led to accumulation of intermediate products in high Arg corresponding to the Met_9-AAP and Met_9-AAP-Globin-AAP products (positions indicated by filled arrowhead and open arrowhead respectively), and the synthesis of the full-length product was delayed by high Arg (Figure 4.5G and H).

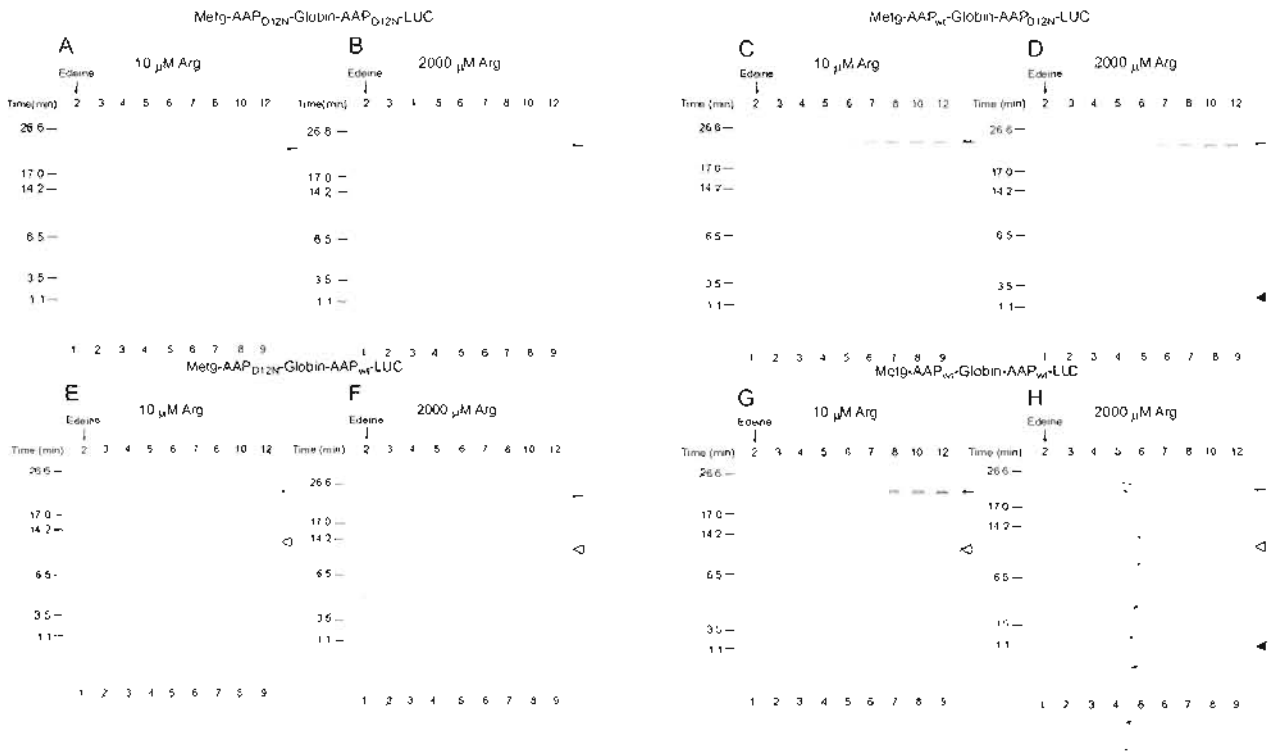

Figure 4.5. Translation time-course for Met$_9$-AAP-Globin-AAP-LUC transcripts in *N. crassa* cell-free extracts. (A) – (H). Micrococcal nuclease-treated *N. crassa* extracts (50 µl containing 50 µCi of [^{35}S]methionine) were programmed with 600 ng of indicated mRNA, and incubated at 25°C in the presence of either 10 µM or 2000 µM Arg and 10 µM each of the other 19 amino acids. Edeine was added at 2 min and 10 µl of reaction mixtures were removed at the indicated timepoints and analyzed as in Figure 4.3. The arrow indicates the position of polypeptides corresponding to Met$_9$-AAP-Globin-AAP-LUC, the open arrowhead indicates the position of polypeptides corresponding to Met$_9$-AAP-Globin-AAP, and the filled arrowhead indicates the position of polypeptides corresponding to Met$_9$-AAP.

The delayed synthesis of longer products in high Arg and the inverse correlation between the loss of intermediate products and the gain of the longer products in these experiments indicated that the ribosomes stalled at N-terminus or internal wild-type AAP domains and could resume translation subsequently.

4.3.5 The Half-Life of the Nascent AAP-Ribosome Complex Increased as the Arg Concentration Increased

We measured the decay-rate of the intermediate products observed in the [^{35}S]Met pulse-chase experiments as the loss of radiolabeled material corresponding to these products as a function of time. The decay rate reflects the stability of the nascent AAP-ribosome complex. Translation reactions containing 10 μM, 500 μM, 2000 μM or 5000 μM Arg were analyzed as described above (Figure 4.5 and data not shown). The amount of radiolabel in each intermediate product was quantitated as described in Materials and Methods. The first-order regression plots for the normalized intensity data are shown in Figure 4.6 and the half-lives for the different intermediate products calculated from these plots are summarized in Table 4.3.

The half-lives of the Met_9-AAP_{wt} products obtained from the translation of mRNAs encoding polypeptides containing an N-terminal wild-type AAP domain increased with the increase of the concentration of arginine (Figure 4.6, A and B; and Table 4.3). The half-lives at each concentration of arginine was similar for the Met_9-AAP_{wt} product regardless of whether the polypeptide contained a wild-type or D12N internal domain (Table 4.3). The half-life of the Met_9-AAP_{D12N}-Globin-AAP_{wt} product also increased with increased arginine (Figure 4.6C, and Table 4.3). The half-life of this product at each concentration of arginine was longer than the corresponding half-life of the Met_9-AAP product (Table 4.3). These experiments and analyses indicated that increasing concentrations of arginine stabilized the nascent AAP-ribosome complex.

4.3.6 Arg-Stalled Nascent Peptides Appear to Be in Peptidyl-tRNA Form

To confirm that the two species of the accumulated intermediate products observed in [^{35}S]Met pulse-chase experiment were nascent peptides capable of being further extended, as the pulse-chase studies indicate, we determined whether these

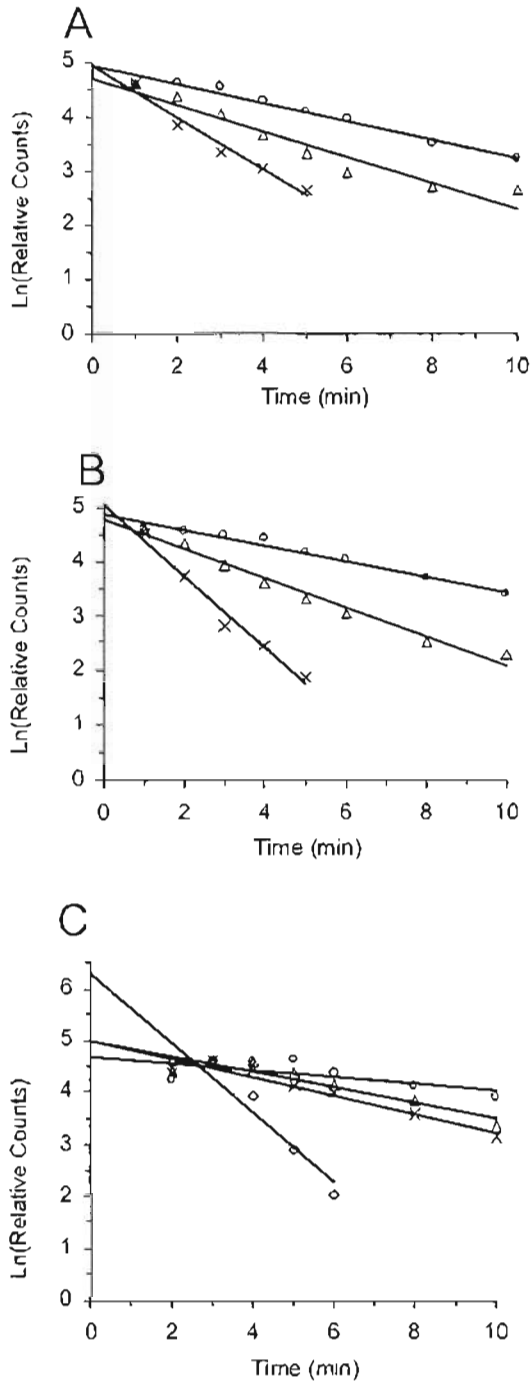

Figure 4.6. Effect of arginine concentration on the decay rate of the nascent AAP-ribosome complex. (A). The decay rate of the Met_9-AAP_{wt}-ribosome complex on the synthesis of Met_9-AAP_{wt}-Globin-AAP_{D12N}-LUC (see Figure 4.5, C and D). (B). The decay rate of the Met_9-AAP_{wt}-ribosome complex on the synthesis of Met_9-AAP_{wt}-Globin-AAP_{wt}-LUC (see Figure 4.5,G and H). (C). The decay rate of the Met_9-AAP_{D12N}-Globin-AAP_{wt}-ribosome complex on the synthesis of Met_9-AAP_{D12N}-Globin-AAP_{wt}-LUC (see Figure 4.5,E and F). The [^{35}S]Met pulse-chase experiments were carried out as described in Figure 4.5, the gels were dried and exposed under PhosphorImager plates for 16 hours. The intensity counts of each band were quantitated with ImageQuant® 5.1 from Molecular Dynamics, the count were then normalized within each data series to the largest value, which was set as 100. The natural Log of the relative counts was plotted *versus* time of translation after the addition of edeine. The first rate regression was simulated based on the trend of each data series. Diamond label was for the data series obtained from the translation in 10 μM Arg, cross label for 500 μM Arg, triangle label for 2000 μM Arg, and circle label for 5000 μM Arg.

Table 4.3

The Half-Lives of the Nascent Peptide-Ribosome Complexes

	Met_9-AAP		Met_9-AAP-Globin-AAP
Arg (μM)	wt-D12N[1]	wt-wt[1]	D12N-wt[1]
10	N/A	N/A	1.0 min
500	1.5 min	1.1 min	3.9 min
2000	2.9 min	2.6 min	4.6 min
5000	4.1 min	4.9 min	10.9 min

[1] The structure of two AAP domains in the Met_9-AAP-Globin-AAP-LUC polypeptide

peptides were present in peptidyl-tRNA form. Several approaches have been used to examine whether the nascent peptides are in peptidyl-tRNA form when they cause ribosome stalling (Cao and Geballe, 1996; Gong et al., 2001; Raney et al., 2002), including immunoprecipitating translation products and analyzing them by tricine SDS-PAGE (Cao and Geballe, 1996), analyzing translation products using acid-urea gels (Raney et al., 2002), and analyzing resuspended ribosome pellets by tricine SDS-PAGE (Gong et al., 2001). The peptidyl-tRNAs can also be selectively precipitated by CTAB (cetyltrimethylammonium bromide) and the radiolabeled peptides hydrolyzed from the precipitated peptidyl-tRNAs examined by SDS-PAGE (Gilmore and Blobel, 1985). We chose this latter approach to test whether the nascent peptides that stalled ribosomes were in peptidyl-tRNA form because of its reliability and simplicity. Time-course for the translation of mRNA specifying Met_9-AAP_{wt}-Globin-AAP_{wt}-LUC in a reaction containing 2000 μM Arg was accomplished as described above. The aliquots that were removed at intervals were either prepared for direct analysis by the addition of SDS loading buffer as in the previous experiments or subject to CTAB precipitation prior to analysis. Samples were analyzed in parallel by SDS-PAGE (Figure 4.7). CTAB precipitation yielded enrichment for two intermediate products whose migration on SDS-PAGE corresponding to Met_9-AAP (Figure 4.7B, filled arrowhead) and Met_9-AAP-Globin-AAP respectively (Figure 4.7B, open arrowhead). The relative intensities of these intermediate products changed over time in a manner consistent with their existing

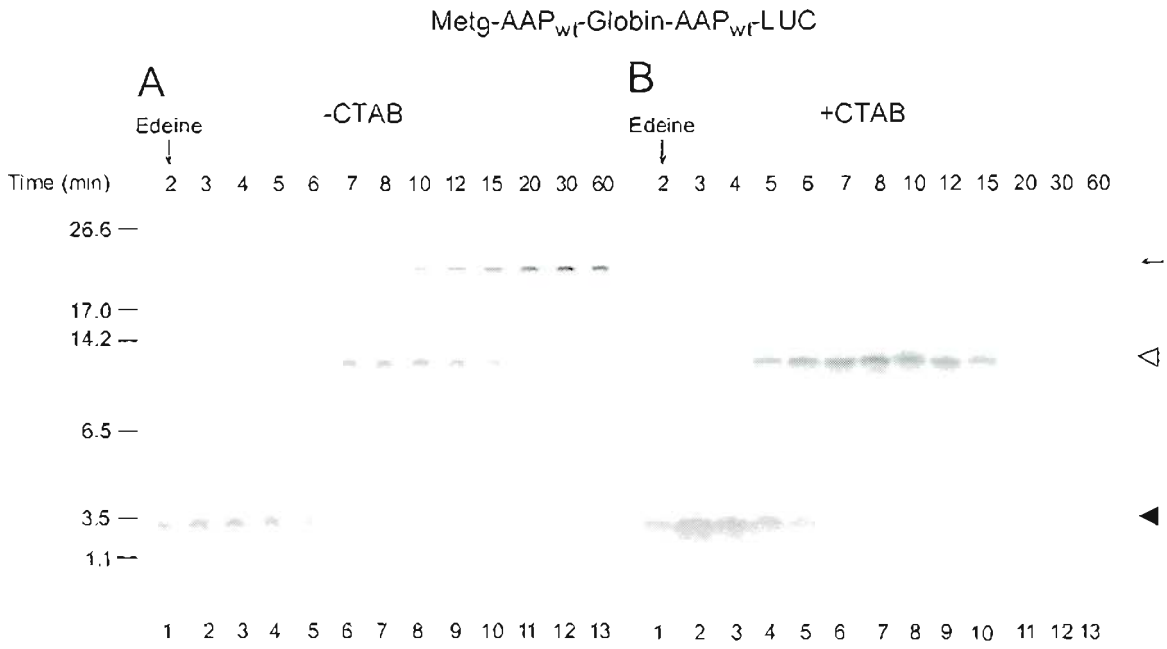

Figure 4.7. Cetyltrimethylammonium bromide (CTAB) precipitation of peptidyl-tRNA from cell-free translation reactions. Micrococcal nuclease-treated *N. crassa* extracts (75 µl containing 75 µCi of [^{35}S]methionine) were programmed with 900 ng of indicated mRNA, and incubated at 25°C in the presence of 2000 µM Arg and 10 µM each of the other 19 amino acids. Edeine was added at 2 min. For analysis of translation products without CTAB precipitation (A), 10 µl of reaction mixtures were removed at the indicated timepoints and analyzed as in Figure 4.3. For analysis of translation products precipitated with CTAB (B), 10 µl of reaction mixtures were removed into 250 µl of 2% (w/v) CTAB solution at the indicated timepoints and vortexed quickly, 50 ng yeast tRNA and 250 µl of 0.5 M sodium acetate (pH5.4) were added into the mixture, the mixture was incubated at 30°C for 10 minutes and then centrifuged. The pellet was washed twice with acetone:HCl (19:1), vacuum-dried, resuspended in SDS-PAGE loading buffer, and examined by Tricine-SDS-PAGE in 16.5% polyacrylamide gels. The arrow, open arrowhead and filled arrowhead are as in Figure 4.5.

as intermediates in translation. The CTAB precipitation yielded much less full-length product than intermediates (Figure 4.7, compare the products indicated by arrow in panel A and panel B), as expected because this product should be released from tRNA through the normal termination process. These results strongly indicated that the Arg-stalled nascent peptides observed in [^{35}S]Met pulse-chase experiments are in peptidyl-tRNA form and those translation intermediates, not dead-end products.

4.3.7 Primer-Extension Inhibition (Toeprinting) Analyses Show That Ribosomes Stall Immediately after Translating Each of the AAP Coding Regions

The toeprinting assay enables mapping of the positions of the stalled ribosomes on mRNA in *N. crassa* cell-free translation systems (Wang and Sachs, 1997; Sachs et al., 2002). We used this assay to map precisely the positions of ribosomes that the [^{35}S]Met data indicated had stalled after synthesizing either N-terminal or internal wild-type AAP domains. *In vitro* translation reaction mixtures containing 10 μM, 500 μM, 2000 μM or 5000 μM Arg were programmed with the mRNAs containing two AAP coding regions as used in the [^{35}S]Met pulse-chase experiments; toeprint analyses were accomplished with radiolabeled primers FP94 or FP93 following 20 minutes translation (Figure 4.8). The toeprinting results showed that ribosomes stalled immediately following both N-terminal (Figure 4.8A, filled arrowhead) and internal wild-type AAP coding regions (Figure 4.8B, open arrowhead) in response to Arg (Figure 4.8, A and B, lane 2, 3, 4). Stalling increased as the concentration of Arg increased, and stalling after the N-terminal AAP appeared stronger than stalling after the internal AAP. The results with the other constructs were also consistent with [^{35}S]Met pulse-chase labeling data. Ribosome stalled in response to Arg only after the internal AAP on mRNA that encoded the N-terminal D12N AAP and an internal wild-type AAP (Figure 4.8B, lane 7, 8, 9); ribosomes stalled only after the N-terminal AAP on mRNA that encoded the N-terminal wild-type AAP and an internal D12N AAP (Figure 4.8A, lane 12,13,14); ribosomes did not stall on mRNA containing D12N N-terminal & internal AAPs. These data confirmed that the synthesis of either N-terminal or internal AAP domains with wild-type sequence but not D12N sequence could cause ribosome stalling.

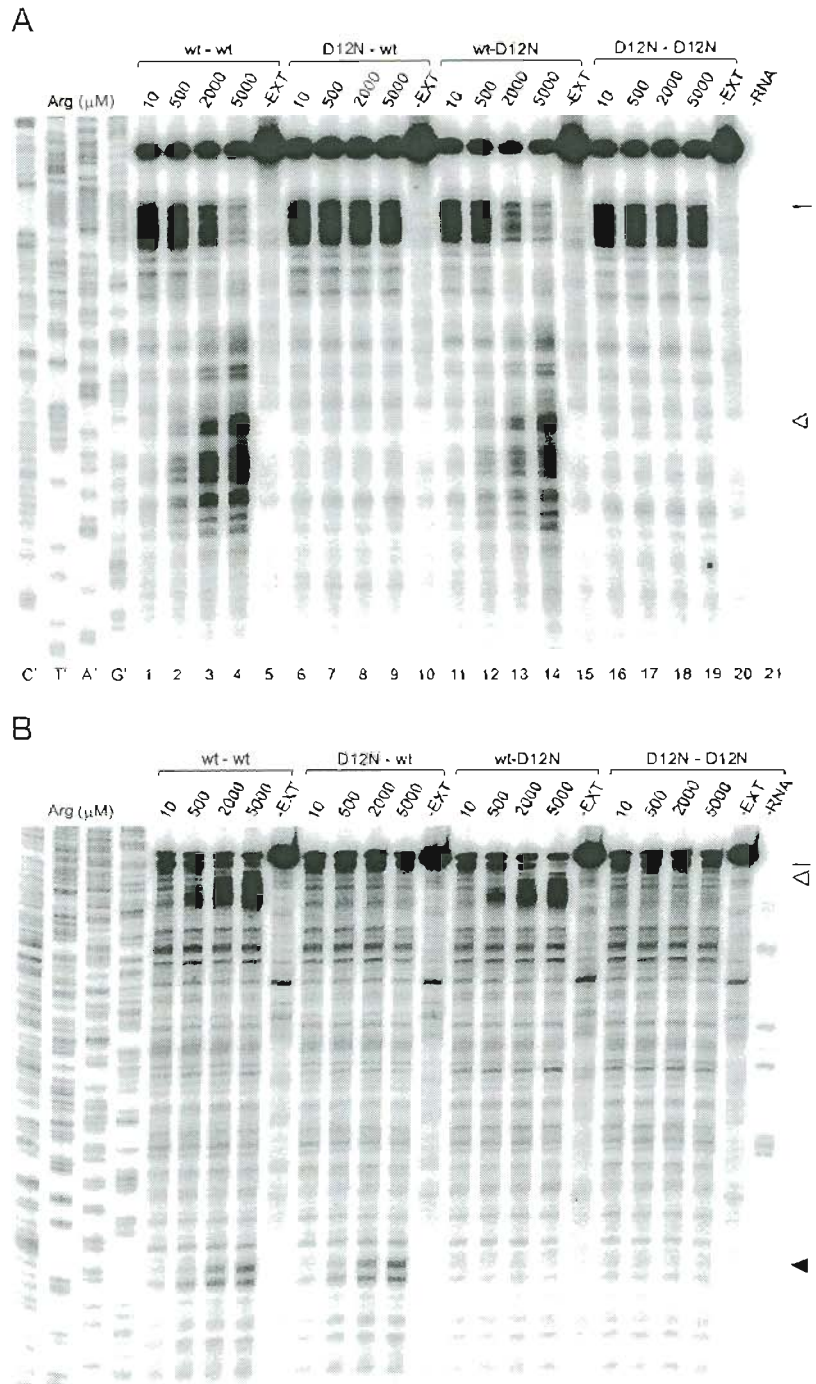

Figure 4.8. Toeprinting analyses of ribosome stalling caused by the translation of the AAP coding motifs in Met_9-AAP-Globin-AAP-LUC mRNA. (A). Toeprinting analysis of ribosome stalling by the translation of Met_9-AAP with radiolabeled primer FP94 (Figure 4.1C). (B) Toeprinting analysis of ribosome stalling by the translation of Met_9-AAP-Globin-AAP with radiolabeled primer FP93 (Figure 4.1C). The transcripts examined (Figure 4.1B) are indicated at the top. Equal amounts of transcripts (120 ng) were translated in 20 μl reaction mixtures at 25°C. Reaction mixtures contained 10 μM,

500 μM, 2000 μM, or 5000 μM Arg and 10 μM of the other 19 amino acids. After 20 min of translation, 3 μl of each translation mixture was used for toeprinting with primer FP93 or FP94. Dideoxynucleotide sequencing reactions for the template containing Met_9-AAP_{wt}-Globin-AAP_{wt}-LUC are on the left. For dideoxynucleotide sequencing, the nucleotide complementary to the dideoxynucleotide added in each reaction is indicated above the corresponding lane so that the sequence of the template can be directly deduced. The sequence reads 5' to 3' from top to bottom. The products obtained from primer extension of pure RNAs (18 ng) in the absence of translation reaction mixture (-EXT, lane 5, 10, 15, 20) and from translation reaction mixture not programmed with RNA (-RNA, lane 21) are shown for comparison. The arrow indicates the positions of premature transcription termination products corresponding to ribosomes at the ORF initiation codons. The open arrowhead indicates the positions of premature transcription termination products corresponding to ribosomes at codons right after the N-terminal AAP coding region. The filled arrowhead indicates the positions of premature transcription termination products corresponding to ribosomes at codons right after the internal AAP coding region.

The cluster signals corresponding to the nine Met codons (indicated by arrow), suggested that translation at these 9 methionine-codons was slow. Whether one or more of those codons was used for initiation could be determined by the addition of cycloheximide to translation reactions before elongation can begin (Gaba et al., 2001). The results from such an experiment using related constructs indicated that ribosomes could initiate translation at each of those codons, although the initiation efficiency decreased at each subsequent codon (data not shown).

4.4 DISCUSSION

The nascent Arginine Attenuator Peptide regulates the movement of the translating ribosome in response to the level of Arg. Our previous studies with toeprinting analyses indicated that high Arg causes ribosome stalling at the termination codon when the AAP is encoded by a uORF, or at the coding region right after the AAP coding region when the AAP is encoded by a N-terminal coding motif in the mRNA (Wang et al., 1998). In this study, using a [^{35}S]Met pulse-chase technique along with toeprinting analyses, we showed that the synthesis of an internal AAP domain within a heterologous polypeptide stalls the ribosome in high Arg. To our knowledge, this is the first example that the synthesis of an internal domain within a nascent polypeptide regulates the movement of the translating ribosome in response to the level of a small molecule.

The control of ribosome movement by the AAP in high Arg is dependent on its peptide sequence but not its mRNA-coding sequence (Fang et al., 2000), and on Arg, but not the level of aminoacylated arginyl-tRNA (Wang et al., 1999). Therefore, we have hypothesized that the nascent AAP together with free Arg interferes with a step in translation common to elongation and termination, resulting in ribosome stalling. The results from the studies described here show that, in high Arg the nascent AAP was still associated with the stalled ribosome (Figure 4.4). Furthermore, the stalling at an N-terminal AAP domain or an internal AAP domain was associated with the accumulation of a peptidyl-tRNA complex (Figure 4.7), indicating the blockage of a step in translational elongation. So far, we have not obtained any direct evidence that the

nascent AAP encoded by a uORF is in its peptidyl-tRNA form when the ribosome stalls at a termination codon.

Based on our current understanding in translation process and ribosomal structure (Noller et al., 2002; Ramakrishnan, 2002), each elongation cycle basically comprises of: occupation of the ribosomal A site by aminoacylated tRNA, decoding by correct codon-anticodon interactions, peptide bond formation at the peptidyl-transferase center (PTC), and translocation. During the process of peptide bond formation, the P-site tRNA is deacylated and the peptide chain is transferred to A-site tRNA spontaneously. Translocation then occurs: the deacylated tRNA moves from the P-site to the E site (the exit site), the peptidyl-tRNA moves from A site to P-site, the A-site becomes empty and able to receive the next cognate aminoacylated tRNA, and the mRNA advances by three bases, exposing the next codon in the A-site. If the mRNA exposes a stop codon in the A-site following the translocation, then termination starts. Release factor eRF1 enters the A-site instead of an aminoacylated tRNA, triggering the hydrolysis of the peptidyl-tRNA and the release of the nascent peptide.

Considering the common steps shared by the processes of elongation and termination, and that ribosome stalling in high Arg occurs right after the synthesis of the intact AAP domain, we think it likely that the nascent peptide and Arg interfere with translocation.

The translocation problem (or other problem if this hypothesis is incorrect) during elongation caused by the synthesis of the AAP domain in high Arg appears to be temporary based on quantitative analyses. The amount of [^{35}S]Met incorporated into the full-length heterologous polypeptides containing the wild type N-terminal and internal AAP domains corresponds to the amount of [^{35}S]Met incorporated into the accumulated intermediate stalled after the synthesis of the N-terminal AAP domain. The radiolabel also transiently appears in the intermediate corresponding to a stall after the internal AAP before it accumulated in full-length product (Figure 4.7A and data not shown). Therefore, the stalled ribosome, after synthesizing either the N-terminal or the internal AAP domain in high Arg, resumes translation.

Ribosome stalling caused by nascent peptides containing specific amino acid sequences is also observed in other eukaryotic and bacterial systems (reviewed in Lovett

and Rogers, 1996; Geballe and Sachs, 2000; Morris and Geballe, 2000; Tenson and Ehrenberg, 2002). Although the AAP appears to be so-far unique in its specific mechanism, it seems that other nascent peptides which cause ribosome stalling during elongation may share some common regulatory features with the AAP. *E. coli secM* encodes a peptide in the 5' leader of the *secA* gene. The translation of a 17aa internal domain (149aa downstream from the N-terminus) in SecM polypeptide causes ribosome stalling when the cellular level of SecA protein is low. The peptidyl-tRNA accumulates and the nascent peptide interacts with the ribosome exit tunnel to block translocation (Nakatogawa and Ito, 2001; Nakatogawa and Ito, 2002). The translation of *cat, cmlA* and *ermC* 5' leader peptides also causes ribosome stalling during elongation in response to antibiotics (Lovett and Rogers, 1996). Recent structural analyses indicate that erythromycin binds adjacent to peptidyl-transferase center in the exit tunnel to interfere with the nascent peptide chain passing through the exit tunnel (Schlunzen et al., 2001; Hansen et al., 2002).

The regulatory mechanisms used by nascent peptides to cause ribosome stalling at termination codons must also be considered when evaluating how the AAP could function. The examples include the 6-codon peptide encoded by uORF in the transcript of S-adenosylmethionine decarboxylase (AdoMetDC), the 22-codon peptide encoded by uORF2 in the transcript of human cytomegalovirus gpUL4, and the 24-codon peptide encoded by *E. coli tnaC* in the 5' leader of the tryptophanase mRNA. In all cases, the accumulation of peptidyl-tRNA has been observed at termination. The peptide encoded by the uORF of S-AdoMetDC causes ribosome stalling in the presence of high levels of polyamines; an elevated level of spermidine could stabilize the peptidyl-tRNA in the stalling ribosome at termination (Raney et al., 2002). The nascent peptide encoded by uORF2 of gpUL4 remains associated with the ribosome in the form of peptidyl-tRNA even when stalling ribosome disengaged from the mRNA, and the peptidyl-tRNA-ribosome complex is insensitive to puromycin (Cao and Geballe, 1998). This peptidyl-tRNA interacts with eRF1 to inhibit peptide release (Janzen et al., 2002). Similarly, the synthesis of the TnaC peptide in high Trp causes ribosome stalling at termination by interfering with release factor-mediated peptidyl-tRNA hydrolysis, and the ribosome complex is again puromycin-insensitive (Gong et al., 2001). By replacing the stop codon

with the Trp codon, a recent study showed that the synthesis of the TnaC peptide causes constitutive ribosome stalling during elongation only when the Trp-tRNA occupies ribosomal A-site. Thus the nascent TnaC peptide in its peptidyl-tRNA form induces some conformational change in the ribosome to accommodate a Trp-tRNA entering A-site to block release factor-mediated peptidyl-tRNA hydrolysis (Gong and Yanofsky, 2002).

Despite the obvious similarity to the AAP in that the nascent TnaC peptide could cause ribosome stalling during both elongation and termination in response to an amino acid, the regulatory mechanism used by this nascent peptide likely differs from that used by nascent AAP, because regulation by the nascent AAP is not context-dependent (there is no special requirement for specific codons following the AAP coding region), and replacing the stop codon with an Arg codon does not cause similar constitutive ribosome stalling after the synthesis of the AAP domain (data not shown).

How would the nascent AAP domain together with Arg interfere with translocation? By default, the nascent peptide chain extends from the PTC into an exit tunnel, which consists of both protein and rRNA, and which is filled with water (Ban et al., 2000; Nissen et al., 2000; Kramer et al., 2001; Jenni and Ban, 2003). The tunnel may cover 30 to 72 amino acid residues; the size of the tunnel as well as the physical and chemical properties of its walls seems not to allow any complex folding of the nascent peptide (Kramer et al., 2001). Although different folding conformations may exist for different nascent peptides (Tsalkova et al., 1998; Hardesty and Kramer, 2001; Kramer et al., 2001), the C-terminal part of the nascent peptide appears to form as an alpha-helix, since the peptidyl transferase reaction generates a peptide in the form of an alpha-helical turn (Lim and Spirin, 1986).

Interestingly, if the AAP folded into an alpha-helix, D12 and D16 of the AAP would be on the same side of the alpha-helix. These two aspartic acid residues in nascent AAP are the potential candidates to interact specifically with Arg when the Arg concentration is high enough. The results from mutagenesis studies stress the absolute requirement for the D12 residue in the AAP-mediated regulation: the D12N mutation abolishes the Arg-specific regulation both *in vivo* and *in vitro*; the D12E mutation also largely abolishes regulation; the other mutations introduces so far at D12 abolish

regulation, too. On the contrary, although D16N mutation also abolished the regulation, the other mutations at D16 cause either a less severe effect as those mutations at D12 do, or even maintain regulation, indicating D16 is not absolutely required for this Arg-specific regulation (Fang et al., 2000 and references therein)(Spevak, Fang and Sachs, unpublished data and see discussion below).

The observation of the free accessibility of the tunnel interior to water and the requirement of D12 for Arg-specific regulation, leads to a hypothesis that free arginine may directly interact with the D12 (possible aided by D16) on the nascent AAP by electrostatic interactions, which has been observed in the formation of arginine repressor (ArgR) hexamer (Maas, 1994; Van Duyne et al., 1996; Kueh et al., 2003) and in the binding of arginine to arginyl-tRNA synthetase (Delagoutte et al., 2000). The consequence of this interaction either leads to distorting nascent AAP conformation to interferes normal peptide movement in the tunnel, which would most likely involves D12. Alternatively, as for ArgR, Arg might serve as a molecular glue to bind the AAP to some residues in the tunnel that faces D12 (rRNA and/or protein) so that the nascent chain can't move forward. Either of the two scenarios could cause ribosome stalling at translocation. Several lines of the evidence seem to more support the second scenario: Arginine seems not be able to directly bind to synthetic AAP in aqueous environment (Wu and Sachs, unpublished data), and last (24^{th}) amino acid residue in the wild-type AAP must be incorporated into the nascent AAP, but the species of this residue is flexible (Wang and Sachs, 1997; Fang et al., 2000), suggesting that the nascent chain may have to reach a certain position inside the tunnel.

The other evolutionarily conserved residues in the AAP may be required to maintain a functional alpha-helical conformation and/or balance electrostatic interactions. It would be interesting to test if D12K(R) and/or D16K(R) mutation (and it is better that the same wild- type alpha-helix conformation is maintained after the mutations) could mimic the wild type AAP function in low Arg to cause constitutive ribosome stalling.

In addition to the strict requirement for the amino acid residues of the AAP domain, the interactions among the AAP domain, Arg and the tunnel seems to impose an absolute requirement for L-Arg, since neither D-Arg nor other close analogs of L-Arg function as does L-Arg (data not shown). On the other hand, if the nascent AAP and L-

Arg do interact with some components in the exit tunnel, these components seems to be conserved among different eukaryotic ribosomes, since the studies indicated that the nascent AAP domain could function in a variety of well-established cell-free eukaryotic systems including *N. crassa* extracts, *S. cerevisiae* extracts, wheat germ extracts and rabbit reticulocyte lysates (Wang et al., 1999 and Fang, Spevak, Wu and Sachs, unpublished data). It remains to be determined whether the AAP also functions in prokaryotic systems. Finally, the interaction site between the AAP and the components of the tunnel may not be limited to a single site but may span a region along the tunnel, since the synthesis of extended AAP could cause Arg-specific ribosome stalling up to adding six more amino acid residues at C-terminus (Fang et al., 2002).

Elevated Arg level appears to stabilize the interactions that cause stalling, as indicated by the fact that the half-lives of the nascent peptide-ribosome complex increased with the increased level of Arg when measured by [^{35}S]Met pulse-chase experiments (Figure 4.6 and Table 4.3) and toeprinting experiment (data not shown). The enhanced interaction between the nascent AAP and the components inside the tunnel in the presence of an increasing concentration of arginine may either because arginine gains the concentration advantage over the water molecule by expelling water out of the channel and /or arginine binds to multiple components in the translation machinery to enhance the ribosome stalling. Interestingly, the stability of the nascent peptide-ribosome complex after the synthesis of the internal AAP domain is different from that after the synthesis of the N-terminal AAP domain (Figure 4.6 and Table 4.3); this difference may be caused by the additional interactions between the peptide folding machinery and the nascent peptide at the tunnel exit. The stability of the nascent AAP-ribosome complex formed after the synthesis of the uORF-encoded AAP at termination appears to also differ from the one after the synthesis of an AAP domain within a polypeptide during elongation, possibly reflecting the functional and kinetic difference between termination and elongation.

In summary, the synthesis of the AAP domain in high Arg causes the accumulation of peptidyl-tRNA, which may be attributed to the temporary impeded translocation, likely resulting from the interaction among Arg, the nascent AAP domain and the components in the ribosome exit tunnel. The unique ability of the evolutionarily

conserved nascent AAP domain to achieve such an arginine-specific regulation within ribosome regardless of its position within a nascent polypeptide has raised many interesting and critical questions regarding to the regulatory mechanism deployed by the nascent AAP; the fundamental process of eukaryotic translation and how it is controlled; and the structural and functional differences between eukaryotic and prokaryotic translation machinery. The controlled ribosomal movement by nascent AAP domain in response to the level of Arg could also serve as a tool to investigate the mechanisms by which the ribosome elongates the nascent peptide. The further elucidation of the detailed mechanism underlying this AAP-mediated Arg-specific regulation thus will greatly contribute to our understanding in the specific nascent peptide-mediate regulation and general translation process.

CHAPTER 5
CONCLUSIONS AND FUTURE DIRECTIONS

5.1 SUMMARY OF RESULTS

The following results have been obtained from the research work described in this thesis:

5.1.1 Evolutionarily Conserved Features of the Arginine Attenuator Peptide Provide the Necessary Requirements for Its Function in Translational Regulation

AAPs encoded by uORFs in four fungal mRNAs each conferred negative regulation in response to Arg by causing ribosome stalling at the uORF termination codon. Deletion of the non-conserved N-terminus of the AAP did not impair regulation, but deletions extending into the conserved region eliminated it. Introduction of many silent mutations into a functional AAP coding region (26/63 nt changes) did not eliminate regulation, but a single additional nucleotide change altering the conserved AAP sequence abolished regulation. Extension of the AAP at its C-terminus resulted in Arg-mediated ribosomal stalling during either translational elongation or termination within the extended region. Comparison of Arg-mediated stalling at a rare or a common codon revealed more stalling at the rare codon. Taken together, these data indicate that the conserved peptide sequence, but not the mRNA sequence, is responsible for regulation; the highly evolutionarily conserved core of the peptide functions within the ribosome to cause stalling. Translational events at a potential stall sites can influence the extent of stalling at these sites.

5.1.2 *N. crassa* Super-Suppressor Strains Are Still Arg Regulated, and Are Amber-Codon Specific

Seven *ssu* strains (*ssu-1*, *-2*, *-3*, *-4*, *- 5*, *-9*, and *–10*) were tested for their translational regulation by arginine with AAP-LUC reporter genes in cell-free translation extracts. None of them lost arginine-specific regulation. The translational suppression at stop codons is tested using cell-free translation extracts by requiring production of firefly luciferase from a reading frame containing premature UAA, UGA, or UAG terminators. All mutants except *ssu-3* suppressed UAG codons. Maximal UAG-suppression ranged from 15% to 30% relative to controls containing sense codons at the corresponding position. Production from constructs containing UAA or UGA was 1-2%, similar to levels observed with all nonsense codons in wild-type and *ssu-3* extracts. UAG-suppression was also seen using [^{35}S]Met to radiolabel polypeptides. Suppression enabled ribosomes to continue translation elongation as determined using the toeprint assay. tRNA from supersuppressors strains showed suppressor activity when added to wild-type extracts. Thus, these supersuppressors produce amber suppressor-tRNA.

5.1.3 The AAP Domain Can Transiently Stall Ribosomes When Placed Internally in a Polypeptide Sequence and the Duration of the Stall Depends on the Level of Arg

Templates specifying 215-residue reporters containing wild type or D12N AAP domains near their N-termini and between residues 101-123 were constructed for testing which AAP domains could regulate the movement of ribosomes. These reporters contained 9 Met-codons at their N-termini but no other Met codons, either in-frame or out-of-frame. [^{35}S]Met pulse-chase labeling experiments indicated that, in high Arg, the synthesis of the N-terminal or internal AAP domains with wild-type but not D12N sequences caused stalling, and that the half-life of the stalled complex increased when the Arg concentration increased. Precipitation with CTAB (cetyltrimethylammonium bromide) showed that the Arg-stalled nascent peptides observed in pulse-chase experiments were in peptidyl-tRNA form and thus not end-products of an aberrant release event. All of the data were consistent with ribosomes resuming translation after release

from the stall. Primer-extension inhibition (toeprint) analyses confirmed that ribosomes stalled immediately after translating each of the AAP coding regions.

5.2 FUTURE WORK

The ultimate goal of this research is to understand the molecular basis of how the AAP regulates the movement of the ribosome on the mRNA in response to arginine. Based on what we know now, further understanding of the detailed mechanism by which the AAP interacts with the ribosome can be obtained from the following studies:

5.2.1 Determining the Components with Which the AAP Interacts with Inside Ribosome in the Presence of High Arg

The components with which nascent AAP interacts inside the ribosome must be detected *in situ*. Photocross-linking technique has been successfully used in detecting components in translational machinery with which nascent peptides interact during translation (Johnson et al., 1978; Krieg et al., 1986; Do et al., 1996; Dey et al., 1998; Nilsson et al., 1999; Pariyarath et al., 2001). We will adapt this technique to our system to investigate what components the AAP interacts with inside the ribosome in the presence of high Arg. The DNA templates used in the studies described in Chapter 4 (Figure 4.1B) will serve as the starting templates for making synthetic mRNA, and mutations to amber codons will be introduced into different codons in the coding region. Photoaffinity cross-linkers can be incorporated into the nascent chain by adding amber-suppressor tRNA charged with appropriate amino acid analogs.

Traditionally, truncated mRNAs are used in order to keep the ribosome associated with the mRNA (the ribosome stays at the end of such mRNA). Since we have shown that the half-lives of the nascent peptide-ribosome complex are quite long in the presence of high Arg (section 4.3.5 and Figure 4.6), we may not need to make truncated mRNA for photocross-linking experiments. For these studies, the photo-affinity label will be covalently linked to the lysine which is charged on the amber suppressor tRNA, and incorporated into nascent peptide after site-specific suppression by the amber suppressor tRNA during translation elongation. After translation in the dark, the reaction mixture

will be irradiated with UV light to obtain cross-linked product. The products are then analyzed to determine what components are present. First, ribosomes can be separated from the extracts as described in Chapter 4, the separated ribosomes are then dissociated into large and small subunits, and are subject to velocity sedimentation through sucrose gradients to collect different fractions (Johnson and Cantor, 1980; Choi et al., 1998). If the AAP is cross-linked to rRNA, the sites of cross-linking can be determined by primer-extension inhibition assays with selected primers complementary to different regions of the rRNAs (Choi et al., 1998 and references therein; Choi and Brimacombe, 1998). If the AAP is cross-linked to a polypeptide, the conjugated products can be first separated by two-dimensional gel system, the species of the polypeptides can then be identified either through analyses of the peptide sequence with mass spectrometry, or through immunoblotting with antibodies against putative ribosomal proteins.

5.2.2 Determine if Arginine Interacts with Nascent AAP

The detection of the direct binding of arginine to the synthetic AAP *in vitro* was not successfully (Wu, C and Sachs, MS, unpublished data). Mostly likely such binding, if it were to occur, would need the presence of the other components involved in AAP-mediated Arg-specific ribosome stalling. One approach to detect this binding could be to use ^{125}I-labeled L-arginine analogs covalently attached with a cross-linker to obtain cross-linked products. Such an approach would require that we filter out the signals resulted from non-specific reactions; this could be achieved by using D-arginine in excess, since it does not competitively inhibit L-arginine-mediated stalling.

5.2.3 Identifying Additional *Trans*-Acting Factors Involved in Arg-Specific Regulation

We will use genetic approaches to obtain *trans*-acting mutants that affect Arg-specific translational regulation *in vivo*. The mutants can be selected in the *arg-12^s pyr-3* genetic background, which affects the expression of the endogenous *arg-2* gene and an *arg-2-hph* reporter gene (Freitag et al., 1996). The mutants will show increased expression of *arg-2* gene and the *arg-2-hph* reporter gene in the medium containing uridine, Arg and hygromycin. The spectrum of the mutations could be broadened by

using chemical mutagens as well as UV light or by using instructional mutagenesis . The cell-free extracts from these mutant strains will be made for analyzing the effects of the mutations on AAP-mediated ribosome stalling *in vitro*. Alternatively, if we have identified some components in translational machinery are involved in Arg regulation from *in vitro* photocross-linking experiments, we can make mutations on the genes encoding these components, and verify that such mutations cause the loss of the Arg-specific regulation both *in vivo* and *in vitro*.

5.2.4 Concluding Remarks

The prevailing presence of uORFs in the transcripts encoding growth factors and their receptors, proto-oncogenes, and transcription factors, has implicated uORFs in the control of cell growth and differentiation, though the functions of these uORFs are largely unknown. Our understanding of the uORFs responsible for in AAP-mediated arginine-specific regulation, as well as the approaches and techniques developed in the course of our studies will contribute to research on uORF-control in translational control by other uORFs, and expand our knowledge of gene regulation and ribosome function.

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BIOGRAPHICAL SKETCH

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