THE EVOLUTIONARILY CONSERVED ARGININE ATTENUATOR PEPTIDE REGULATES THE MOVEMENT OF RIBOSOMES THAT HAVE TRANSLATED IT

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Abstract

The Evolutionarily Conserved Arginine Attenuator Peptide Regulates the Movement of Ribosomes That Have Translated It

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The major focus of this study is to elucidate the mechanism of translational control of *arg-2*. *arg-2* is a gene involved in *Neurospora crassa* arginine (Arg) biosynthesis, and translation of ARG2 is negatively regulated by the availability of Arg. This regulation is mediated by a short upstream open reading frame (uORF) in the *arg-2* transcript. The mechanism for this specific regulation has been addressed:

(i) We developed an amino acid-dependent cell-free translation system in which the Arg-specific regulation is reconstituted.

(ii) We introduced a sensitive assay called "toeprinting" (primer extension inhibition) into this cell-free system. We showed that, in high Arg, the translation of the *arg-2* uORF-encoded Arg attenuator peptide (AAP) causes ribosomes to stall precisely at the position in which the uORF termination codon is in the ribosomal A site. Point mutations which changed single amino acids within the conserved AAP region abolish this Arg-specific ribosomal stalling.

(iii) We elucidated the role of the AAP by directly fusing it to the N-terminus of a longer polypeptide. In high Arg, elongating ribosomes are stalled just after they

have translated the AAP region. Thus, the AAP appears to function as a nascent peptide that acts in *cis* to cause regulated stalling of ribosomes.

(iv) We investigated the generality of AAP-mediated regulation. Both the *N. crassa arg-2* and *Saccharomyces cerevisiae CPA1* AAP retain their regulatory effects in *N. crassa*, *S. cerevisiae*, and wheat germ extracts. These studies further revealed that AAP-mediated Arg-specific regulation appears independent of the charging status of arginyl-tRNAs.

This work represents the first instance in which translational control in response to the availability of a single amino acid has been reconstituted in a eukaryotic cell-free translation system. It represents one of a handful of examples of how a uORF-encoded peptide controls protein synthesis is understood. Many of the genes involved in growth control, development, and cancer encode such short peptides in uORFs. Understanding how the fungal AAPs mediate Arg-specific regulation provides key insights into fundamental aspects of translation and helps advance our understanding of how such uORFs mediate translational control.

CHAPTER 1
INTRODUCTION

The coordinate and timely regulation of gene expression is essential to the proper growth and development of all organisms. Regulation of gene expression occurs at multiple levels: transcription, mRNA processing and stability, translation, and protein modification and turnover. Translational control is defined narrowly as the modulation of the efficiency of polypeptide synthesis from mRNAs (adapted in this chapter) and more broadly to include translation-coupled regulation of mRNA stability. Control at the level of protein synthesis allows cells to respond rapidly to changes in physiological conditions, since activation or repression of translation from pre-existing mRNA can occur essentially instantaneously. In contrast, regulation at the level of transcription can entail a delay before a precursor RNA is processed and mRNA accumulated in the cytoplasm [Hershey et al., 1996].

This thesis describes the function of the evolutionarily conserved Arg attenuator peptide (AAP) in the translational control of the expression of the small subunit of the carbamoyl phosphate synthetase, the first enzyme in fungal Arg biosynthesis. The AAP is encoded as an upstream open reading frame (ORF) in the 5′ leader of the transcript specifying the enzyme. It mediates ribosomal stalling in response to Arg, causing a reduction in translation of the enzyme when Arg is plentiful, and thus provides a clear example of how cells can swiftly control the translation of a metabolic enzyme in response to changes in the availability of the end-product of that pathway. To introduce this work, this chapter will first focus on the basic mechanism of eukaryotic translation, then provide a description of some key general aspects of translational control, and finally discuss the role of the AAPs in the regulation of *Neurospora crassa arg-2* and *Saccharomyces cerevisiae CPA1*.

1.1 The Mechanism of Eukaryotic Translation

Both biochemistry and genetics have contributed to our knowledge of translation; early on, before work in model systems, biochemistry was of primary importance [Hershey et al., 1996]. One of the most fruitful techniques to establish translational mechanisms has been to reconstitute these processes *in vitro*. Fractionated and unfractionated cell-free translation systems derived from mammalian, plant, and fungal sources have allowed the identification and purification of many of the major factors with roles in translation (Table 1.1). More recent genetic approaches, mainly using *S. cerevisiae*, have extended our knowledge of the roles of these factors in the living organism.

The structures of mRNA play key roles in both the process of translation and translational control. A single mRNA can contain several different types of signal elements that contribute to translation or translational control (Fig. 1.1). A distinguishing feature of a eukaryotic mRNA is that it contains a m7GpppG cap structure (or a close variant) at its 5′ end and a poly(A) tail at its 3′ end. Apart from its role in nuclear export [Hamm and Mattaj, 1990], the cap is required for efficient translation [Merrick and Hershey, 1996; Sachs et al., 1997] and also influences mRNA stability [Furuichi et al., 1977]. At the 3′ end, the mRNA carries a poly(A) tail (about 55–90 in yeast [Sachs, 1990], 20–70 in *Neurospora* [Sachs and Yanofsky, 1991], and 150–250 in mammals [Brawerman, 1981]). In yeast, this tail also influences the cytoplasmic expression and fate of yeast mRNAs [Sachs and Wahle, 1993; Jacobson, 1996]. *In vivo* and *in vitro* studies indicate that the cap and poly(A) tail function synergistically to promote ribosome loading at the 5′ end of the mRNA [Gallie, 1991; Tarun and Sachs, 1995; Wang and Sachs, 1997a; Wells et al., 1998]. In addition to a cap and a poly(A) tail, the translation of eukaryotic mRNAs can also be modulated by elements in the 5′ and 3′ regions flanking the coding region for the mRNA. Finally, elements within the coding region can influence translation.

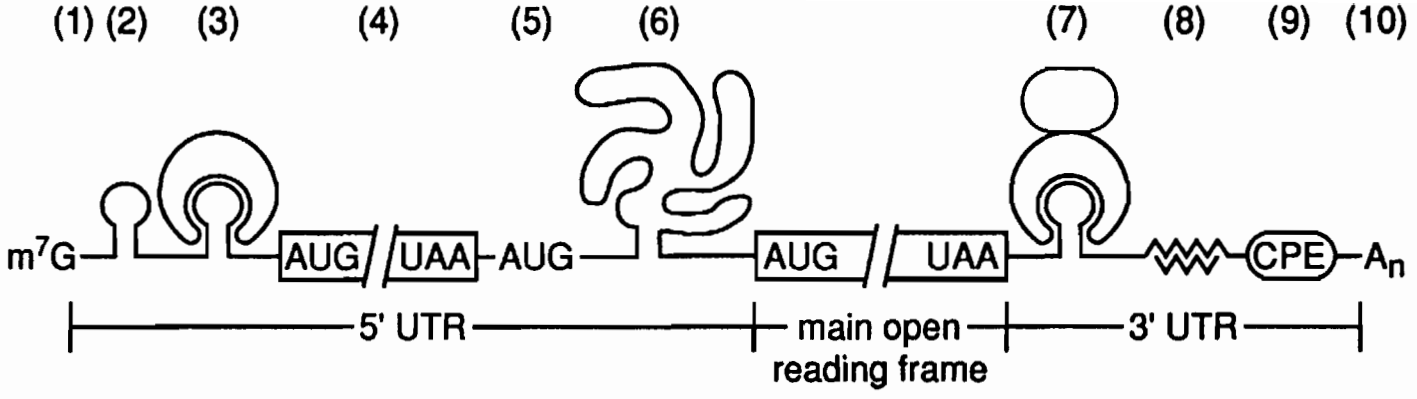

Figure 1.1 A diagram of structural elements in eukaryotic mRNAs that can influence the process of translation and translational control. 5′ UTR elements include: (1) cap structure, (2) secondary structure, (3) RNA-protein interaction, (4) upstream open reading frames, (5) upstream AUGs, and (6) IRES elements. 3′ UTR elements include: (7) RNA-protein interactions, (8) RNA-RNA interactions, (9) cytoplasmic polyadenylation elements, and (10) changes in poly(A) tail length.

1.1.1 Initiation: Scanning Model

For polypeptide synthesis to initiate, a ribosome initiation complex must form in which the anticodon of the initiator Met-tRNA$_i$ interacts with the initiator codon. This interaction precisely establishes the reading frame of the encoded polypeptide. In prokaryotes, the small subunit of the ribosome binds directly to a Shine-Dalgarno sequence in front of the initiation codon and then initiates translation at that start codon [Calogero et al., 1988]. In eukaryotes, it is generally accepted that the 40S small ribosomal subunit first binds to the cap structure and then scans in a 5′ to 3′ direction until it finds an initiator codon [Kozak, 1989c]. The binding of Met-tRNA$_i$ and mRNA to ribosomes is promoted by at least 11 initiation factors (eIFs); energy in the form of ATP or GTP hydrolysis is required for initiation. The initiation pathway discussed below can be summarized as follows: dissociation of 80S ribosomes into 40S and 60S subunits; binding of an active ternary complex containing eIF2, GTP, and Met-tRNA$_i$ to the 40S subunit; binding of this complex to mRNA; recognition of the initiator codon; and junction with the 60S subunit to form an elongation-competent 80S ribosome (Fig. 1.2).

1.1.1.1 Dissociation of ribosomes into subunits. At physiological Mg^{2+} concentrations ($\sim$1–2 mM), 80S ribosomes are the predominant species; they are in dynamic equilibrium with their subunits, as determined by sucrose gradient centrifugation [Russell and Spremulli, 1978, 1979; Moldave, 1985]. Because free 40S subunits are required to interact with the ternary complex prior to initiating translation (and Met-tRNA$_i$ bound to 40S ribosomes in the absence of mRNA is detected in cell lysates [Smith and Henshaw, 1975]), mechanisms exist to actively dissociate 80S ribosomes into subunits. Three initiation factors are thought to contribute to dissociation: (i) eIF1A and eIF3 bind to the 40S ribosomal subunit; eIF1A also reduces the formation of 40S dimers [Goumans et al., 1980]. (ii) eIF6 binds to the 60S ribosomal subunit and inhibits its association with the 40S subunit [Goumans et al., 1980]. (iii) The molar levels of eIF1A [Wei et al., 1995] and eIF3 [Duncan and Hershey, 1983] in HeLa cells are 20–50% of that of total ribosomes and therefore are sufficient to interact stoichiometrically with all of the 40S and 60S subunits not engaged in peptide synthesis under most conditions.

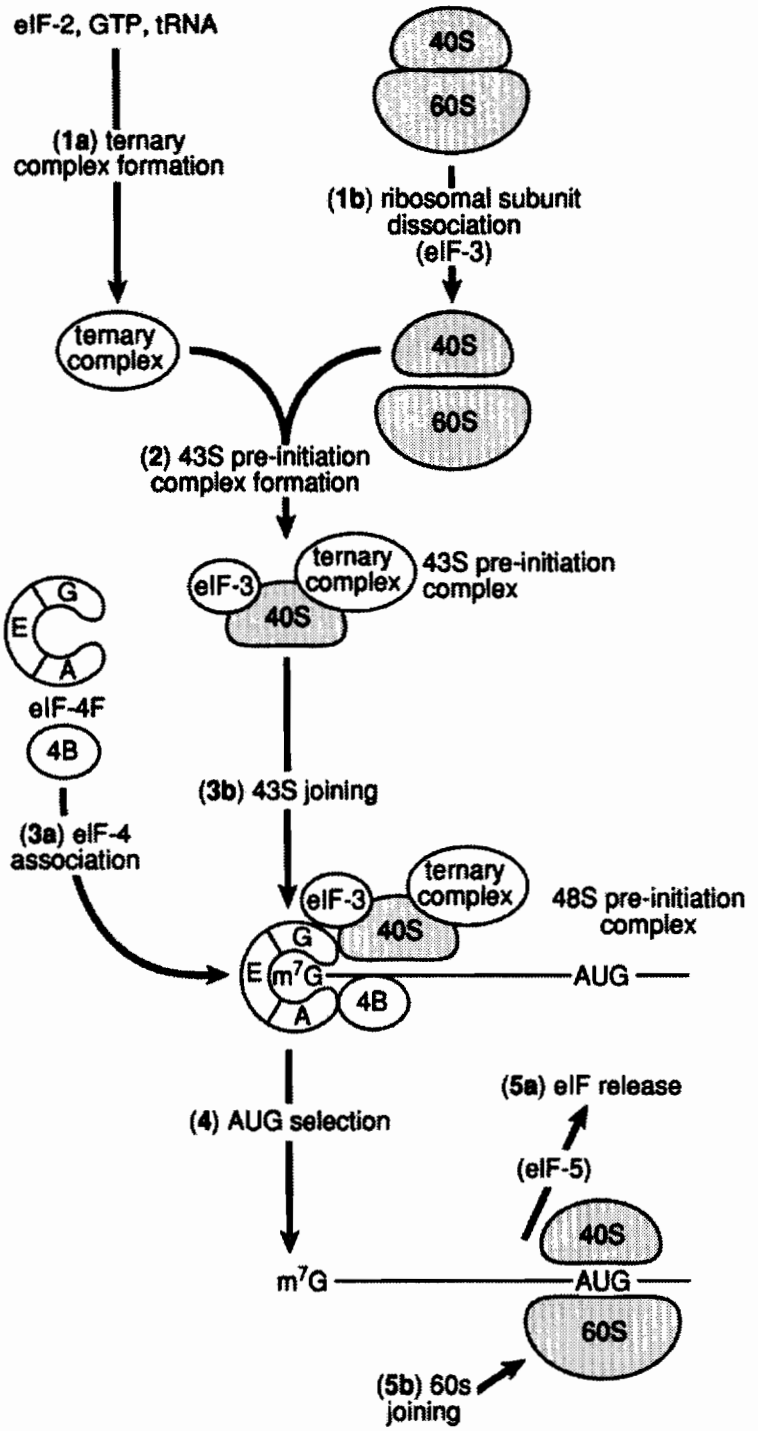

Figure 1.2 Model of initiation pathway in eukaryotes.

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1.1.1.2 Binding of an active ternary complex to the ribosome. Two initiation factors, eIF2C and eIF3, act stoichiometrically to stabilize the ternary complex at low concentrations ($<0.1\ \mu M$) [Gupta et al., 1990]. eIF2C also prevents ternary complex disruption by RNA [Roy et al., 1988]. Whether these two factors can interact with the ternary complex when it is associated with the 40S subunit is not clear. In any case, when a round of initiation is complete, eIF2 is released from the ribosome as a binary complex with GDP. The GDP must then be exchanged for GTP in order for eIF2 to be in the proper form to enable a subsequent round of ternary complex formation. This exchange reaction occurs slowly and requires catalysis by eIF2B, which binds to eIF2 · GDP and promotes the exchange reaction [Trachsel, 1996]. Since GDP binds 400-fold more tightly than GTP to eIF2 [Rowlands et al., 1988], ternary complex formation in theory may be partly influenced by the GTP/GDP ratio and thus by the energy charge of the cell.

A 40S pre-initiation complex is formed after binding of the ternary complex to 40S ribosomal subunits. The Met-$tRNA_i$ · 40S complex is moderately stable and its formation can be measured by sucrose gradient centrifugation. eIF1A has been shown to be essential for transfer of the ternary complex to 40S subunits [Chaudhuri et al., 1997]. Both eIF4A and eIF3 stabilize ternary complex binding and are present in the 40S pre-initiation complex [Trachsel et al., 1977; Benne and Hershey, 1978; Goumans et al., 1980]. $tRNA_I^{Met}$ binding can also be promoted by nonhydrolyzable GTP analogs, indicating that GTP carried on eIF2 is not hydrolyzed at the binding step. GTP hydrolysis is actually not required for any of the subsequent steps of initiation involving eIF2 and Met-$tRNA_I$ until ribosomal subunit joining [Trachsel, 1996]. The formation of ternary complexes and their binding to 40S ribosomal subunits is one of the most important sites of translational control [Trachsel, 1996].

1.1.1.3 Association of mRNA with the pre-initiation complex. It is commonly accepted that the most common first general step in the binding of the ribosome to mRNA is mediated by the recognition of the m7G cap structure by eIF4E [Sonenberg, 1996]. eIF4E, together with eIF4A and eIF4G, is a subunit of the cap-binding protein complex, eIF4F. The affinity of eIF4F for capped mRNA is about

15-fold greater than that of eIF-4E alone [Lawson et al., 1988], suggesting that eIF4F also may interact with other regions of the mRNA. Bound eIF4F, together with eIF4B, possesses ATP-dependent RNA helicase activity which can melt moderate amounts of secondary structure in the mRNA 5′ leader. Through interaction of eIF4G with eIF3, which is already associated with the 40S subunit, the small ribosomal subunit binds to the unfolded mRNA. Although mRNA binding to ribosomes is potentially an important step for translational control, the molecular details of this step are poorly understood. For example, it is not clear whether the RNA helicase activity occurs while the eIF4F–eIF4B complex is bound to the 40S subunit, or if initiation factors alone move along the mRNA, with the 40S complex following passively. Also, it is not clear if eIF4F and eIF2 bind simultaneously to eIF3 on the 40S subunit.

Recent evidence also implicates the poly(A) tail in the recruitment of ribosomes to the mRNA. The level of polyadenylation can affect mRNA translational efficiency and the time of onset of mRNA decay [Jacobson, 1996]. Only the former function will be discussed here. Poly(A) has been shown to stimulate translation both *in vitro* and *in vivo* [Gallie, 1991; Iizuka et al., 1994; Sheets et al., 1994; Tarun and Sachs, 1995; Jacobson, 1996; Wang and Sachs, 1997a]. The cytoplasmic poly(A) binding protein (PAB) is the most likely mediator of the translational effects of poly(A) [Tarun and Sachs, 1995; Sachs et al., 1997]. The translational effects of poly(A) can be accounted for by a closed-loop model in which the 5′ and 3′ ends of the mRNA are bought together [Jacobson and Favreau, 1983; Palatnik et al., 1984]. Experiments using *S. cerevisiae* provide direct support for such a closed-loop model. eIF4G1 and eIF4G2 are two isoforms of eIF4G which contain N-terminal binding sites for PAB [Tarun and Sachs, 1996; Tarun et al., 1997]. The PAB contains four RNA recognition motifs; the second of these is required for eIF4G binding [Kessler and Sachs, 1998]. Through the simultaneous interaction of PAB with initiation factors and poly(A), mRNAs form a closed loop in the process of translation [Wells et al., 1998]. Therefore, PAB can help mediate 43S pre-initiation complex binding to the 5′ end of mRNA (Fig. 1.3).

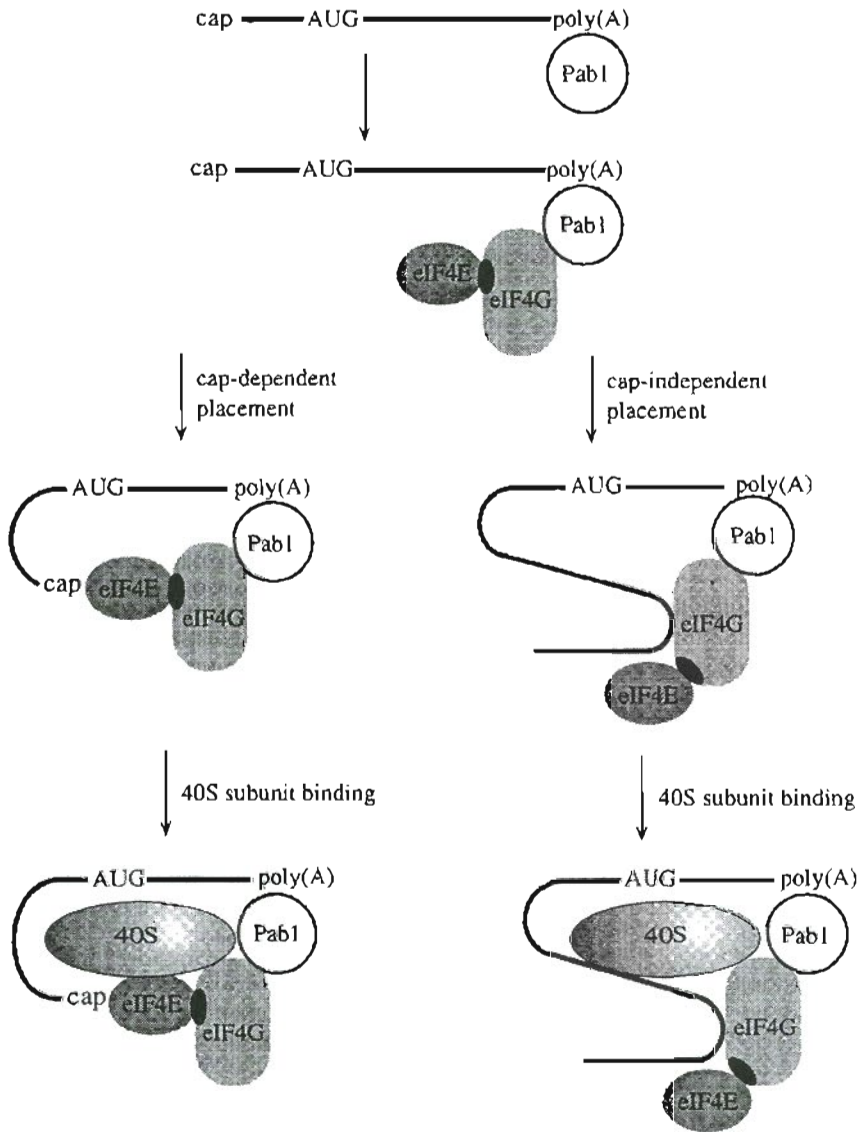

Figure 1.3 Poly(A)-mediated translation initiation and a revised model for the mechanism of 40S subunit binding to mRNA. Through interaction with the eIF4G–eIF4E complex, PAB (Pab1p) stimulates translation initiation. For cap-dependent initiation, both eIF4E and Pab1p can recruit eIF4G. The eIF4E–eIF4G–Pab1p complex forms a closed loop for the mRNA and then recruits the 40S ribosome to initiate translation. For cap-independent translation, e.g. internal initiation, the eIF4G–Pab1p complex could bind to an IRES (not shown here), which would lead to placement of the 40S subunit at a unique position within the mRNA.

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1.1.1.4 Selection of the translation start site and initiation of polypeptide synthesis. The scanning model predicts that the 43S pre-initiation complex will scan from the cap in a 5′ to 3′ direction until it selects an initiation codon. This model predicts that the 5′ proximal AUG should serve as an initiation codon, and this is true for more than 90% of mRNAs [Kozak, 1989c]. Further study indicates that the nucleotide sequence around AUG serves a critical function for efficient recognition of the initiation codon (initiation context). A "good" initiation context in mammalian cells will closely match the ACCAUGG, with the purine at -3 having the most impact. When an AUG is located in a relatively "poor" context, the initiation machinery can scan past it; this is known as "leaky scanning" [Kozak, 1989b] and will be considered in more detail later. Efficient initiation also requires distance between the 5′ end and the initiation codon; AUG codons less than 12 nucleotides (nt) from the 5′ terminus cannot be recognized efficiently, regardless of their context [Kozak, 1991c; Slusher et al., 1991].

The eukaryotic ribosome is directed to an initiation site by the anticodon–codon specificity of Met-tRNA$_i$ [Cigan et al., 1988b]. eIF2 and eIF5 have roles in determining the fidelity of start-site selection [Donahue et al., 1988; Huang et al., 1997]. The β subunit of eIF2 carries a binding site for eIF5, suggesting that the selection of the start site involves direct interactions between the two factors [Das et al., 1997].

Once the 43S subunit has located a start codon, the 60S subunit joins it to form the 80S initiation complex, which can then begin with peptide bond formation between the initial Met and the second encoded amino acid. This ribosome-joining step is promoted by eIF5, which, as mentioned above, also determines the fidelity of start site selection [Chakravarti and Maitra, 1993; Merrick and Hershey, 1996; Huang et al., 1997]. When the ribosome switches from initiation to elongation, eIF2 is released as a binary complex with GDP, which will have to be recycled back to the GTP form by eIF2B in preparation for a further round of initiation [Trachsel, 1996].

While the scanning model accounts for many eukaryotic initiation events, ribosomes can load at initiation codons through other well-documented mechanisms. These include internal initiation and discontinuous scanning (hopping). Internal

initiation is discussed later. Hopping is best understood from studies on a small number of viral mRNAs containing structures within their 5′ leaders that are unfavorable to the progression of scanning complexes but yet do not promote internal initiation [Futterer et al., 1993; Yueh and Schneider, 1996; Dominguez et al., 1998]. These mRNAs, exemplified by the cauliflower mosaic virus (CaMV) 35S mRNA, are translated by a discontinuous scanning mechanism [Futterer et al., 1993]. The pre-initiation complex begins scanning at the 5′ end of the mRNA but bypasses a strong secondary structure present in the 5′-leader structure by hopping. The mechanism of the hop is unclear but requires specialized sequence elements [Futterer et al., 1993; Yueh and Schneider, 1996; Dominguez et al., 1998; Hemmings-Mieszczak et al., 1998].

1.1.1.5 Internal initiation. Internal initiation was first discovered in translation studies in picornaviruses, a family with positive-sense, single-stranded RNA genomes [Ehrenfeld, 1996]. A typical mRNA contains 650–1300 nt in its 5′ noncoding region that are rich in secondary structures and many AUG codons yet can be efficiently translated in infected cells [Meerovitch and Sonenberg, 1993]. Meanwhile, picornavirus infection usually leads to a specific inhibition of host cell translation [Sonenberg, 1990]. Efficient initiation on the viral template was therefore difficult to reconcile with the scanning model, which posits cap-binding and relatively short leaders with little or moderate secondary structure for efficient translation. Studies on encephalomyocarditis virus RNA indicated the existence of mechanisms enabling internal initiation, where translation is mediated by an internal ribosome entry site (IRES) [Jang et al., 1988; Pelletier and Sonenberg, 1988]. Subsequently, IRESs have been identified in other viral transcripts and even cellular mRNAs [Jackson and Kaminski, 1995; Ehrenfeld, 1996]. IRES-mediated translation does not require a free 5′ end, as demonstrated by the translation of circular IRES-containing RNAs [Chen and Sarnow, 1995]. IRESs are functionally different from other 5′-leader secondary structures in their ability to mediate translation of the downstream ORF of a bi-cistronic reporter mRNA, independent of the translational status of the first ORF [Jang et al., 1988; Pelletier and Sonenberg, 1988].

Why does a picornavirus use an IRES to initiate translation? At least one rationale for this came from studying several picornaviral proteases that cleave eIF4G to yield an N-terminal eIF4G/eIF4E complex and a C-terminal eIF4G/eIF4A-eIF3 complex [Lamphear et al., 1995; Ziegler et al., 1995]. Based on our knowledge of cap-dependent initiation discussed previously, the cleavage separates the cap-binding function of eIF-4G from its RNA-helicase and ribosome-binding activities, so that when eIF-4G is cleaved, the cap cannot bring the pre-initiation complex to the mRNA. Cleavage of eIF-4G thus inactivates translation of most cellular mRNAs. However, the C-terminal fragment of eIF-4G can substitute for intact eIF-4G in IRES-mediated translation and in fact may be more efficient [Buckley and Ehrenfeld, 1987; Liebig et al., 1993; Ziegler et al., 1995; Ohlmann et al., 1996; Pestova et al., 1996b]. An interesting question is how poly(A) and PAB in animal cells are involved in this process (Fig. 1.3).

1.1.2 Elongation and Termination

The elongation phase is a cyclic process involved in the sequential addition of amino acid residues to the carboxy-terminal end of the nascent peptide. It can be divided into three major steps: binding of the aminoacyl-tRNA in the ribosomal A site, peptide bond formation, and translocation of the mRNA and peptidyl-tRNA on the ribosomal surface. The ribosome has three tRNA-binding sites: the A-site, where the aminoacyl-tRNA first binds; the P-site, where peptidyl-tRNA binds after the translocation reaction; and the E-site, where the stripped tRNA binds before it is ejected from the ribosome. The mechanism of this cyclic process has been studied in great detail in prokaryotes [Moldave, 1985; Slobin, 1991; Wilson and Noller, 1998]. It is thought, but hardly proven, that the process is very similar in eukaryotes.

1.1.2.1 Binding of aminoacyl-tRNA to the A site of ribosomes. eEF1A catalyzes the binding of aminoacyl-tRNA to the A site of ribosomes [Moldave, 1985]. In a similar fashion to Met-tRNA$_i$, aminoacyl-tRNAs form a ternary complex with eEF1A and GTP prior to interacting with the ribosome. Ternary complexes bind to the A-site, with the cognate ternary complex (those whose tRNA anticodon matches

the mRNA's codon exposed in the A-site) binding at a rate comparable or slightly faster than that of noncognate complexes. However, cognate complex formation facilitates GTP hydrolysis to GDP more so than noncognate complex formation. After hydrolysis, the complex dissociates from the ribosome and peptide bond formation occurs. eEF1A again catalyzes the GTPase reaction. It's very possible that eEF1A changes its conformation like its prokaryotic counterpart EF-Tu when it hydrolyzes GTP, and so the context of the codon-anticodon interaction is altered. The correct aminoacyl-tRNA interacts strongly with mRNA in both states, but an incorrect one does not. This double-check mechanism ensures the fidelity of the protein synthesis by putting the correct aminoacyl-tRNA in the A site. For eEF1A to promote another round of aminoacyl-tRNA binding, the GDP must be exchanged for GTP. The exchange reaction is slow and is facilitated by eEF1B [Janssen and Moller, 1988].

1.1.2.2 Peptide bond formation and translocation. Following hydrolysis of GTP and release of eEF1A · GDP, the aminoacyl-tRNA is bound to the A site. A catalytic activity inside the ribosome called peptidyl transferase catalyzes the reaction of aminoacyl-tRNA in the A-site with the peptidyl-tRNA in the P-site, resulting in peptide bond formation and transfer of the growing peptide chain to the A-site tRNA. In their aminoacyl ends, the newly synthesized peptidyl-tRNA moves from the A site to the P site; meanwhile, the deacylated tRNA moves to the E site. This partial translocation occurs without the expenditure of energy and likely is driven by a stronger binding site for the peptidyl portion of the tRNA in the 60S subunit P-site.

eEF2 · GTP promotes the translocation of the mRNA and the anticodon regions of the two tRNAs [Merrick and Hershey, 1996]. Following GTP hydrolysis, eEF2 · GDP leaves the ribosome. Now peptidyl-tRNA lies completely in the P-site, stripped tRNA is in the E-site, and the A-site is vacant, awaiting the binding of the next ternary complex. The presence of stripped tRNA in the E-site appears to decrease the binding affinity of a ternary complex in the A-site. In yeast, ejection of stripped tRNA is promoted by eEF3, thereby enhancing ternary complex binding [Triana et al., 1993]. The reaction cycle is quite rapid; in mammalian cells, up to

eight amino acids are incorporated per second per elongating ribosome [Lodish and Jacobsen, 1972; Palmiter, 1974]. A detailed description of the whole translocation process for prokaryotic ribosomes can be found in an article by Wilson and Noller [1998].

1.1.2.3 Termination. When a termination codon (UAA, UGA, or UAG) is exposed in the A-site, normal cells do not contain tRNAs with anticodons complementary to these stop codons. Instead, proteins called release factors bind and promote hydrolysis of the peptidyl-tRNA in the P-site, presumably by the peptidyl transferase center. Only a single release factor, eRF1 (55 kDa), is found in mammalian cells, and this factor must therefore recognize all three termination codons (UAA, UAG, UGA). In fact, eRF1 recognizes 4 nt in the A-site [Brown et al., 1990]: the termination codon triplet plus an adjacent downstream nucleotide. In bacteria, RF1 and RF2 each functions as a heterodimer with RF3, a protein with GTPase activity that stimulates the rate of peptide release. The binding of a release factor to a termination codon in the A site somehow activates peptidyl transferase so that it hydrolyzes the bond between the polypeptide and the tRNA in the P site, and a water molecule rather than an amino group is the acceptor of the activated peptidyl moiety.

Following termination, the 80S ribosome is thought to be released from the mRNA and then to dissociate for participation in another round of protein synthesis. However, exceptions have been found in work on *GCN4* expression in *S. cerevisiae*, indicating that termination does not necessarily lead to rapid release of ribosomal subunits from the mRNA [Hinnebusch, 1996]. Reinitiation after translation of short upstream ORFs (uORFs) is known to occur in the case of *GCN4* and in a number of other eukaryotic mRNAs [Geballe, 1996; McCarthy, 1998]. Detailed description for these types of initiation will be provided later.

1.2 Translational Control in Eukaryotes. Why Control Translation?

Gene expression can be regulated at multiple levels. Regulation of a given gene is possible at the levels of transcription and transcript metabolism, as well as translation, protein translocation, modification, folding and assembly, and degradation. Transcription and translation are especially critical for the cell. Both are biosynthetic steps with large consumption of energy, and both steps are subject to general energy resource checkpoints of the cell. Transcription is the first step for gene expression (chromatin modification is largely considered to be part of the control of transcription), and transcription is controlled in many ways. It is considered to be the predominant control mechanism. So what are the advantages of controlling translation instead of transcription?

There are several reasons for cells to deploy translational control in their arsenal of regulatory mechanisms. Compared to transcription or other steps prior to translation, control at translation can be quick. No extra time is needed for splicing, nuclear transport, or other nuclear events to take place.

Another important feature for translational control is that it can take place in systems lacking transcriptional control, such as reticulocytes, oocytes, and cells infected with RNA viruses. In fact, the initial studies of translational control were accomplished using these systems [Hershey et al., 1996]. A related feature discovered in the study of egg fertilization and development is the spatial control of translation [Wickens et al., 1996]. Regulation of the site of protein synthesis within the cell can generate concentration gradients of proteins. Such gradients are known to affect the translational efficiency of other specific mRNAs that determine patterning in early development.

Flexibility is another important feature for translational control. Because of the wide variety of mechanisms for translational control, it can be focused by specific effector mechanisms on a single or a few gene(s) or cistrons (such as the coat protein and replicase of RNA phages, antizyme, and ferritin). Alternatively, by influencing general factors, it can encompass whole classes of mRNAs (as in the heat-shock translational response and in the virus-induced shutoff of host-cell mRNA translation).

1.3 Global and Specific Control of Translation

Translational control can be roughly divided into two categories. First, a specific mRNA or subset of mRNAs can be regulated. Such regulation may be quantitative, determining the amount of protein produced. It may be an all-or-none response or a graded response. Specific regulation can also be qualitative, enabling a single mRNA to produce several different proteins. Variations in individual mRNA sequences (Fig. 1.1) are linked to gene-specific translational regulatory events, which may involve site-specific protein binding or, as with *GCN4*, mRNA-specific responses to changes in translational factor activity.

Alternatively, translational regulation may be global, modulating rates and patterns of protein synthesis and thereby contributing to the overall regulation of cell growth and metabolism. Global regulation occurs through modulation of the activities of components of the translational machinery. Phosphorylation plays a major role in this kind of regulation.

General features of eukaryotic mRNAs (e.g., cap and poly(A)) or specific features of individual mRNAs are important in consideration of general and specific translational control phenomena. In addition, such features can strongly influence the stability of mRNAs [Beelman and Parker, 1995; Jacobson, 1996; Richter, 1996; Gray and Wickens, 1998]. The stability of mRNA, either dependent or independent of the process of translation, is an important research area in its own right but beyond the scope of this chapter.

1.3.1 Specific Control of Translation

1.3.1.1 Regulation via 5′ leaders. As demonstrated in Fig. 1.1, the leader regions of eukaryotic mRNAs can vary in length. They can also possess secondary structural features, upstream AUG codons, and uORFs. These features can play important roles in regulation. The majority of eukaryotic mRNAs have 5′ leaders of 20–100 nt [Kozak, 1987a]. As mentioned previously, shortening the 5′ leaders of reporter mRNAs to less than 12 nt impairs the efficiency of translation from the first AUG [Sedman et al., 1990; Kozak, 1991c]. Increasing the length of a 5′ leader can

increase the efficiency of translation, as additional 43S pre-initiation complexes can be loaded. This is sometimes described as pre-loading [Kozak, 1991b]. However, many cellular mRNAs with unusually long 5′ leaders are poorly translated owing to the presence of upstream AUGs, uORFs, and/or secondary structures [Kozak, 1987b, 1991a]. This appears to be especially common in mRNAs encoding proto-oncogenes, transcription factors, growth factors, and their receptors [Kozak, 1987b, 1991a], which suggests that their translation is tightly controlled.

1.3.1.1.1 Secondary structure. Many of the effects of secondary structure on translation have been examined by introducing artificial structures into the 5′ leaders of reporter mRNAs [Pelletier and Sonenberg, 1985a; Kozak, 1986b, 1989a, 1998]. When a moderately stable structure is introduced immediately behind the cap, it blocks translation [Kozak, 1989a]; however, if the distance between the cap and the structure is greater (14 nt or more), the structure is no longer sufficient to block translation.

This position effect can be explained if the cap-proximal structure blocks the access of 43S pre-initiation complexes [Kozak, 1989a] and initiation factors eIF-4A and eIF-4B to the mRNA. Once the distance between the cap and the structure is sufficient to allow 43S entry, the effect becomes minimal [Pelletier and Sonenberg, 1985b; Lawson et al., 1986; Kozak, 1989a]. The translational machinery appears to progress linearly through the stem-loop rather than by hopping, since an AUG introduced into the distal side of the stem can be utilized as an initiation site [Kozak, 1986b].

Secondary structure can also increase the use of a particular initiation site when inserted downstream of that site. An inefficient initiation site, due to either a poor context, a non-AUG initiator, or a very short 5′ leader, is needed for this effect [Kozak, 1989b, 1990, 1991c]. The optimal placement for the structure is again about 14 nt downstream from the initiation codon. The structure may pause the 43S pre-initiation complex at or near the initiation codon, allowing more time for its recognition [Kozak, 1990].

In contrast to moderate secondary structures, more stable stem-loop structures (ΔG = -50/61 kcal/mol) block translation even when located further downstream of

the cap [Kozak, 1986b, 1989b]. RNase protection experiments suggest the stem-loop forms an impenetrable barrier to the migration of the 43S pre-initiation complex [Kozak, 1989a].

There are clear examples of secondary structure in the 5′ leader regulating translation of cellular mRNAs. Ornithine decarboxylase (ODC) is involved in the synthesis of polyamines required for cell proliferation. Translation of its mRNA is normally inefficient but can be stimulated by growth factors and mitogens such as insulin. An approximately 140-nt region close to the cap of rat and hamster ODC mRNAs is responsible for its inefficient translation [Grens and Scheffler, 1990; Manzella and Blackshear, 1990]. This G-C-rich region forms an inhibitory secondary structure and does not appear to be a protein binding site, because inverting the region does not diminish repression [Grens and Scheffler, 1990]. The stimulation of ODC mRNA translation by mitogens might be achieved through elevated eIF-4E activity, because overexpression of eIF-4E can increase the translation of reporter mRNAs with 5′ secondary structures [Koromilas et al., 1992]. Consistent with this, insulin can promote phosphorylation of eIF-4E and eIF-4B [Manzella et al., 1991] which increases activity (discussed below), and overexpression of eIF-4E increases ODC translation [Shantz and Pegg, 1994]. Another well-characterized example of mRNA translationally regulated through secondary structure is human platelet-derived growth factor 2 (PDGF2) mRNA [Rao et al., 1988].

1.3.1.1.2 Regulation via selective use of alternative initiation sites. Many mRNAs with long 5′ leaders can contain multiple in-frame AUGs that are not followed by in-frame stop codons, and thus might produce multiple polypeptides with different N-termini but similar internal and C-terminal domains. LIP (liver-enriched transcriptional inhibitor protein) and LAP (liver-enriched transcriptional activator protein) provide an elegant example of how translational control can regulate the forms of polypeptide produced from such an mRNA in a given cell [Descombes and Schibler, 1991]. LAP is a transcriptional activator most abundant in liver. The mRNA that encodes LAP also gives rise to a shorter protein product, LIP, owing to leaky scanning at the LAP initiation codon. LIP does not contain the transcriptional activation domain and is therefore thought to impede the activity of LAP by occlusion

of the promoter [Descombes and Schibler, 1991]. During postnatal development, a shift in the LAP/LIP ratio coincides with the function of LAP in terminal liver differentiation. Other examples of such mRNAs include Int-2, an EGF-related protein in mouse, which uses both an initiator AUG and an upstream initiator CUG to synthesize an N-terminal extended protein [Acland et al., 1990], human bFGF [Saris et al., 1991], and human c-myc [Hann et al., 1988, 1992].

1.3.1.1.3 Internal initiation of cellular mRNAs. IRES elements, as well as being present in certain viral RNAs, are found in some cellular transcripts [Jang et al., 1988; Pelletier and Sonenberg, 1988]. IRES elements in cellular mRNAs appear similar but less complex than those of viruses, consisting of as little as 55 nt [Oh and Sarnow, 1993]. The specialized *trans*-acting factors involved in cellular IRES-mediated translation may differ from those used by their viral counterparts [Vagner et al., 1996; Yang and Sarnow, 1997]. IRES-mediated translation of cellular mRNAs could provide a simple way to allow translation of a specific mRNA in circumstances in which the cap-dependent mechanism is impaired (see Fig. 1.3). mRNAs that may utilize the internal initiation mechanism include human immunoglobulin heavy-chain-binding protein [Macejak and Sarnow, 1991], human IGF-II [Teerink et al., 1995], human FGF-2 [Vagner et al., 1995a], human PDGF2 [Bernstein et al., 1997], Drosophila Antennapedia [Oh et al., 1992; Oh and Sarnow, 1993], and c-myc [Stoneley et al., 1998]. Interestingly, human eIF4G also contains an IRES in its messenger's 5′ non-coding region [Gan and Rhoads, 1996].

1.3.1.1.4 RNA-protein interactions. Transcriptional factors bind to promoter regions of their target genes and exert their effects on transcription. In a similar fashion but with fewer documented examples, proteins can regulate translation by interacting with target sequences within 5′ leaders. The best example of such a translational control mechanism is iron regulatory protein (IRP)-mediated regulation. First identified in mammalian cells, IRP-1 and IRP-2 regulate the translation of a number of mRNAs including ferritin (reviewed in Hentze and Kuhn [1996]), erythroid 5-aminolevulinate synthase [Cox et al., 1991; Dandekar et al., 1991; Bhasker et al., 1993; Melefors et al., 1993], mitochondrial aconitase [Gray et al., 1996; Schalinske et al., 1998], and succinate dehydrogenease-iron protein [Kohler et al., 1995; Gray et

al., 1996; Melefors, 1996] in response to a number of physiological stimuli. The binding site for IRP, the iron responsive element (IRE) [Aziz and Munro, 1987; Hentze et al., 1987] is typically located close to the 5′ cap. When the IRE is moved to a more distal position, IRP-mediated regulation is diminished [Goossen et al., 1990; Goossen and Hentze, 1992]. The IRE–IRP complex appears to reduce translation in a manner similar to that of the moderately stable secondary structure in the mRNA 5′ leader (discussed above). That is, binding of the pre-initiation complex is prevented by the presence of IRP-1 [Gray and Hentze, 1994a], and moving the IRE distally allows the scanning complex to load and displace IRP-1 [Paraskeva et al., 1999].

Other mRNAs whose translational regulation may also involve 5′-leader-bound repressor proteins include Mst87F and related genes in Drosophila spermatogenesis [Schafer et al., 1990; Kempe et al., 1993], mouse superoxide dismutase mRNA [Gu and Hecht, 1996], and the autoregulation of PAB [de Melo Neto et al., 1995]. Proteins also have been implicated in the 5′-leader-mediated control of TOP mRNAs (see ribosomal protein S6). Further information can be found in a review by Gray and Hentze [1994b].

*1.3.1.2 **Regulation by 3′ UTRs.*** Sequences in mRNAs' 3′ UTR can also mediate translational control (reviewed in Sonenberg [1994], Curtis et al. [1995], Macdonald and Smibert [1996], and Wickens et al. [1996]). Numerous studies have shown sequences in 3′ UTRs serve important biological functions, yet relatively little is known concerning their mechanisms. Nevertheless, an important feature for 3′ UTR regulation is that their functions are often related to the role of poly(A). For example, during early development, specific mRNAs undergo changes in poly(A) tail length generally correlated with increases in translational activity [Richter, 1996; Wickens et al., 1997]. Sequences in 3′ UTRs identified genetically by their effects on translation often govern poly(A) length (e.g., elimination of a negative regulatory element may cause both translational activation and a longer poly(A) tail).

Developmental genetics in Drosophila and *Caenorhabditis elegans* have revealed cascades of 3′-UTR-based translational control. For example, in *C. elegans*,

early in gametogenesis, *tra-2* must be repressed to make sperm, and *fem-3* must be repressed to make oocytes (reviewed in Puoti et al. [1997]). Regulation of both of these genes is achieved through their 3′ UTRs; however, although *fem-3* is post-transcriptionally regulated, it has not been conclusively shown to be at the level of translation.

Relatively few of the cognate repressors that bind to repressive elements in the 3′ UTR have been identified. The control of Drosophila *hunchback*, which encodes a transcriptional factor, exemplifies this situation (reviewed by St. Johnston and Nusslein-Volhard [1992] and Macdonald and Smibert [1996]). *hunchback* mRNA, which is present throughout the early syncitial embryo, is initially repressed by Nanos protein in the posterior, where it is later degraded. Nanos-dependent repression is mediated via Nanos response elements in the 3′ UTR [Wharton and Struhl, 1991]. Yet it is another protein, Pumilio, that binds to the Nanos response elements [Murata and Wharton, 1995]. In one simple model, Pumilio binds to Nanos response elements throughout the embryo and Nanos, which is restricted to the posterior, either binds to or modifies the Pumilio/*hunchback* mRNA complex.

Translation repressors that interact with the 3′ UTR may be RNA as well as proteins. *lin-14* is required for the proper timing of a range of developmental events in *C. elegans* [Ambros and Horvitz, 1987]. Temporal repression of *lin-14* requires sequences in its 3′ UTR and the *lin-4* RNA [Wightman et al., 1991, 1993; Lee et al., 1993]. *lin-4* RNA can potentially base-pair with seven sequence elements of 14–19 nt within the *lin-14* 3′ UTR, prompting the proposal that *lin-4/lin-14* duplexes cause translational repression [Lee et al., 1993; Wightman et al., 1993].

Regulation via 3′ UTRs can also involve activators. In Drosophila, Staufen appears to contribute to the expression of *oskar* mRNA by establishing and maintaining its localization and is a double-stranded RNA-binding protein [St. Johnston et al., 1992]. Positive elements in the 3′ UTR that enhance translation of uncapped mRNA have been observed for certain plant viruses [Gallie and Walbot, 1990; Hann et al., 1997]. Studies suggest that these positive elements might be the functional equivalents of the cap or poly(A) tail and may bind basal initiation factors.

A poly(A) tail can functionally replace a positive element in the 3′ UTR of tobacco mosaic virus [Gallie and Walbot, 1990], whereas a cap can substitute for the elements in BYDV-PAV and satellite tobacco necrosis virus [Timmer et al., 1993; Wang and Miller, 1995].

1.3.1.3 The poly(A) tail. Most eukaryotic mRNAs have poly(A) at their 3′ end; the length of the tail ranges from 50 to 250 nt [Jacobson, 1996]. The poly(A) tail is synthesized in the nucleus in a reaction considered to be tightly coupled to RNA polymerase II transcription [Dantonel et al., 1997; McCracken et al., 1997; Wahle and Kuhn, 1997]. Addition of poly(A) requires endonucleolytic cleavage of the precursor RNA, creating a new RNA 3′ end which serves as a substrate for the poly(A) polymerase [Colgan and Manley, 1997; Wahle and Kuhn, 1997]. The primary poly(A) tail length is thought to be longer than its mature length in the cytoplasm. Interestingly, a recent study of the Pab1p-dependent poly(A) nuclease in *S. cerevisiae* provides evidence for a model that, after addition of poly(A) tails for mRNAs in the nucleus, the poly(A) tails are quickly matured to a message-specific length in a poly(A) nuclease-dependent manner [Brown and Sachs, 1998].

In addition to its direct role in stimulating translation mediated by PAB as discussed above, changes in the length of the poly(A) tail of specific mRNAs can also be very important for translational control [Wickens et al., 1996] as has been elegantly demonstrated in oogenesis and early embryogenesis [Richter, 1996; Wickens et al., 1997]. For example, the *c-mos* transcript, which encodes a serine-threonine kinase and is required for the resumption of meiosis and initiation of oocyte maturation in *Xenopus* [Gebauer and Richter, 1997; Sagata, 1997], receives poly(A) in the cytoplasm as its translation increases [Sheets et al., 1994]. Further studies indicate that polyadenylation is critical both for the translation of *c-mos* and maturation of *Xenopus* oocytes [Sheets et al., 1995]. Another example is Drosophila Bicoid mRNA, whose translational activation is accompanied by polyadenylation and is required for the determination of anterior structures in the embryo [Salles et al., 1994].

Poly(A) tail length can decrease and cause translational repression. For example, in Drosophila, translational repression of *hunchback* mRNA by Nanos and Pumilio involves rapid deadenylation mediated by regulatory elements in the *hunchback* 3′ UTR [Wreden et al., 1997].

The effects of the poly(A) tail during development may be especially important because of competition among mRNAs for the translational machinery, as exists in *Xenopus oocytes* [Laskey et al., 1977]. This is supported by studies showing that the effects of poly(A) are greatest under competitive conditions [Proweller and Butler, 1994, 1997; Preiss and Hentze, 1998]. Competition, and hence the effects of poly(A), may also be modulated by modification of the translational apparatus.

While changes in poly(A) tail length can cause changes in translational activity, it is also clear that changes in poly(A) tail length can be secondary effects of repression. In some cases, changes in poly(A) tail length may sustain the change in translational activity achieved by independent mechanisms, including relief of sequence-specific repression (reviewed by Standart and Jackson [1994] and Wickens et al. [1997]). For example, derepression may be perpetuated or enhanced by elongation of the poly(A) tail.

1.3.1.4 Localization and translation. Evidence for mRNA localization is provided by studies in oogenesis and early embryogenesis. The elements determining localized expression are often found within the mRNAs' 3′ UTRs [Wickens et al., 1996]. mRNAs are sometimes localized to produce protein in only one region of the cell. Mechanisms exist to repress mRNAs that have not yet reached their proper destination or are not properly anchored there. *oskar* and *nanos* are required for formation of the posterior region of Drosophila. Both are localized to the presumptive posterior of the oocyte and early embryo [St. Johnston, 1995; Macdonald and Smibert, 1996]. To reach the destination, the mRNAs must move across the oocyte, since they enter the anterior end of the oocyte from nurse cells. Translational repression of *oskar* mRNA during its transit is mediated at least partially by a protein, Bruno, which binds to Bruno responsive elements in the *oskar* 3′ UTR [Kim-Ha et

al., 1995; Webster et al., 1997]. *nanos* mRNA also contains signals within its 3′ UTR that direct it to the posterior and control its translation [Gavis and Lehmann, 1994].

The cytoskeleton may have several functions in the regulation of mRNA expression (reviewed in Bassell and Singer [1997]). It may provide a surface for the interaction of cellular components, allow mRNAs to be spatially organized where their products are to be utilized, provide an opportunity for feedback regulation, and/or sequester mRNAs from the translational machinery until they reach their destination. Proteins that may mediate the interaction of mRNAs with the cytoskeleton have been identified. For example, Drosophila Staufen appears to form a cytoskeletally associated complex with *oskar* mRNA that leads to its localization and translation [Ephrussi et al., 1991; Kim-Ha et al., 1991; St. Johnston et al., 1991; Ferrandon et al., 1994; Manseau et al., 1996].

1.3.1.5 Nuclear and cytoplasmic cross-talk. Transcription occurs in the nucleus, while translation occurs in the cytoplasm; thus, mRNAs have to be transported to cytoplasm. Previously, nuclear events were thought to be entirely unrelated to its control. Recently, the studies of mRNA maturation, export, and mRNA turnover have begun to suggest otherwise.

Two key features of mRNAs, the cap and poly(A) tail, are recognized in both the nucleus and cytoplasm. In the nucleus, the cap is recognized by the CBC-α complex of two proteins involved in pre-mRNA processing [Izaurralde et al., 1994], while in the cytoplasm, a different cap-binding complex, eIF-4F, is bound [Sonenberg, 1996]. PAB and poly(A) nuclease are both found in nucleus and cytoplasm. They might be involved both in mRNA processing and export in addition to translation and mRNA degradation [Amrani et al., 1997; Minvielle-Sebastia et al., 1997; Brown and Sachs, 1998].

In some cases, sequence-specific translational repression may be established in the nucleus and carried to the cytoplasm. For example, hnRNP K, an abundant nuclear protein that can shuttle to and from the cytoplasm, represses translation of LOX mRNA [Michael et al., 1997]. Proteins that regulate translation in the

cytoplasm may also have nuclear functions. These may include Drosophila Sex Lethal [Green, 1991], Bicoid [Driever, 1992], and the yeast ribosomal protein L32 [Dabeva and Warner, 1993].

Recently, a report provided evidence that aminoacylation of the corresponding tRNA promotes its export from nucleus to cytoplasm, while a defective tRNA retards its appearance in cytoplasm [Lund and Dahlberg, 1998]. Translationally coupled nonsense mRNA degradation in mammalian cells may occur in the nucleus or concomitant with its transport to the cytoplasm [Theodorakis and Cleveland, 1996]. Considering that ribosomes are assembled in the nucleolus, and translation factors are often found in the nucleus, it is interesting to consider what translation-related events occur in the nucleus.

1.3.2 Global Control of Translation

Phosphorylation and dephosphorylation of translation factors appear to play important roles in controlling the overall rate of protein synthesis in eukaryotic cells [Hershey, 1989]. Numerous components of the translational machinery are phosphoproteins, including at least 13 initiation factors as well as three elongation factor subunits, three ribosomal proteins, and many aminoacyl tRNA synthetases [Hershey, 1991]. Phosphorylation of some of these polypeptides inhibits translation, whereas phosphorylation of others can stimulate translation. For initiation factors, the phosphorylation state of eIF-4B, -4E, -4G, eIF-2, and eIF-3 can all be modulated *in vivo* (reviewed by Merrick [1992] and Morley [1994]). A detailed understanding of the effect of these modifications is available only for eIF-2 and eIF-4E.

1.3.2.1 Initiation factors.

1.3.2.1.1 EIF-4E. Regulation of eIF-4E activity is expected to allow the cell to modulate the 5′-end-dependent binding of 40S ribosomal subunits to mRNA (discussed above). Two types of regulatory mechanism have been proposed: modulation of eIF-4E cap-binding activity mediated by changes in the phosphorylation state of this protein and regulation mediated by interactions between eIF-4E and eIF-4E-binding proteins (4E-BPs).

TABLE 1.1

Translation Factors and Their Functions

Name	Function
Initiation factors	
eIF1	Enhances initiation complex formation
eIF1A	Ribosomal dissociation; promotes Met-tRNA$_i$ binding
eIF2	Binds Met-tRNA$_i$ and GTP
α	Site of phosphorylation on Ser-51
β	Binds Met-tRNA$_i$
γ	Binds GTP, Met-tRNA$_i$
eIF2B	Guanine nucleotide exchange factor for eIF2
eIF2C	Stabilizes ternary complex in the presence of RNA
eIF3	Dissociates ribosomes; promotes Met-tRNA$_i$ and mRNA binding
eIF4A	ATPase, helicase, binds RNA
eIF4B	Binds RNA, promotes helicase activity
eIF4F	Binds m7G caps, binds PABP, helicase
eIF4E	Cap-binding subunit
eIF4A	ATPase, helicase
eIF4G	Binds eIF4E, PABP, eIF4A, and eIF3; promotes cap and poly(A)s synergistic stimulation on initiation
eIF5	Promotes GTPase with eIF2 and ejection of eIF2
eIF6	Binds to 60S ribosomes, promotes dissociation
Elongation factors	
eEF1A	GTP-dependent binding of aminoacyl-tRNAs; GTPase
eEF1B	Guanine nucleotide exchange on eEF1A
eEF2	Promotes translocation; GTPase
Termination factors	
eRF1	Recognizes UAA, UAG, and UGA; promotes peptide hydrolysis
eRF3	GTPase; stimulates eRF1 activity

Phosphorylation increases both eIF-4E's affinity for the cap [Minich et al., 1994] and its association with eIF-4A and eIF-4G to form eIF-4F complexes [Lamphear and Panniers, 1990; Bu et al., 1993; Morley et al., 1993; Morley and Pain, 1995]. The phosphorylated form of eIF-4E is also found predominately in 48S pre-initiation complexes [Joshi-Barve et al., 1990]. Since eIF-4F has been reported to have a greater affinity for the cap than eIF-4E alone [Bu et al., 1993; Haghighat and Sonenberg, 1997], effects of eIF-4E phosphorylation may result from a combination of an increase in the amount of eIF-4F and enhanced cap-binding capacity.

eIF-4E has been suggested to be limiting for translation *in vivo* in mammalian cells [Sonenberg, 1996]. Limiting eIF-4E levels may regulate translation of specific mRNAs by forcing all mRNAs to compete for the translation apparatus [Hiremath et al., 1985; Duncan et al., 1987]. In this view, when eIF-4E (and therefore eIF-4F) is limiting, mRNAs that have structure-rich 5′ leaders will be most poorly translated. The increased availability of eIF-4F is anticipated to result in an increased delivery of RNA helicase activity to mRNAs, increasing the likelihood that the secondary structures that hinder initiation will be disrupted. Consistent with this, in mammalian cells, overexpression of eIF-4E results in a more efficient translation of reporter mRNAs containing structured 5′ leaders [Koromilas et al., 1992] and also mRNAs encoding growth factors and mitogenic proteins that have structure-rich 5′ leaders. In yeast, however, overexpression of eIF-4E is without pronounced effect and does not enhance translation of mRNAs with structured 5′ leaders [Lang et al., 1994].

1.3.2.1.2 4E-BPs. A set of mammalian 4E-BPs which can act as regulators of eIF-4E function have been identified. For example, treatment of rat adipose cells with insulin leads to enhanced phosphorylation of the 4E-BP designated PHAS-I (phosphorylated heat- and acid-stable protein regulated by insulin) [Belsham and Denton, 1980; Hu et al., 1994]. PHAS-I phosphorylation correlates with enhanced translation [Lin et al., 1994, 1995; Pause et al., 1994]. There are sequence similarities between the eIF-4E-binding domain of eIF-4G and a region in PHAS-I and other 4E-BPs [Mader et al., 1995; Sonenberg and Gingras, 1998]. Since 4E-BPs can compete with eIF-4G for binding to eIF-4E [Haghighat et al., 1995], a model for 4E-BP function has been proposed in which eIF-4G–eIF-4E binding is regulated by

the eIF-4E–4E-BP interaction, which in turn is subject to modulation by phosphorylation of the 4E-BP. Serum, growth factors, hormones, and stress can all modulate 4E-BP binding via a number of signal transduction pathways [Vries et al., 1997; Gingras et al., 1998; Sonenberg and Gingras, 1998].

1.3.2.1.3 eIF-2. eIF-2 binds GTP and the initiator tRNA and delivers this complex to the small ribosomal subunit (discussed above; see Fig. 1.1). Phosphorylation of eIF-2, which consists of three subunits (α, β, and γ), results in an inhibition of translation and is a central control point in the initiation pathway [Clemens, 1996]. Of key importance for regulation of eIF-2 activity are the kinases that act upon eIF-2 and the guanine nucleotide exchange factor eIF-2B that exchanges GTP for GDP on eIF-2. This is because the phosphorylation of eIF-2 prevents eIF-2B from exchanging GTP for GDP. Furthermore, since phosphorylated eIF-2 has a greatly increased affinity for eIF-2B, and eIF-2B is present in lower quantities than eIF2, phosphorylated eIF2 acts as an effective competitive inhibitor for cellular regeneration of active eIF-2-GTP-Met-tRNA$_i$ and leads to a general inhibition of translation.

There are currently four known eukaryotic kinases that phosphorylate the α subunit of eIF-2: Three—HRI (hemin-controlled repressor kinase) [Clemens, 1996], PKR (double-stranded RNA-regulated protein kinase) [Clemens, 1996; Mathews, 1996], and PERK/PEK (PKR-like ER kinase) [Harding et al., 1999; Kaufman, 1999; Shi et al., 1998]—are found in mammals, and the fourth, GCN2, is found in *S. cerevisiae* [Hinnebusch, 1996, 1997]. These kinases phosphorylate eIF-2α to inhibit translation in response to different threats, e.g., heme deficiency in reticulocytes (HRI); virus infection (PKR); ER stress–caused protein unfolding (PERK/PEK); and amino acid-deprivation (GCN2). The details of eIF-2 phosphorylation by GCN2 and its relationship to translational control of *GCN4* will be discussed in more detail below.

*1.3.2.2 **Elongation factors.*** Treatment of mammalian cells with the immunosuppressant rapamycin, a bacterial macrolide, selectively suppresses mitogen-induced translation of an essential class of mRNAs which contain an oligopyrimidine

tract (~8 nt) at their transcriptional start (5′ TOP) [Jefferies et al., 1994; Pedersen et al., 1997]. These TOP-containing mRNAs are most notably mRNAs encoding elongation factors and ribosomal proteins [Jefferies and Thomas, 1994; Terada et al., 1994; Meyuhas et al., 1996]. In parallel, rapamycin blocks mitogen-induced p70 ribosomal protein S6 kinase ($p70^{S6k}$) phosphorylation and activation [Jefferies and Thomas, 1996] and prevents phosphorylation of eIF-4EBP1 [Beretta et al., 1996; Graves et al., 1995].

Phosphorylation of elongation factor eEF-2 inhibits protein synthesis [Nairn and Palfrey, 1987; Ryazanov, 1987]. eEF-2 is believed to be phosphorylated primarily at Thr56 by a highly specific kinase, known as Ca^{2+}/calmodulin-dependent protein kinase III or eEF-2 kinase [Ovchinnikov et al., 1990; Price et al., 1991]. Thr-53 and Thr-58 of eEF-2 are also phosphorylated on prolonged treatment. These Thr residues reside in a region that may be involved in eEF-2 binding to ribosomes [Peter et al., 1990]. Phosphorylated eEF-2 binds to 80S ribosomes but does not promote the translocation reaction with GTP *in vitro* [Ryazanov and Davydova, 1989]. Therefore, the phosphorylation of eEF-2 can function to inactivate eEF-2 and inhibit the elongation phase of protein synthesis.

Phosphorylation of other elongation factors is also observed. eEF-1β can be phosphorylated at Ser-89, perhaps by casein kinase II [Janssen and Moller, 1988]. eEF-1γ is phosphorylated in *Xenopus laevis* oocytes by the cell division control protein kinase $p34^{cdc2}$ [Belle et al., 1989]. Both $p34^{cdc2}$ kinase and casein kinase II are activated during meiotic cell division, a stage in which changes in protein synthesis occur [Wasserman et al., 1982].

1.3.2.3 Ribosomal proteins. Studies of a number of ribosomal protein mRNAs suggest that proteins that bind to their 5′ leaders may control translation; however, binding does not correlate with translational regulation [Gray and Hentze, 1994b; Amaldi et al., 1995; Meyuhas et al., 1996]. One assumption is that control is accomplished by an interaction between specific and general factors. However, studies on S6 protein, one among 30 distinct proteins which comprise a mature 40S ribosomal subunit with one molecule of 18S rRNA [Wool et al., 1996], suggest that

phosphorylation of S6 by $p70^{S6k}$ directly enhances TOP mRNA translation [Jefferies and Thomas, 1994; Terada et al., 1994; Jefferies et al., 1997]. Modification of S6, which lies within the mRNA binding site of the ribosome [Jefferies and Thomas, 1996], increases the affinity of the small ribosomal subunits for poly(U) [Gressner and van de Leur, 1980]. This indicates that S6 modification may result in more efficient interactions with TOP mRNAs via the polypyrimidine tract. Detailed mechanisms of TOP mediated translational control await more investigation.

1.4 uORF-Mediated Translational Control

uORFs, together with upstream AUGs and secondary structures (Fig. 1.1), are common features in transcripts encoding proto-oncogenes, transcription factors, growth factors, and their receptors [Kozak, 1987a, 1991a]. In general, since the first AUG encountered by a scanning ribosome is selected as the site of initiation [Kozak, 1983; Kozak, 1987a], uORFs and upstream AUGs can modulate initiation from the main ORF simply by exploiting this polarity. Certain uORFs also provide a translation termination site preceding the main ORF, providing additional means for regulating translation.

A common feature of uORF function in translational regulation is that uORFs only act in *cis*. There are two mechanisms that can account for uORF effects on translation of the main ORF: leaky scanning and re-initiation [Geballe, 1996]. Factors and *cis*-acting elements that influence the efficiency of these processes determine the magnitude of inhibition as discussed below. Analyses of the role of uORFs that are known to affect the translation of the downstream major ORF indicate that they can be placed into two groups: uORFs whose polypeptide-coding sequence is not important for function and uORFs whose coding sequences are important for function.

1.4.1 Coding Sequence-Independent uORFs

1.4.1.1 Yeast GCN4. Studies on *S. cerevisiae GCN4* provided the best-understood example of how uORFs regulate translation in a relatively coding-

sequence-independent way [Hinnebusch, 1996, 1997]. *GCN4* encodes a transcriptional factor that regulates the expression of at least 40 amino acid biosynthetic enzymes in response to amino acid starvation (cross-pathway control) [Hinnebusch, 1996]. Studies on *S. cerevisiae* have shown that eIF-2α becomes phosphorylated when cells are deprived of an amino acid or purine and that this event leads to an inhibition of translation by the mechanism discussed previously. The phosphorylation of eIF-2α also regulates the translation of *GCN4*, so that increased synthesis of the GCN4 polypeptide is observed under conditions in which the translation of other yeast mRNAs is reduced. Increased translation of the *GCN4* gene product helps alleviate the limitation for nutrients that triggers phosphorylation of eIF-2α.

Four uORFs in the leader of *GCN4* mRNA mediate translational derepression in response to eIF2 phosphorylation under starvation conditions. These uORFs are located in the 5′ leader of the mRNA, between 150 and 360 nt upstream of the Gcn4p initiation codon. Eliminating the start codons of all four uORFs results in high level *GCN4* expression under both starvation and nonstarvation conditions without substantially altering the level of mRNA [Mueller and Hinnebusch, 1986]. The uORFs inhibit *GCN4* translation in nonstarved cells by restricting the progression of scanning ribosomes through the leader to the *GCN4* start codon. The first and fourth of these small uORFs are sufficient for nearly wild-type regulation. These two uORFs have different effects on *GCN4* translation. uORF1 must be present upstream of uORF4 for the induction of *GCN4* translation that occurs in response to eIF2 phosphorylation [Mueller and Hinnebusch, 1986]. uORF1 alone reduces *GCN4* translation by only 50%, presumably because half of the ribosomes that translate uORF1 resume scanning and reinitiate at *GCN4*. Efficient reinitiation after translation of uORF1 depends not only on the sequence context of its stop codon but on sequences upstream of the uORF [Grant and Hinnebusch, 1994; Grant et al., 1995].

When present alone in a *GCN4* construct in which ORFs 1–3 have been eliminated by point mutations, uORF4 reduces *GCN4* translation to only 1% of the value seen in the absence of all four uORFs, whether or not cells are starved for an

amino acid [Mueller and Hinnebusch, 1986]. According to the scanning mechanism described earlier, essentially all ribosomes that bind to the 5′ end of *GCN4* mRNA are expected to translate uORF4 when no other uORFs are present in the leader. To account for the strong inhibitory properties of uORF4, it has been proposed that, in contrast to the situation with uORF1 in which ribosomes can reinitiate translation after finishing uORF1 translation, none of the ribosomes that translate uORF4 can reinitiate downstream; presumably, they dissociate from the mRNA following peptide chain termination at uORF4 [Mueller and Hinnebusch, 1986].

Replacing the last codon and 10 nt 3′ to the uORF1 stop codon with the corresponding nucleotides from uORF4 converts uORF1 into a strong translational barrier and destroys its ability to stimulate *GCN4* translation when situated upstream from uORF4 [Miller and Hinnebusch, 1989]. It has been suggested that base pairing between the mRNA surrounding the uORF4 stop codon and the rRNA could lengthen the time spent by the ribosome in the termination region and increase the probability of ribosome release from the mRNA [Miller and Hinnebusch, 1989; Hinnebusch, 1996].

How does phosphorylation of eIF2 affect Gcn4p synthesis? According to the current model (Fig. 1.4), essentially all ribosomes that bind to the 5′ end of GCN4 mRNA translate uORF1 under both starvation and nonstarvation conditions. About 50% of these ribosomes will remain attached to the mRNA and resume scanning downstream, presumably as 40S subunits. Under nonstarvation conditions, when the active form of eIF2 is abundant, these 40S subunits will rapidly rebind the eIF2 · GTP · Met-tRNA$_i$ ternary complexes and regain the ability to recognize an AUG codon as a translational start site. Consequently, most of these ribosomes will be forced to reinitiate at uORFs 2, 3, or 4, dissociate from the mRNA following chain termination, and thus fail to reach the Gcn4p start codon. Under starvation conditions, when one or more amino acids are limiting, uncharged tRNA(s) will bind the HisRS-like domain of Gcn2p and activate its kinase activity. Recent data suggests that a Gcn1p–Gcn20 complex binds near the A site of the ribosome and functions in an EF3-like manner to stimulate the binding of uncharged tRNA to the A site or its delivery to the HisRS-like domain in Gcn2p [Hinnebusch, 1997]. When eIF2α is

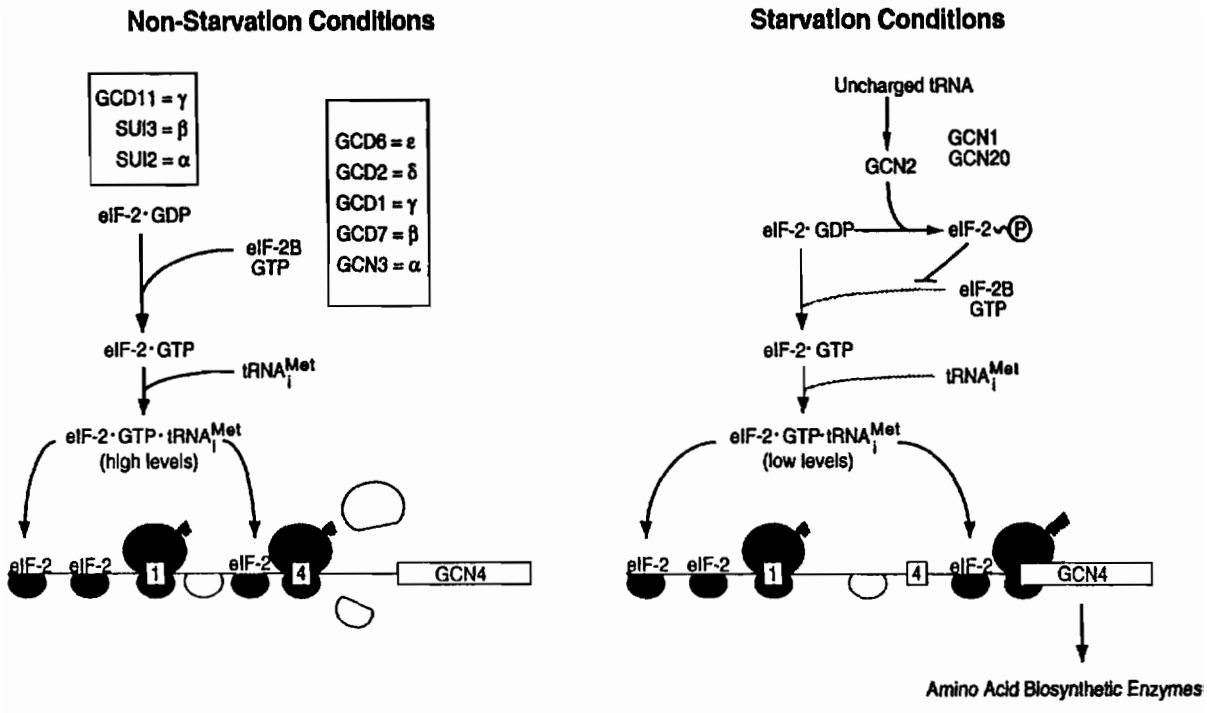

Figure 1.4 A model for translational control of *GCN4* mediated by eIF2α phosphorylation. Only uORFs 1 and 4 are shown in the *GCN4* mRNA and *GCN4* coding sequences indicated as boxes. Shaded ribosomal subunits indicate that they are competent for initiation or reinitiation (associated with ternary complex) or in the process of translation. Unshaded 40S subunit lacks the ternary complex and cannot reinitiate. Coils attached to the 60S subunits of the 80S ribosomes indicate the synthesis of peptides. Free 40S and 60S subunits (unshaded) are shown to leave the mRNA after termination at uORF4 (left panel). In yeast, *SUI2*, *SUI3*, and *GCD11* encode the α, β, and γ subunits of eIF2α, respectively. *GCD6*, *GCD2*, *GCD1*, *GCD7*, and *GCN3* encode the subunits of eIF2B. GCN1 and GCN20 are proteins required for increased eIF2α phosphorylation by GCN2 under conditions of amino acid deprivation. Additional data in text.

From Hinnebusch [1997], and used with permission of the American Society for Biochemistry and Molecular Biology.

phosphorylated on Ser-51 by Gcn2p, then the level of eIF2 · GTP · Met-tRNA$_i$ ternary complexes in the cell is reduced due to inhibition of the recycling factor eIF2B. Following translation of uORF1, many ribosomes will scan the entire distance between uORF1and uORF4 without rebinding ternary complexes. Lacking the initiator tRNA^{Met}, they cannot recognize the AUG start codons at uORFs 2, 3, and 4 [Cigan et al., 1988a] and will continue scanning downstream. While traversing the remaining leader segment between uORF4 and *GCN4*, most of these ribosomes will bind the ternary complex and reinitiation translation at *GCN4*. Thus, reducing the level of ternary complexes by phosphorylation of eIF2α will decrease the rate at which ribosomes become competent to reinitiation following translation of uORF1. This will enable ribosomes to bypass uORFs 2-4 and reinitiate further downstream at Gcn4p instead [Abastado et al., 1991; Dever et al., 1992]. Therefore, while phosphorylation of eIF2α decreases the general translation of the cell, translation of *GCN4* is stimulated.

1.4.1.2 Cauliflower mosaic virus 35S RNA. CaMV has an 8-kb genome which is replicated by reverse transcription of an 8.2-kb 35S RNA containing a 600-nt leader sequence and 7–8 tightly arranged long ORFs encoding all of the viral proteins [Mason et al., 1987]. The 600-nt 5′ leader is quite complex. It contains seven uORFs, several positively acting response elements, and many possible stable secondary structures. The translation of the downstream ORF within the transcript of the 35S RNA is enhanced by the action of uORFs and is dependent on a trans-activator protein encoded by the virus [Futterer and Hohn, 1991, 1992]. A mechanism for shunting scanning ribosomes has been proposed to explain the regulatory effects that link upstream sequences and the downstream ORF [Futterer et al., 1993; Dominguez et al., 1998].

1.4.2 Coding Sequence-Dependent uORFs.

1.4.2.1 Human cytomegalovirus gp48 (UL4). Human cytomegalovirus is a medically important herpes virus responsible for severe infections in newborns and immunocompromised patients [Alford and Britt, 1990]. The glycoprotein gp48 (UL4)

gene encodes a protein product which is synthesized as a β (early) protein that is present in virions [Chang et al., 1989b]. The gp48 ORF is contained in two β (E1 and E2) and one γ (late) transcript with different 5′ ends and identical 3′ ends [Chang et al., 1989a]. The 5′ leader of the most abundant of these transcripts (E1) contains three upstream AUGs with associated short uORFs, and this leader inhibits downstream translation in cell extracts [Chang et al., 1989b]. Removal of the initiator codon (AUG→AAG) for uORF2 abolishes inhibition. The sequence of uORF2, particularly the carboxy-terminal codons, are important for inhibition, as demonstrated by frame-shift mutations [Schleiss et al., 1991] and single missense mutations [Degnin et al., 1993]. Mutations that preserve the coding content of uORF2 uniformly retain the inhibitory signal. The uORF2 termination codon is required for the inhibitory effect [Degnin et al., 1993].

The primer extension inhibition (toeprinting) assay [Hartz et al., 1988, 1989] was applied to demonstrate that ribosomes stall at the termination codon of uORF2 and, remarkably, that the coding information of uORF2 is required for both the translational repression and ribosomal stalling at the uORF2 termination codon [Cao and Geballe, 1996a]. The peptide product of uORF2 is synthesized and is retained in the ribosome in the form of a peptidyl-tRNA [Cao and Geballe, 1996b]. Translation of the gp48 transcript leader in cell extracts produces the 2.4-kDa uORF2 peptide and a second product that represents the uORF2 peptide covalently linked to $tRNA^{Pro}$, which is predicted to decode the carboxy-terminal codon of uORF2 [Cao and Geballe, 1996b]. These data support a model in which the nascent uORF2 peptide blocks translation termination prior to hydrolysis of the peptidyl-tRNA bond. This blockade results in ribosomal stalling on the transcript leader which in turn impedes the access of ribosomes to the downstream cistron [Cao and Geballe, 1996a,b].

1.4.2.2 Mammalian AdoMetDC. S-Adenosylmethionine decarboxylase (AdoMetDC) is a key regulated enzyme of polyamine biosynthesis [Heby and Persson, 1990]. The *AdoMetDC* 5′-transcript leader region is highly conserved between human and bovine mRNAs. It has a length of approximately 330 nt and contains a six-codon uORF, MAGDIS, that represses downstream translation in

normal T cells and T-cell lines [Hill and Morris, 1992]. Mutations that alter the sequence of the peptide enhance translation of the main ORF, whereas those that preserve the peptide do not [Hill and Morris, 1992, 1993; Mize et al., 1998]. The uORF restricts the intracellular distribution of *AdoMetDC* mRNA primarily to monosomes in normal T-lymphocytes and T-cell lines. In contrast, nonlymphoid cells normally carry an average of 7–9 ribosomes per *AdoMetDC* mRNA molecule [Hill and Morris, 1992]. A decrease in intracellular polyamine concentration may disrupt interactions between the peptide and translational machinery, allowing translation of the main ORF to proceed [Ruan et al., 1994, 1996].

1.4.3 Other uORFs That Have Important Biological Functions

1.4.3.1 Maize Lc. The pigmentation pattern of maize is determined by the genetic constitution of the R/B gene family, composed of *R* locus on chromosome 10 and *B* locus on chromosome 2 [Damiani and Wessler, 1993, and references therein]. The *Lc* gene was the first R/B family member to be cloned and sequenced [Ludwig et al., 1989]. Its transcript has a 5′ leader region of 235 nt containing three upstream AUGs that are all part of a 38-codon uORF [Damiani and Wessler, 1993]. Mutational analysis indicates that elimination of all the upstream AUGs derepressed the expression of downstream ORFs by 30-fold; further analysis shows that the first AUG is primarily responsible for the derepression. *In vitro* assays demonstrate directly that translation of the uORF is required for repression [Wang and Wessler, 1998]. An increase of translation of the uORF peptide is accompanied by a decrease of downstream gene expression, and repression is unaffected by either subtle or gross changes in the uORF peptide. In addition, ribosomes that translate the uORF reinitiate inefficiently, and the intercistronic sequence downstream of the uORF mediates this effect.

1.4.3.2 CLN3. *CLN3* has recently been identified as an mRNA whose translation is restricted by a four-codon uORF [Polymenis and Schmidt, 1997]. The cyclin encoded by *CLN3* is required for the normal passage of the cell cycle through the G1→S transition. By attenuating the translation of CLN3, the uORF contributes to

the control of the cell cycle at this step [Yaglom et al., 1996]. Elimination of the uORF start codon, for example, increased budding in late-log phase, most probably due to accelerated progress through start [Polymenis and Schmidt, 1997].

Other examples of uORFs for which there is evidence for a role in affecting translation include Her-2, C/EBPs, and the transcripts listed in Table 1.2.

1.4.4 *N. crassa arg-2* and *S. cerevisiae CPA1*

N. crassa arg-2 and *S. cerevisiae CPA1* specify the small subunit (glutamine amidotransfer activity) of Arg-specific carbamoyl phosphate synthetase [Nyunoya and Lusty, 1984; Orbach et al., 1990] in the arginine synthesis pathway. One difference between these two genes' products is that the yeast enzyme is cytoplasmic, while the *Neurospora* enzyme is mitochondrial. Synthesis of both enzymes is subject to cross-pathway-mediated induction under conditions of amino acid limitation [Davis, 1986; Sachs, 1998]. They are also subject to Arg-specific negative regulation.

1.4.4.1 CPA1. *CPA1* is among the first yeast mRNAs to be found to have an uORF [Nyunoya and Lusty, 1984; Werner et al., 1985]. Expression of *CPA1* is repressed approximately five-fold by Arg. This effect is likely to arise through both transcriptional and post-transcriptional components [Crabeel et al., 1990].

The 250-nt *CPA1* leader has a single uORF composed of 25 codons [Nyunoya and Lusty, 1984; Werner et al., 1985]. Genetic studies provide support for the importance of this uORF [Thuriaux et al., 1972; Werner et al., 1987] in regulation. Three classes of constitutive mutations were identified. The first lost the uORF AUG initiator codon (AUG→AUA). The second contained a single nonsense mutation at either codon 8 (CAA→UAA) or codon 20 (UGG→UAG, or UGG→UGA). These mutations result in truncated uORFs. The third involved a single missense mutation at codon 11 (UGC→UAC, Cys to Tyr) or codon 13 (GAC→AAC, Asp to Asn).

Removal of the uORF AUG start codon (AUG→UAG) by site-directed mutagenesis leads to derepression of *CPA1* enzyme expression in the presence of high Arg, while having a limited effect on the *CPA1* mRNA level [Werner et al., 1987].

TABLE 1.2

uORFs That Are Under Investigation for Their Possible Roles in Translational Control

Organism	Gene	References
RNA viruses	Rous sarcoma virus	Donze and Spahr [1992]; Moustakas et al. [1993]
	reovirus S1 RNA	Fajardo and Shatkin [1990]; Belli and Samuel [1993]
	HIV tat	Luukkonen et al. [1995]; Gunnery et al. [1997]
	yellow dwarf virus	Brault and Miller [1992]
	Cauliflower Mosaic Virus 35S RNA	Futterer et al. [1993]; Dominguez et al. [1998]; Pooggin et al. [1998]
DNA viruses	cytomegalovirus gp48	Cao and Geballe [1996a,b, 1998]; Geballe [1996]
	SV40 - 16S RNA	Grass and Manley [1987]; Perez et al. [1987]; Sedman et al. [1989]
	SV40 - 19S RNA	Sedman and Mertz [1988]; Sedman et al. [1989]
Yeast	*GCN4*	Hinnebusch [1996, 1997]
	CPA1	Werner et al. [1987]; Delbecq et al. [1994]
	CLN3	Polymenis and Schmidt [1997]
	YAP1, *YAP2*	Vilela et al. [1998]
	HOL1	Wright et al. [1996]
	SCH9	di Blasi et al. [1993]
	cyc-1	Pinto et al. [1992]
Neurospora	*arg-2*	Luo et al. [1995]; Freitag et al. [1996]; Luo and Sachs [1996]; Wang and Sachs [1997a,b]; Wang et al. [1998]
	cpc-1	Ebbole et al. [1991]
	rgb-1	Yatzkan and Yarden [1999]
	mt A-2 and *mt A-3*	Ferreira et al. [1996]
	frq	Garceau et al. [1997]; Liu et al. [1997]

other fungi	*arg-2*	Shen and Ebbole [1997]; Baek and Kenerley [1998]
	brlA α & β	Han et al. [1993]
Plants	*Opaque-2*	Lohmer et al. [1993]
	Lc	Damiani and Wessler [1993]
	CaMV 35S RNA	Futterer and Hohn [1992]; Futterer et al. [1993]
	pma-1	Michelet et al. [1994]
	maize R	Wang and Wessler [1998]
	bZIP proteins	Martinez-Garcia et al. [1998]; Smidt et al. [1998]
	CpPHY2	Pasentsis et al. [1998]
	pma3	Lukaszewicz et al. [1998]
	SAMDC (AdoMetDC)	Lee et al. [1997]
Mammals	transforming growth factor β3	Arrick et al. [1991]
	AdoMetDC (S-adenosylmethionine decarboxylase)	Hill and Morris [1992, 1993]; Ruan et al. [1994, 1996]; Mize et al. [1998]
	HER-2	Child et al. [1999a]
	β2-adrenergic receptor	Parola and Kobilka [1994]
	retinoic acid receptor-β2	Zimmer et al. [1994]
	lck	Marth et al. [1988]
	BTEB	Imataka et al. [1994]
	erythrocyte carbonic anhydrase inhibitor	Bergenhem et al. [1992]
	fibroblast growth factor 5	Bates et al. [1991]
	bax	Salomons et al. [1998]
	bcl-2	Harigai et al. [1996]; Salomons et al. 1998]
	C/EBP αβ	Lincoln et al. [1998]
	c-mos	Steel et al. [1996]
	RAR β2	Reynolds et al. [1996]
	human muscle acylphosphatase	Fiaschi et al. [1997]
	lbp	Mittag et al. [1997]
	Atf4	Mielnicki et al. [1996]
	serine hydroxymethyltransferase	Byrne et al. [1995]

(Mammals)	insulin-like growth factor I	Kajimoto and Rotwein [1990]
	insulin-like growth factor II	Teerink et al. [1995]
	testicular cytochrome c	Yiu et al. [1994]
	FGF-5	Bates et al. [1991]
	PIGF	Maglione et al. [1993]
	ODC (ornithine decarboxylase)	Manzella and Blackshear [1990]
	MG-160	Gonatas et al. [1995]

Arg-specific repression is also abolished by frame-shift mutations in the uORF, or by introduction of a nonsense mutation at codon 5 (UCG→UAG) [Werner et al., 1987]. Interestingly, for the two frame-shift mutants encoding 30 codon peptides, one with a better initiation context than wild-type and another with the same context as wild-type, the Cpa1p protein level of the former was five-fold less than the latter. This suggests that increasing initiation of translation at the upstream AUG reduces the number of ribosomes able to translate the second ORF.

Deletions created either upstream or downstream from the uORF in the leader region had little effect on the Arg-dependent regulation of CPA1 expression [Delbecq et al., 1994]. A set of substitution mutations which modify the uORF sequence while leaving unchanged the corresponding amino acid sequence did not significantly affect the repression of *CPA1* by Arg [Delbecq et al., 1994]. The mRNA segment encoding the leader peptide of CPA1 is sufficient to confer Arg-specific regulation when inserted in the leader region of a heterologous yeast transcript [Delbecq et al., 1994].

1.4.4.2 Neuropora arg-2 and its regulation. The *N. crassa arg-2* gene also specifies the small subunit of Arg-specific carbamoyl phosphate synthetase [Orbach et al., 1990] and was among the first metabolic genes identified [Srb and Horowitz, 1944]. Control of *arg-2* expression regulates the flux of metabolites through the Arg biosynthetic pathway under most growth conditions [Davis et al., 1981; Davis, 1986]. At least three separate control mechanisms regulate *arg-2* expression. *arg-2* is

positively regulated in response to amino acid starvation, as are many genes involved in *N. crassa* amino acid metabolism [Sachs and Yanofsky, 1991; Luo et al., 1995]. The level of *arg-2* transcript increases in response to amino acid starvation; Cpc1p, the product of the cross-pathway control gene *cpc-1*, is important for this response [Paluh et al., 1988; Ebbole et al., 1991; Sachs and Yanofsky, 1991; Luo et al., 1995]. *arg-2* is also developmentally regulated; the highest level of *arg-2* mRNA is found during spore germination and early exponential growth [Sachs and Yanofsky, 1991]. Most importantly related to this study, the *arg-2* gene is the only gene in the Arg biosynthetic pathway known to be negatively regulated by Arg.

Comparison of the genomic and cDNA sequences of *arg-2* revealed that there is a 24-codon uORF present in addition to the major ORF coding for the Arg2p polypeptide [Orbach et al., 1990]. The predicted peptide sequence comparison with yeast *CPA1* and *arg-2* from other fungi indicates that this uORF coded peptide is evolutionarily conserved [Werner et al., 1987; Shen and Ebbole, 1997; Baek and Kenerley, 1998].

Previous work performed in the Sachs lab has shown long-term exposure of wild-type cells to Arg reduced concomitantly the steady-state level of Arg2p, the rate of Arg2p synthesis, and the level of *arg-2* transcripts [Luo et al., 1995]; the reduction in the level of mRNA appeared sufficient to account for most of the reduction in the rate of Arg2p synthesis and the level of Arg2p within the precision of these measurements [Luo et al., 1995]. However, polysome analysis indicated that the average size of the polysomes associated with *arg-2* mRNA was also affected. A clear response of *arg-2* expression to Arg at the translational level was shown by exposing wild-type cells to Arg for a short time. Short-term exposure to Arg reduced the rate of Arg2p synthesis but did not affect the level of *arg-2* transcript, suggesting that a negative translational control mechanism is responsible for modulating Arg2p expression as an immediate response to Arg exposure. Consistent with this, a reduction of the average size of polysomes translating *arg-2* mRNA was also observed in cells exposed briefly to Arg [Luo et al., 1995].

The importance of the *arg-2* uORF in Arg-specific translational control was demonstrated by changing the sequence of the uORF *in vivo* [Freitag et al., 1996; Luo and Sachs, 1996]. Elimination of the *arg-2* uORF by removing the translation initiation codon increased the expression of the reporter gene in both minimal and Arg-containing media, and Arg-specific regulation was lost. A 21-codon uORF initiating at the same site as the wild-type uORF, but frameshifted so that it had an altered coding sequence, did not confer Arg-specific regulation to the fusion gene [Luo and Sachs, 1996]. A mutation in the uORF changed the predicted peptide sequence at codon 12 from Asp to Asn and abolished translational regulation [Freitag et al., 1996], highly consistent with the D13N mutant in *S. cerevisiae CPA1* uORF. Because the peptides encoded by the uORFs of *arg-2* and *CPA1* are critical for the regulation for reasons provided above and in the following chapters, we have named them "Arg attenuator peptides" (AAPs).

In my thesis work, to elucidate the mechanism of translational control mediated by AAP, an amino acid-dependent cell-free translation system was developed in *N. crassa* in which the Arg-specific regulation of *arg-2* is fully reconstituted (Chapter 2). Then the toeprinting assay was introduced into this system, and it is demonstrated that ribosomal stalling at the uORF termination codon is responsible for the regulation (Chapter 3). Further studies showed that the wild type uORF-encoded peptide sequence alone is critical for the ribosomal stalling. In fact, when fused directly to the N-terminus of the reporter firefly luciferase, the AAP still functions. In high Arg, ribosomes involved in elongation stall immediately after they have synthesized the AAP (Chapter 4). Finally, both the *N. crassa* and *S. cerevisiae* AAPs mediate regulation in *N. crassa*, *S. cerevisiae*, and wheat germ extracts, and, more importantly, the charging status of arginyl-tRNAs appears not to have a role for AAP-mediated ribosomal stalling (Chapter 5). In closing, conclusions and future directions are provided in Chapter 6.

CHAPTER 2

ARGININE-SPECIFIC REGULATION MEDIATED BY THE *NEUROSPORA CRASSA ARG-2* UPSTREAM OPEN READING FRAME IN A HOMOLOGOUS, CELL-FREE *IN VITRO* TRANSLATION SYSTEM*

2.1 Introduction

Translational control is an important regulatory mechanism that often depends on sequences within the transcript being translated [Hershey et al., 1996]. A significant subset of eukaryotic mRNAs, including many encoding polypeptides involved in growth control and development, contain upstream open reading frames (uORFs) in their 5′ leader regions. In many cases, these uORFs influence the level of translation [Geballe and Morris, 1994; Geballe, 1996; Hinnebusch, 1996; Lovett and Rogers, 1996]. The mechanisms by which uORFs regulate translation in eukaryotes are mostly unknown.

In several instances, uORF-mediated translational control has been demonstrated to occur in response to specific environmental conditions. Expression of *Saccharomyces cerevisiae GCN4* is regulated by limitation for many different amino acids through a mechanism involving the translation of multiple uORFs in the mRNA [Hinnebusch, 1992, 1996; Sachs, 1996]. The predicted peptide sequences encoded in the *GCN4* uORFs are not important for control. Expression of *Neurospora crassa arg-2*, which specifies the small subunit of arginine-specific

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

carbamoyl phosphate synthetase, is subject to unique, arginine (Arg)-specific translational regulation [Luo et al., 1995]. In Arg-containing medium, the translation of mRNA containing the wild-type *arg-2* uORF decreases, and the decrease in translation is associated with a decrease in the number of ribosomes associated with the mRNA. The sequence of an uORF specifying a 24-residue peptide is critical for Arg-specific translational control; an Asp to Asn codon change at codon 12 of the *arg-2* uORF (D12N) abrogates this control [Freitag et al., 1996; Luo and Sachs, 1996]. The corresponding *S. cerevisiae* gene, *CPA1*, contains a similar uORF whose sequence is also important for Arg-specific regulation, which presumably also occurs at the level of translation [Werner et al., 1987; Delbecq et al., 1994].

The relatively common occurrence of uORFs in eukaryotic mRNAs [Kozak, 1986; Geballe and Morris, 1994; Geballe, 1996] suggest that uORFs often have roles in modulating gene expression. As with the *arg-2* and *CPA1* uORFs, the sequences of the peptides specified by uORFs in the transcripts for mammalian β_2 adrenergic receptor [Parola and Kobilka, 1994], human S-adenosyl methionine decarboxylase [Ruan et al., 1994], cytomegalovirus gp48 [Cao and Geballe, 1996a] and maize *Lc* [Damiani and Wessler, 1993] RNAs appear to be important for uORF function. The sequences of uORF-encoded peptides in prokaryotes can also be important for translational control, and common mechanisms may be involved in uORF-mediated translational control in both kingdoms [Lovett and Rogers, 1996].

Cell-free translation systems have been invaluable for addressing many mechanisms of translational control [Hershey et al., 1996]. Here we describe an amino acid-dependent *N. crassa* cell-free translation system that reconstitutes cap, poly(A), and uORF effects on translation. Although there have been previous descriptions of programmable *N. crassa* cell-free translation systems [Szczesna-Skorupa et al., 1981; Addison, 1987; Curle and Kapoor, 1988; Devchand et al., 1988], translational control has not been investigated using such a system. To our knowledge, these data represent the first instance in which translational control in response to the availability of a single amino acid has been reconstituted in a eukaryotic cell-free translation system.

2.2 Experimental Procedures

2.2.1 Preparation of Templates Containing Wild-Type and Mutant *arg-2* Sequences

Megaprimer PCR [Sarkar and Sommer, 1990] was used to obtain wild-type and mutant *arg-2* DNA fragments to which 5′-*Bgl*II and 3′-*Xho*I sites were added. Templates for PCR reactions were plasmids pMF11-wt and pMF11-D12N [Freitag et al., 1996], which contain the wild-type *arg-2* uORF and the D12N (Asp to Asn) mutant uORF, respectively (see Fig. 2.7). The *arg-2* region amplified by PCR and the nucleotide changes in mutant templates are indicated in Fig. 2.7. Primers for megaprimer PCR were: ZW1 (5′-CTGAGATCTAACTTGTCTTGTCGC-3′), which includes the *Bgl*II site; ZW2 (5′-CGCTCGAGCTTGACTTGAATGGT-3′), which includes the *Xho*I site; ZL19 (5′-TTGTCGCAATCTGCCACAATGAACGGGCG-CCC-3′), which puts the uORF initiation codon in a better context (↑ uORF); and ZL17 (5′-ATCTGCCCTTGTGAACGGGC-3′), which removes the predicted uORF translation initiation codon (ΔAUG). Conditions for PCR were as described [Freitag et al., 1996].

*Bgl*II- and *Xho*I-digested megaprimer PCR products were gel-purified and ligated to *Bgl*II- and *Xho*I-digested vectors pHLucS4 (see Fig. 2.8A) and pHLuc+NFS4 (see Fig. 2.8C). pHLucS4 [Oliveira et al., 1993], which was based on pHST7, was from N. I. T. Zanchin and J. E. G. McCarthy (National Biotechnology Research Center, Federal Republic of Germany); pHLuc+NFS4 was constructed by digestion of pHLucS4 with *Nco*I and *Xba*I and replacement of the luciferase coding region with the *Nco*I-*Xba*I fragment of pSPLuc+NF (Promega) which contained the Luc+NF luciferase coding region. The sequences of plasmid constructs were confirmed by sequencing both strands of the template plasmids.

2.2.2 Preparation of Synthetic RNA Transcripts

Synthetic RNA for *N. crassa cox-5* (cytochrome oxidase subunit V) was obtained from plasmid pSRCOX5 [Sachs et al., 1989], which had been linearized with

*Hin*dIII. Synthetic RNA for *N. crassa arg-2* was obtained from plasmid pAH4, which had been linearized with *Eco*RI. pAH4 contained the *Pvu*II-*Eco*RI fragment of *arg-2* cDNA obtained from pARCG228 [Orbach et al., 1990] subcloned into *Pvu*II- and *Eco*RI-digested pSP72. Luciferase RNA was obtained from a variety of linearized plasmids. pSPLuc+NF was linearized with *Eco*RI; plasmids T3LUC and T3LUCpA [Iizuka et al., 1994] were linearized with *Bam*HI; and plasmids pHLucS4, pHLuc+NFS4, and their derivatives (see Fig. 2.8) were linearized with *Ppu*10I. Synthetic RNAs from pHLucS4 and pHLuc+NFS4 should contain a poly(A) tail of 30 adenylate residues, as encoded by the template; in practice, the poly(A) tail length is expected to be longer because T7 RNA polymerase slips when synthesizing poly(A) tracts [Groebe and Uhlenbeck, 1988].

Synthetic RNAs were prepared by run-off transcription of linearized DNA templates. Reactions (50 μl) to synthesize capped RNA contained: 2 μg of linearized template, transcription buffer (40 mM Tris-HCl pH 7.5, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl), 10 mM dithiothreitol, 0.5 mM each of ATP, CTP, and UTP, 2 μCi of [α-^{32}P]UTP, 0.05 mM GTP, 0.5 mM m^7G(5′)ppp(5′)G, 50 units of RNasin, and 50 units of T7, T3, or SP6 RNA polymerase as appropriate. These synthesis conditions yield high levels of capped RNA [Yisraeli and Melton, 1989] as borne out by analyses of the effects of exogenous cap on the translation of capped RNA compared with uncapped RNA (see Fig. 2.4). Water was substituted for cap when uncapped RNA was synthesized. For RNA that was used in chemical stability experiments, the concentration of unlabeled UTP was lowered to 0.05 mM, and 20 μCi of [α-^{32}P]UTP was added. In all experiments, a common stock of reactants without DNA or enzyme was prepared and aliquoted to separate tubes to which DNA and enzyme were then added. This ensured that RNAs prepared in parallel would be radiolabeled with [α-^{32}P]UTP to the same specific activity. Reactions (50 μl) were incubated for 1 hr at 37°C. After incubation, 150 μl of water and 200 μl of 5 M NH_4OAc were added, and the reactions were placed on ice for 15 min to selectively precipitate the RNA. Precipitated RNA was recovered by centrifugation and dissolved in 200 μl of water. RNAs were reprecipitated with KOAc and ethanol, washed with 70% ethanol, and dissolved in 200 μl of water.

Yields of RNA, and verification that the purified RNA was free of unincorporated nucleotides were determined by polyethyleneimine chromatography and by trichloroacetic acid precipitation. Intactness of RNA and further proof of successful synthesis was accomplished by ethidium staining of RNA following electrophoresis in formaldehyde agarose gels. Equal amounts of different RNAs that were prepared in parallel were used in translation studies. These amounts were determined by considering the size of each RNA, the fraction of U residues within each RNA, and the amount of each RNA synthesized as measured by incorporation of radiolabeled nucleotide.

2.2.3 Preparation of Cell-Free Extracts for Translation

Wild-type *N. crassa* strain 74A-OR23-1VA was obtained from D. Perkins, Stanford University. *Neurospora* conidia were collected with water and germinated in Vogel's minimal medium/1.5% sucrose at a concentration of 1×10^7 conidia/ml [Luo et al., 1995]. To prepare cell-free extracts that were not dependent on the addition of amino acids, 1-liter cultures were incubated at 34°C with orbital shaking (200 rpm) for 6.5 h, and then mycelia were harvested by vacuum filtration onto Whatman 541 filter paper. To prepare cell-free extracts that were dependent on addition of amino acids, 1-liter cultures were incubated at 32°C for 8 h, and mycelia were harvested by vacuum filtration, resuspended in 1 liter of fresh growth medium, incubated an additional 1 h, and then harvested again. Although we do not understand why this latter growth regimen enables the production of amino acid-dependent cell-free translation extracts, it does so reproducibly. Following harvesting, mycelial pads were rinsed with ice-cold buffer A (30 mM HEPES-KOH, pH 7.6; 100 mM KOAc; 3 mM MgOAc; 2 mM dithiothreitol [Tarun and Sachs, 1995]). In the cold room, mycelia (10 g of wet weight) were combined with 10 g of acid-washed 0.5-mm glass beads and ground with a mortar and pestle with the gradual addition of 10 ml of grinding buffer [buffer A with protease inhibitors: 25 μg/ml *p*-amidinophenylmethylsulfonyl fluoride (Calbiochem), 5 μg/ml each of pepstatin A, antipain, chymostatin and leupeptin (Sigma)]. The homogenized mycelia were centrifuged in a polycarbonate centrifuge tube for 10 min at 31,000 $\times$ *g* at 4°C in an

SS34 rotor. The supernatant was carefully removed, avoiding both the pellet and the fatty upper layer; it was chromatographed on a 2.0× 20-cm Sephadex G-25 Superfine column that was pre-equilibrated with buffer A. Fractions containing the peak A_{260} were pooled. Typical preparations yielded 6–7 ml of extract with an A_{260}/ml = ~50. When extracts were treated with micrococcal nuclease, they were first adjusted to 1 mM $CaCl_2$; then micrococcal nuclease was added to 50 units/ml, and the extracts were incubated for 10 min at 21°C. EGTA was then added to a final concentration of 2.5 mM to inhibit the nuclease. Amino acid-dependent extracts were not treated with micrococcal nuclease. Extracts were aliquoted to Eppendorf tubes, frozen with liquid N_2, and stored at -80°C.

2.2.4 Cell-Free Translation and Analyses of Translation Products

Unless otherwise indicated, standard translation reactions (20 μl) contained: 10 μl of *N. crassa* extract; 30 mM HEPES-KOH, pH 7.6; 3.75 mM MgOAc; 150 mM KOAc; 1 mM dithiothreitol; 1 mM ATP; 0.25 mM GTP; 25 mM creatine phosphate; 3.6 μg (0.9 units) of creatine phosphokinase; 25 μM of each amino acid; 4 units of ribonuclease inhibitor; and protease inhibitors (25 μg/ml *p*-amidinophenylmethylsulfonyl fluoride, and 5 μg/ml each of pepstatin A, antipain, chymostatin, and leupeptin). Standard reactions were incubated at 25°C for 30 min and stopped by freezing in liquid N_2.

Standard reaction conditions were determined by analyses of the effects of different temperatures and different levels of K^+ and Mg^{2+} on translation. Temperatures of 25–30°C appeared optimal for cell-free translation. Varying the concentrations of Mg^{2+} and K^+ affected the translation of capped, polyadenylated *luc* RNA (capLUCpA RNA) and uncapped, unadenylated *luc* RNA (LUC RNA). Concentrations of these ions were chosen to yield the greatest relative translation of capLUCpA RNA compared to LUC RNA.

Translation of RNA in nuclease-treated reticulocyte lysates (Promega) was accomplished according to the supplier's directions. Translation of RNA in *S. cerevisiae* cell-free extracts was as described [Tarun and Sachs, 1995].

Quantitation of [^{35}S]methionine-labeled polypeptides was accomplished using a Molecular Dynamics PhosphorImager following SDS-PAGE separation of the reaction products. The luciferase activity produced in *N. crassa* and reticulocyte translation reactions was measured by thawing translation reactions on ice, adding 5 μl of the thawed reactions (diluted with luciferase reaction buffer if necessary) to 50 μl of LUC assay reagent (Promega), and immediately measuring photon production in a Beckman LS6500 scintillation spectrometer. Luciferase activity in *S. cerevisiae* extracts was measured with a Turner TD-20e luminometer [Tarun and Sachs, 1995]. All of the data presented represent averages of duplicate or triplicate reactions of one experiment; standard errors are indicated for experiments that examined Arg-specific regulation. All experiments were repeated multiple times with similar results.

2.3 Results

2.3.1 Characterization of the *N. crassa* Cell-Free Translation System

Analyses of ^{35}S-methionine-labeled products from *N. crassa* and reticulocyte systems programmed with synthetic, capped *cox-5*, *arg-2* or *luc* RNAs or containing no exogenous RNA indicated that these systems were comparable in activity (Fig. 2.1). The observed sizes of the COX5, ARG2, and LUC translation products were similar in each system and consistent with the predicted masses of these polypeptides. The yields of radiolabeled polypeptides were similar in these systems; measurements of luciferase enzyme activity produced were also comparable when similar amounts of capped, adenylated luciferase RNA (capLUCpA RNA) were added to *N. crassa* and reticulocyte translation systems (see below).

A time-course experiment to measure production of luciferase (Fig. 2.2) from capLUCpA RNA revealed that within the first 10 min, there was little luciferase production; between 10 and 30 min, luciferase production was linear; after 30 min, luciferase production began to level off. [^{35}S]methionine-labeling experiments and analyses of the synthesis of full-length, radiolabeled luciferase polypeptide yielded comparable results (data not shown). Synthesis of the full-length [^{35}S]methionine-labeled COX5 polypeptide was observed earlier (6 min). Because COX5 polypeptide

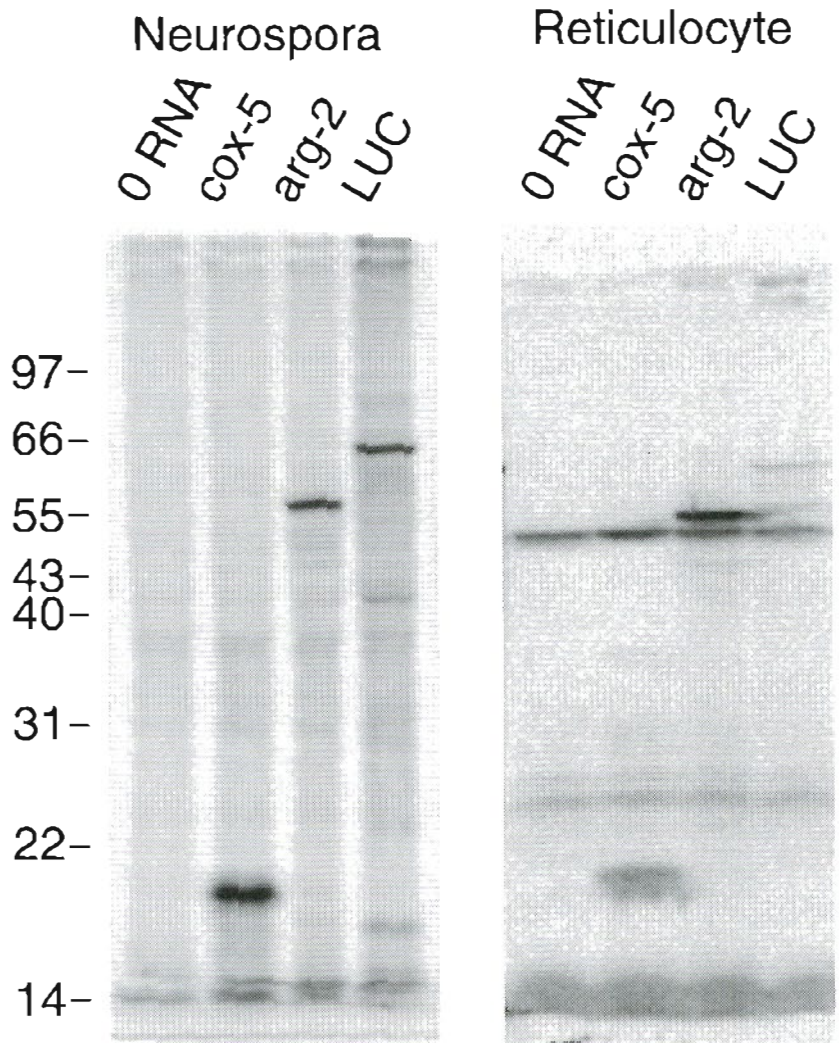

Figure 2.1 Analyses of [^{35}S]methionine-labeled polypeptides produced by *N. crassa* and reticulocyte programmed translation systems. Micrococcal nuclease-treated *Neurospora* extracts and conditions for translation were obtained by modification of previously described procedures [Szczesna-Skorupa et al., 1981; Tarun and Sachs, 1995]. Micrococcal nuclease-treated reticulocyte lysates and conditions for translation were obtained from Promega. *N. crassa* and reticulocyte reactions (20 μl containing 2 μCi of [^{35}S]methionine) that contained no RNA or equal amounts of synthetic, capped RNAs for *cox-5* (*N. crassa* cytochrome oxidase subunit V [Sachs et al., 1989]), *arg-2* (Orbach et al., 1990), or *LUC* (luciferase [Tarun and Sachs, 1995]) were incubated for 30 min at 25°C (*N. crassa*) or 30°C (reticulocyte). Reactions were stopped by immersing tubes in liquid nitrogen and examined by SDS-PAGE in 12.5% polyacrylamide gels. Radiolabeled translation products were visualized by phosphorimaging; the positions of molecular weight markers (kDa) visualized by staining with Coomassie Blue are indicated. The positions of COX5, ARG2, and LUC are consistent with their predicted masses.

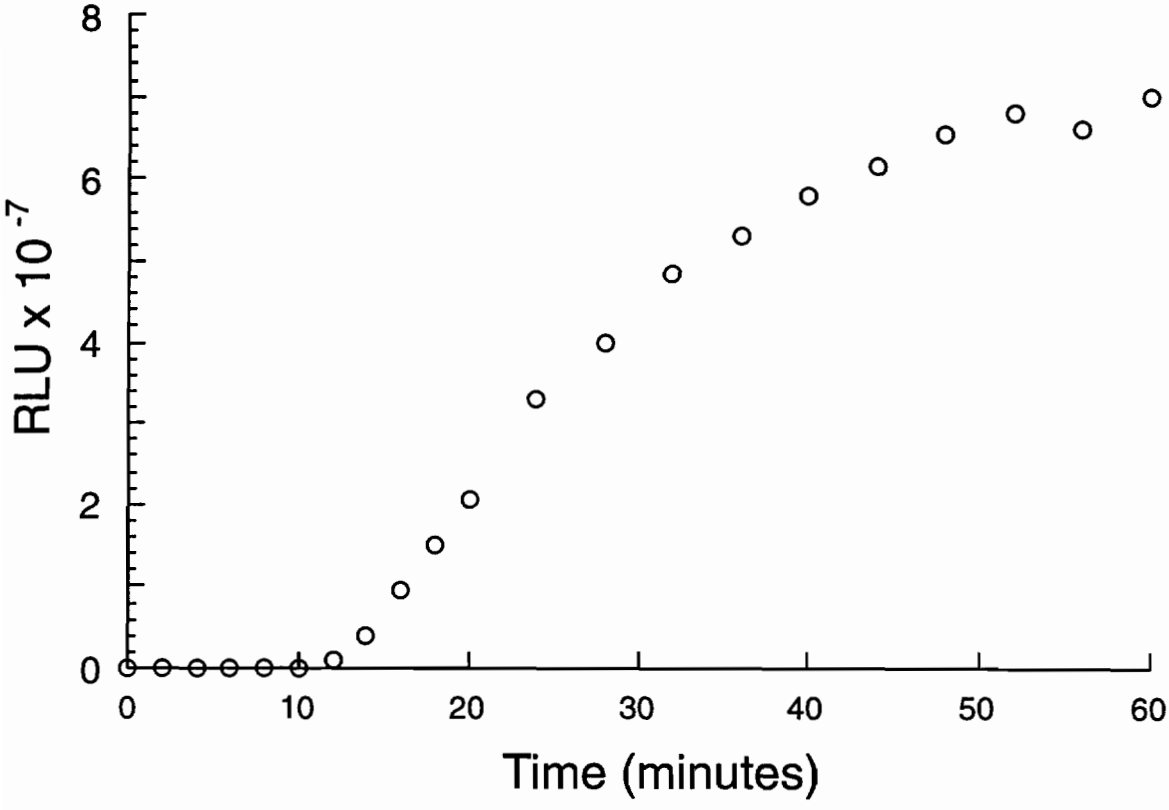

Figure 2.2 The effect of incubation time on the production of luciferase in *N. crassa* extracts. The cell-free translation system was programmed with 60 ng/ml of capped, adenylated *luc* RNA (capLUCpA) RNA and incubated at 25°C for the indicated periods, when samples were taken for luciferase assays. The data represent average values from two independent translation reactions. RLU, relative light units.

is smaller than LUC polypeptide, the lag in polypeptide synthesis was likely to be due in part to the time required for nascent polypeptide elongation (data not shown).

The effects of cap and poly(A) on the efficiency of translation were examined in nuclease-treated *N. crassa* extracts over a wide range of RNA concentrations (Fig. 2.3A). The level of luciferase production was linearly proportional to the level of RNA used to program translation in all cases over RNA concentrations varying by several orders of magnitude. Uncapped, unadenylated RNA (LUC RNA) was least efficiently translated. Addition of cap to RNA (capLUC RNA) increased translation. Addition of poly(A) alone to RNA (LUCpA RNA) stimulated translation more than addition of cap alone. The addition of both cap and poly(A) to mRNA (capLUCpA RNA) had synergistic stimulatory effects on translation, and capLUCpA RNA translated best. CapLUCpA typically translated more than 2 orders of magnitude more efficiently than LUC RNA.

Another observation consistent with the cap- and poly(A)-dependent translation that was seen in nuclease-treated extracts was that the addition of exogenous cap analog to *N. crassa* translation reactions strongly inhibited the translation of capLUC RNA (Fig. 2.4). The addition of poly(A) to RNA relieved inhibition of translation by cap analog; translation of LUCpA RNA was least affected by addition of analog, and translation of capLUCpA RNA was less affected than capLUC RNA (Fig. 2.4).

Removal of endogenous RNA by pretreatment of *N. crassa* extracts with micrococcal nuclease was necessary for detecting [^{35}S]methionine-labeled translation products (Fig. 2.1 and data not shown). However, this was not necessary for luciferase measurements. Thus, addition of cap and poly(A) to RNA had similar, synergistic effects on translation of luciferase in *N. crassa* extracts that were not treated with nuclease (Fig. 2.3B) as extracts that were nuclease-treated (Fig. 2.3A). Commercial reticulocyte lysate did not show cap- and poly(A)-dependent effects on translation under the recommended translation conditions (Fig. 2.3B).

Direct measurement of the chemical stability of translated RNAs showed that RNA was degraded during incubation in *N. crassa* translation reactions (Fig. 2.5). Cap and poly(A) both stabilized the RNA; cap had a greater effect than poly(A).

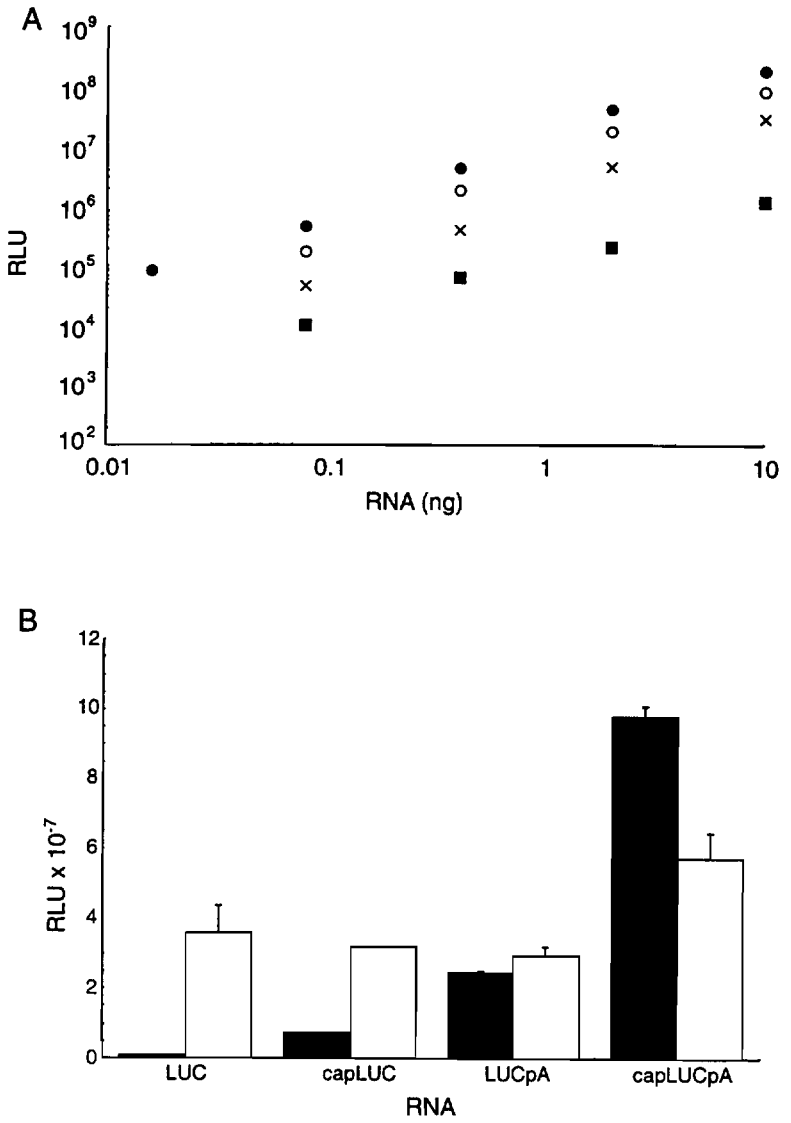

Figure 2.3 Production of luciferase in nuclease-treated *N. crassa* extracts is linearly dependent on RNA concentration and cap and poly(A) stimulate translation. (A) The indicated amounts of capLUCpA (closed circles), LUCpA (open circles), capLUC (crosses) or LUC (squares) were used to program translation reactions (20 μl), and luciferase activity was assayed. The background emission of extracts that contained no exogenous RNA (8 $\times$ 10^4 RLU) was subtracted from the experimental values given in this plot, which represent the average of duplicate samples. Translation of 0.016 ng of capLUC, LUCpA and LUC RNAs did not produce detectable luciferase. (B) Cap and poly(A) on RNA stimulate translation in *Neurospora* but not reticulocyte cell-free systems. Equal amounts of each RNA (0.9 ng) were translated in 20 μl of *N. crassa* extract that was not nuclease-treated (black bars) or micrococcal nuclease-treated reticulocyte lysate (white bars).

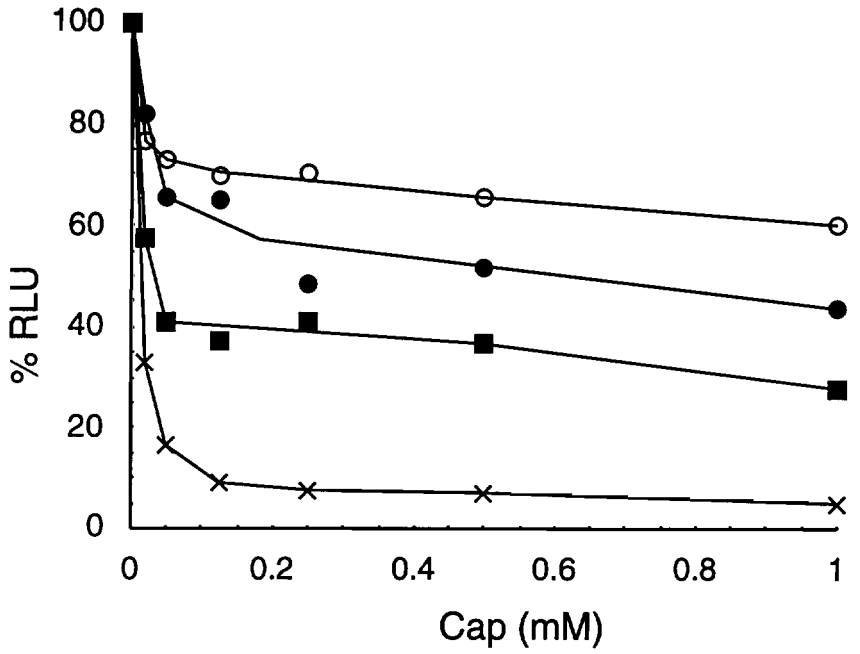

Figure 2.4 Effects of adding exogenous cap ($m^7G(5')ppp(5')G$) on the translation of LUC, capLUC, LUCpA and capLUCpA RNAs in *N. crassa* extracts. Extracts (20 μl) containing the indicated concentrations of exogenous cap were programmed with 1 ng of RNA: capLUC (crosses); LUC (squares); capLUCpA (closed circles); LUCpA (open circles). The production of luciferase was assayed by luminescence and the values were plotted as a percentage of the amount of luciferase produced by each RNA in the absence of exogenous cap.

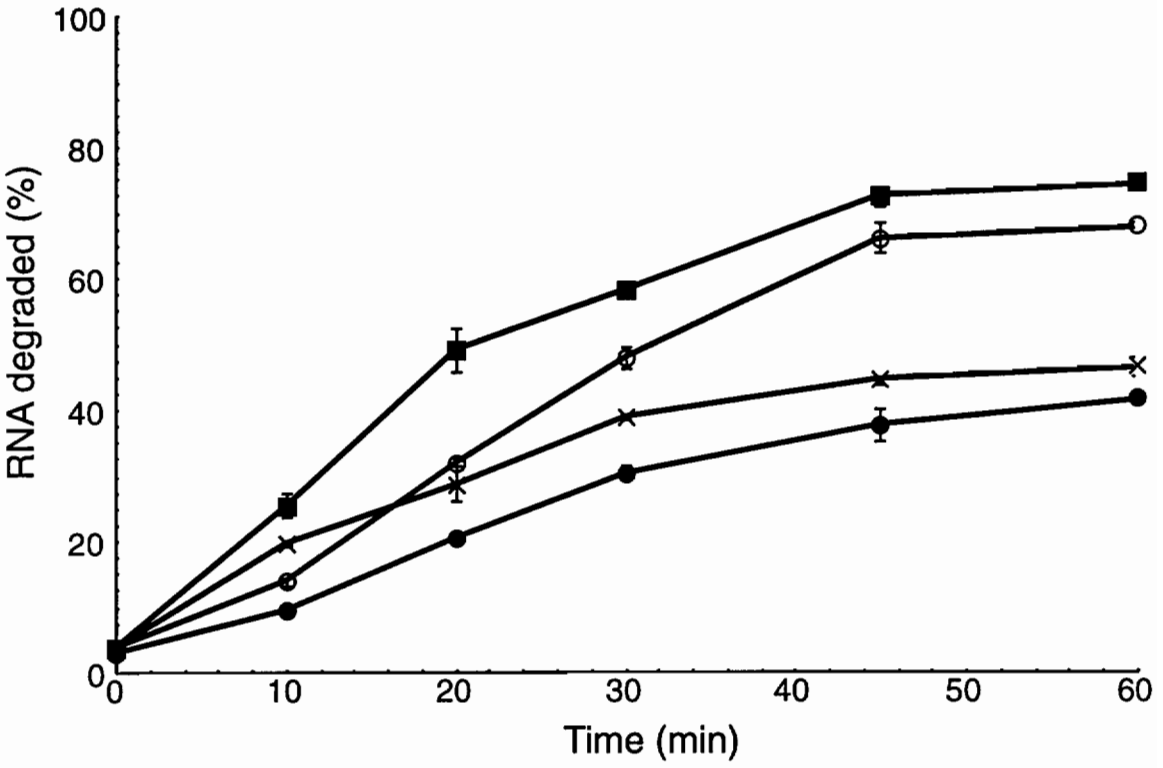

Figure 2.5 Effects of cap and poly(A) on RNA stability in *N. crassa* translation extracts. Radiolabeled RNAs (1.7×10^5 trichloroacetic acid-insoluble cpm representing 25 ng of each RNA) were translated in nuclease-treated *N. crassa* extracts (80 μl) for the indicated periods. Aliquots were removed (8 μl), the trichloroacetic acid-soluble cpm were measured and then were plotted as a percentage of the input cpm per aliquot. LUC (squares), LUCpA (open circles), capLUC (crosses), and capLUCpA (closed circles) RNAs were analyzed.

2.3.2 Effects of Upstream Open Reading Frames on Translation

The standard *N. crassa* translation system prepared from cells growing in minimal medium was not dependent on the addition of exogenous amino acids for translation (Fig. 2.6), and addition of extra Arg did not affect translation of uORF-containing RNAs (data not shown). Therefore, as described in Section 2.2, we used a different set of growth conditions that enabled the preparation of amino acid-dependent *N. crassa* translation extracts to test the effects of adding Arg. The effects of adding different concentrations of 20 amino acids on production of luciferase in amino acid-dependent and -independent *N. crassa* translation reactions is shown in Fig. 2.6. The amino acid-dependent translation system enabled testing of Arg-specific translational regulation *in vitro*.

Both amino acid-independent and amino acid-dependent cell-free translation systems were used to examine the effects of the *arg-2* uORF on translation of the luciferase coding regions in RNA. Wild-type and mutant *arg-2* sequences (Fig. 2.7) were placed upstream of the luciferase coding regions in either of two vectors (Fig. 2.8A and C). One vector contained the wild-type luciferase coding region, LUC; the other contained a luciferase coding region modified by site-specific mutagenesis to alter codon usage and eliminate several restriction enzyme cleavage sites, LUC+NF. Constructs were used to produce capped, polyadenylated RNA that contained the luciferase coding region and (i) no *arg-2* sequence; (ii) the wild-type *arg-2* 5′ leader containing the uORF (wild-type uORF); (iii) an *arg-2* 5′ leader containing the wild-type *arg-2* uORF in a better predicted initiation context (↑ uORF); (iv) the wild-type *arg-2* 5′ leader containing the *arg-2* uORF with the Asp to Asn change that abrogated regulation *in vivo* (D12N); (v) a double mutant containing the D12N uORF in a better predicted initiation context (D12N ↑ uORF); and (vi) a mutated *arg-2* 5′ leader lacking the uORF AUG codon (ΔAUG).

Equal amounts of the capped, adenylated RNAs obtained from the vectors in Fig. 2.8A were compared in the amino acid-independent, micrococcal nuclease-treated *N. crassa in vitro* translation system and nuclease-treated *S. cerevisiae* and reticulocyte systems. Transcripts containing uORFs were translated less well than

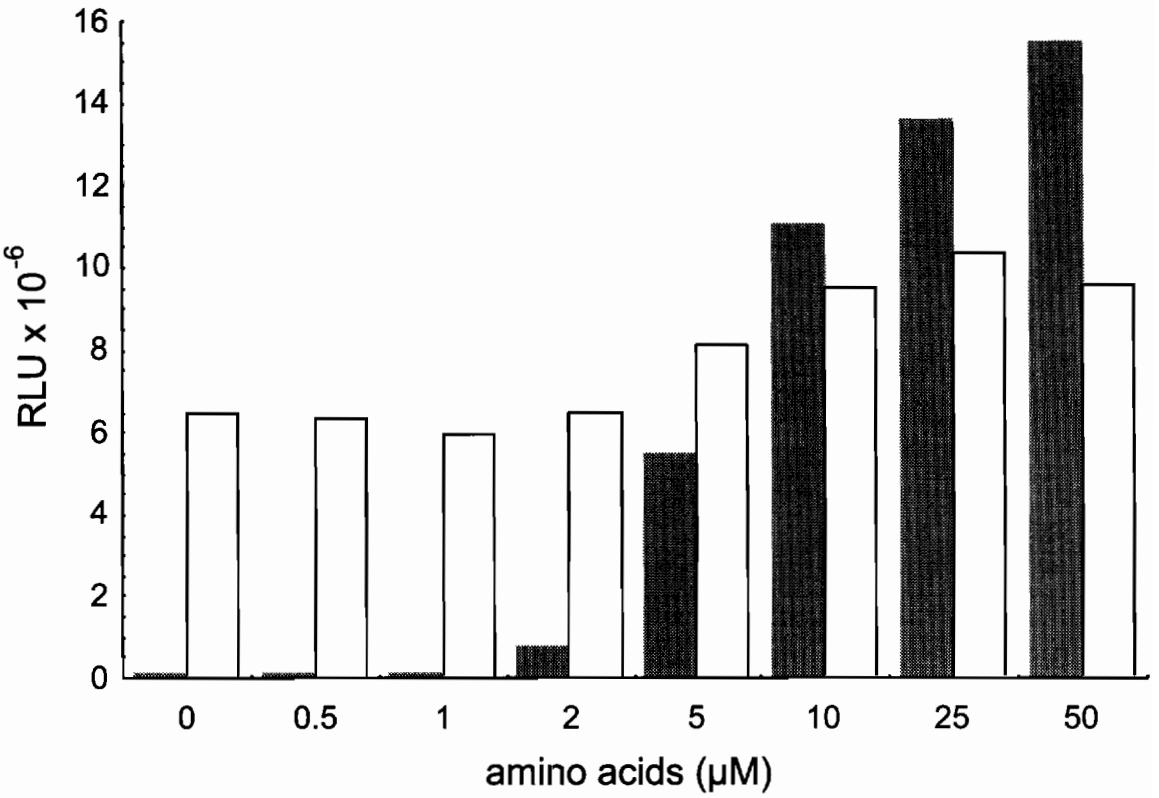

Figure 2.6 Effects of added amino acid concentrations on translation in amino acid-dependent and -independent *N. crassa* translation reactions. CapLUCpA RNA (2 ng) was translated in reaction mixtures (20 μl) containing the indicated concentrations of a mixture containing all amino acids. The data represent average values from two independent translation reactions. Shaded bars, amino acid-dependent translation mixture; white bars, amino acid-independent translation mixture.

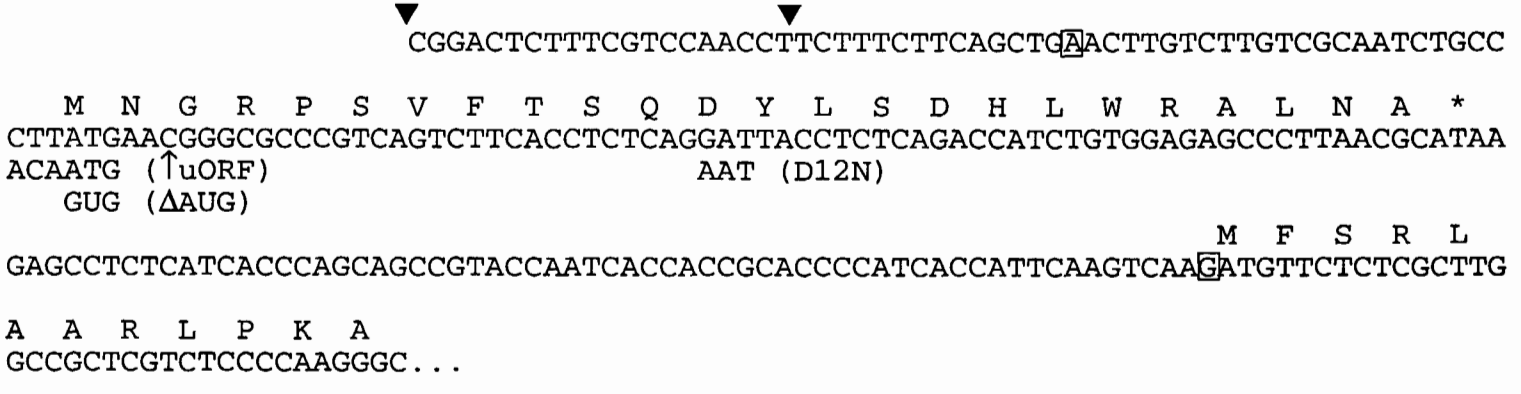

Figure 2.7 Sequence (GenBank accession #J05512) of the *arg-2* 5′ region with introns removed (Orbach, et al., 1990). Triangles indicate the major mRNA 5′ ends *in vivo*. The amino acid sequences of the uORF and the N-terminus of ARG2 are given above the nucleotide sequence. Mutations that were analyzed in the *in vitro* translation system are indicated. These include the change to D12N, changes that improve the translation initiation context of the uORF (↑ uORF), and a change that eliminates the uORF start codon (ΔAUG). The 5′ and 3′ boundaries of the *arg-2* region that was amplified by PCR for testing *in vitro* are boxed.

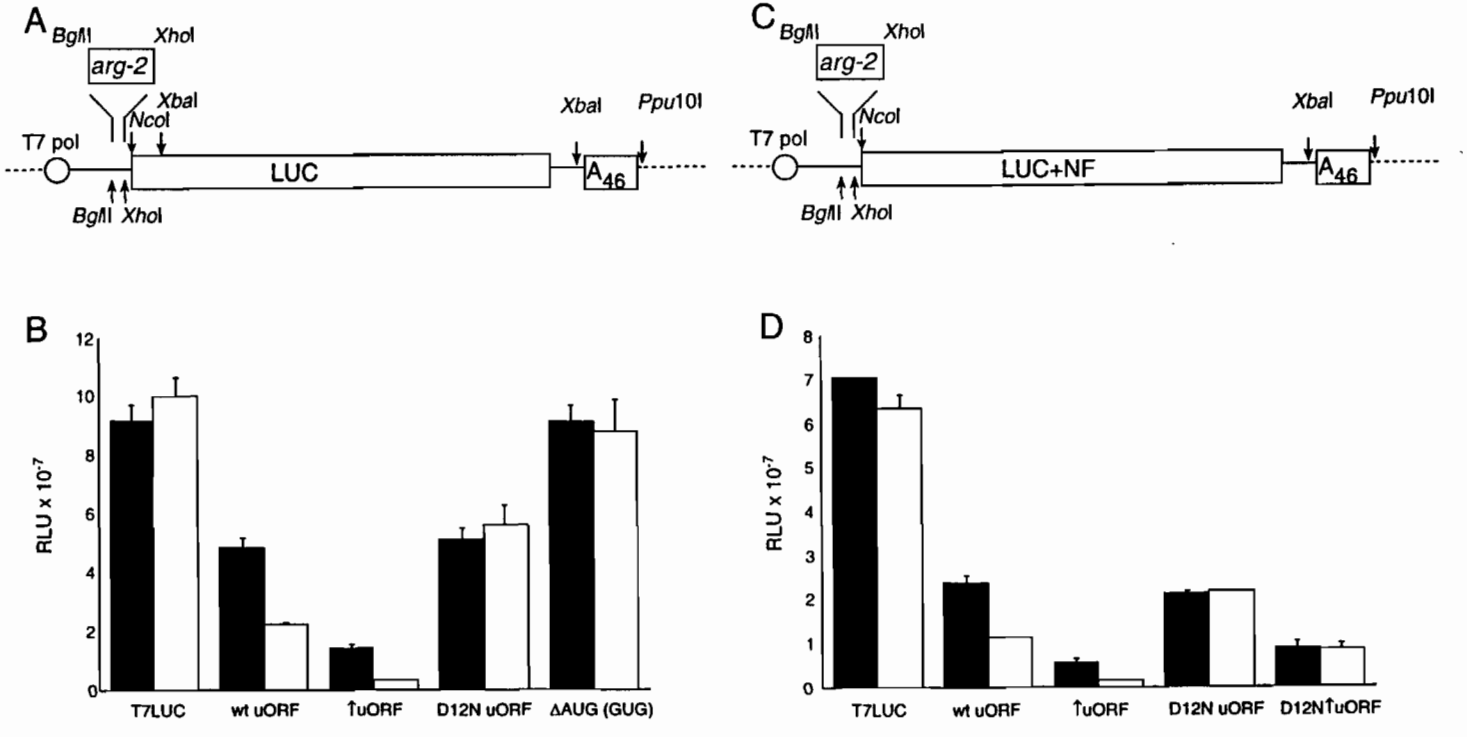

Figure 2.8 Effects of *arg-2* sequences on Arg-specific regulation in the *N. crassa in vitro* translation system. (A, C) Schematic of RNAs used for analyses. Vectors that contained or lacked wild-type and mutant *arg-2* sequences in front of either of two luciferase coding regions were used to synthesize capped, polyadenylated RNA for *in vitro* translation. (A) Vectors derived from pHLucS4 containing the wild-type firefly luciferase coding region (LUC). (C) Vectors derived from pHLuc+NFS4 containing the modified firefly luciferase coding region. (B, D) Effects of Arg on the translation of different RNAs. Equal amounts of each RNA (0.8 ng) were translated in extracts containing 10 μM (black) or 500 μM Arg (white) and 10 μM of the other 19 amino acids. (B) RNAs containing LUC. (D) RNAs containing LUC+NF. Standard deviations from mean values are indicated by error bars.

transcripts lacking uORFs in the *N. crassa* system, as also observed in the amino acid-dependent system (discussed below); similar results were observed with the *S. cerevisiae* translation system but not with the reticulocyte system, which did not appear to respond to uORFs (data not shown).

In amino acid-dependent translation reactions, the addition of Arg to a final concentration of 10 μM and the addition of the other 19 amino acids to final concentrations of 10 μM were sufficient for near maximal translation of luciferase (Fig. 2.6), enabling testing of the effects of adding 10 or 500 μM Arg on the translation of uORF-containing and control RNAs (Fig. 2.8B). Excess Arg did not affect translation of RNA obtained from a construct that lacks all *arg-2* sequences (T7LUC). Arg reduced translation of luciferase in RNA containing the wild-type uORF in its original translation initiation context or in an improved initiation context upstream of LUC (Fig. 2.8B). A reproducible, slight increase in Arg-specific regulation was seen with constructs containing the uORF in an improved initiation context. Improving the translation initiation context of the uORF also decreased production of luciferase from the downstream initiation codon. In contrast to the wild-type uORF constructs, the D12N uORF construct did not show Arg-specific regulation. Eliminating the uORF initiation codon eliminated regulation and increased expression to the level observed in the construct lacking *arg-2* sequences (Fig. 2.8B).

The data shown in Fig. 2.8B were highly reproducible. Considering the T7LUC, wild-type uORF and D12N uORF constructs alone, five different batches of RNA translated in nine independently derived Arg-dependent *N. crassa* extracts gave similar results. Three independent batches of RNA synthesized from all five constructs have been translated in three independently derived Arg-dependent translation extracts with similar results.

The effects of uORFs on Arg regulation were confirmed and extended using a second set of luciferase constructs in which LUC+NF (Promega) has been substituted for LUC (Fig. 2.8D). In *N. crassa* extracts, RNAs containing the LUC+NF polypeptide coding region instead of LUC produced similar yields of luciferase activity. The wild-type uORF construct, the ↑ uORF construct, and the D12N uORF construct showed effects on translation similar to those observed in the original LUC

constructs (Fig. 2.8, compare B and D). An additional construct, in which the D12N mutation was placed in an uORF that had an improved initiation context (D12N ↑ uORF), showed reduced translation of luciferase compared to the D12N mutation in the original uORF initiation context but still showed no Arg-specific regulation (Fig. 2.8D).

The primary mechanism of Arg-specific regulation mediated by the *arg-2* uORF in the *in vitro* translation system does not appear to involve changes to the RNA. First, the chemical stability of each of the types of RNAs used in Fig. 2.8B was measured by determining the amount of trichloroacetic acid-soluble radioactivity released from RNA over the course of translation reactions. There were no discernible differences among RNAs; addition of excess Arg did not appear to affect the stability of any of these RNAs (data not shown). Second, the negative effects of Arg on translation of the wild-type uORF RNA appeared to be reversible. Translation reactions containing excess Arg that were programmed with RNA containing the wild-type uORF translated RNA at a reduced rate compared with extracts without excess Arg (Fig. 2.9). 14-fold dilution of such translation mixtures with additional complete translation mixture lacking Arg after translation was initiated for 20 min resulted in an increased rate of RNA translation (Fig. 2.9). This rate was comparable with that observed in translation reactions initiated without excess Arg that were monitored at a similar time and was substantially higher than the rates observed in reactions diluted with reaction mixture containing excess Arg (Fig. 2.9). Similar results were obtained when reactions were diluted at 15 or 30 min instead of 20 min after incubation (data not shown). These data indicate that (i) degradation or irreversible modification of uORF-containing RNA was unlikely to be responsible for its reduced translation in the presence of excess Arg and (ii) the effects of Arg on translation of uORF-containing RNA appeared reversible.

How specific is Arg-specific regulation? The effects of compounds that might be expected to act similarly to Arg were examined by comparing the addition of 500 μM of Arg or 500 μM of each of these compounds. The addition of 150 μM Arg was sufficient to observe maximal regulatory effects (data not shown). The addition of canavanine, an Arg analog which can be incorporated into polypeptides and, when

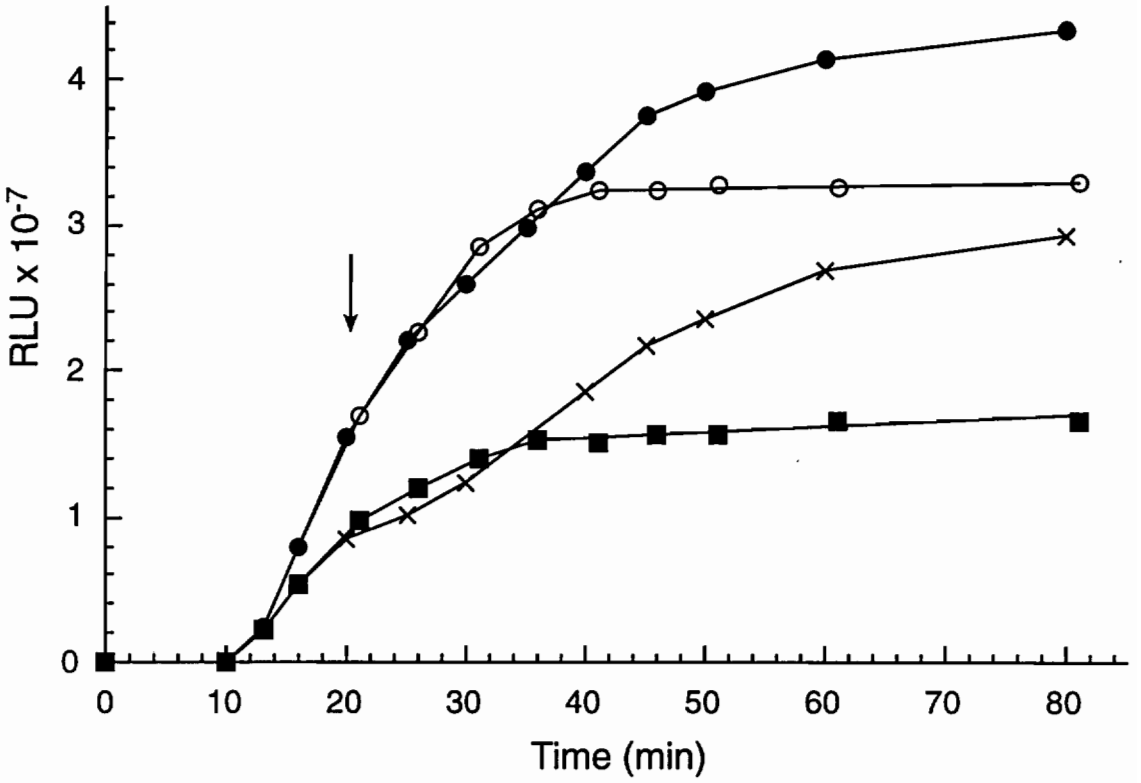

Figure 2.9 Arg-specific regulation *in vitro* is reversible. Translation reactions were initiated using 40 ng capped, polyadenylated RNA containing the wild-type uORF in an 80-μl volume; reaction mixtures contained 10 μM Arg (closed circles) or 150 μM Arg (squares) and 10 μM of the other 19 amino acids at the outset. After 20 min (arrow), 10 μl aliquots of each reaction were diluted with 140 μl of fresh reaction mixture containing 10 μM of amino acids other than Arg and 10 μM Arg (closed circles), 150 μM Arg (open circles and squares), or no Arg (crosses). Luciferase production at each time point is indicated; activity of extracts prior to dilution with fresh reaction mixture were normalized by appropriate dilution with buffer prior to assay.

incorporated, prevents their functional activity, resulted in a loss of luciferase activity in all cases, and its activity was not evaluated. Arginine methyl ester and arginine ethyl ester, which can be hydrolyzed to Arg, conferred regulation, but the Arg biosynthetic precursors citrulline and ornithine did not, nor did the basic amino acids His and Lys. Homoarginine, which has a side chain that is one methyl group longer than Arg, did not confer regulation. The Arg-related compounds agmatine, L-argininamide, phospho-L-arginine, L-argininic acid, and D-arginine did not confer regulation. These data strongly indicate that Arg-specific translational regulation has a high specificity for sensing the level of L-arginine.

2.4 Discussion

In vivo, Arg-specific regulation has effects on the expression of the *N. crassa arg-2* and *S. cerevisiae CPA1* genes specifying the small subunit of Arg-specific carbamoyl phosphate synthetase at both transcriptional and translational levels [Crabeel et al., 1990; Orbach et al., 1990; Sachs and Yanofsky, 1991; Luo et al., 1995]. Here we show that the *N. crassa arg-2* uORF has a role in Arg-specific translational regulation using a homologous cell-free *in vitro* translation system programmed with synthetic RNA. The sequence of the uORF was critical for regulation *in vitro*, but the context of the uORF codon initiation codon was not. Furthermore, Arg-specific translational regulation appeared specific for L-arginine and was not elicited by related amino acids or by biosynthetic precursors to Arg. These data confirm that the *arg-2* uORF modulates gene expression at the level of translation and, to our knowledge, represent the first demonstration of translational control in a eukaryotic *in vitro* system in response to the availability of a single amino acid.

Translation of RNA in the *N. crassa* cell-free translation system also was dependent on important features of eukaryotic RNA, cap, and poly(A). The addition of poly(A) to RNA stimulated translation in the *N. crassa* cell-free system independently of the addition of cap to RNA, while the addition of both to RNA synergistically stimulated translation (Fig. 2.3). Although synergistic interactions

between cap and poly(A) have been demonstrated in electroporated mammalian and fungal cells [Gallie, 1991], only recently have such interactions been observed *in vitro* and only in *S. cerevisiae* cell-free translation systems [Iizuka et al., 1994; Tarun and Sachs, 1995]. The effects of adding cap and poly(A) to RNA used to program *N. crassa* and *S. cerevisiae* translation reactions are similar, and, in both systems, the addition of poly(A) to RNA relieves the inhibitory effects of adding exogenous cap analog to translation reactions. In the *S. cerevisiae* system, these results have been combined with additional studies to support the interpretation that cap and poly(A) stimulate translation initiation by recruiting different RNA-binding proteins [Tarun and Sachs, 1995], and it is likely that a similar situation holds in *N. crassa*.

In the *N. crassa* cell-free system, the presence of cap and poly(A) on mRNA appear to affect its chemical stability (Fig. 2.5 and data not shown). In *S. cerevisiae*, similar effects have been reported in some studies [Gerstel et al., 1992] but not others [Iizuka et al., 1994; Tarun and Sachs, 1995]. The reasons why cap and poly(A) have effects on RNA stability in some *in vitro* studies but not others remains unclear, but a variety of studies have implicated that these features of the RNA molecule are important in modulating RNA stability [Caponigro and Parker, 1996; Jacobson, 1996].

The *in vitro* data indicate that the *arg-2* uORF has sequence-independent and sequence-dependent effects on translation. Thus, either the wild-type uORF or the D12N uORFs in the wild-type translation initiation context reduce translation of the downstream luciferase coding region (Fig. 2.8B and D). The uORF's wild-type initiation context is not typical for *N. crassa* [Luo et al., 1995]; changing the initiation context of either uORF to one resembling preferred *N. crassa* initiation contexts [Edelmann and Staben, 1994] further reduces translation of the downstream luciferase coding region. These data are consistent with the scanning model for translation initiation [Kozak, 1989; Jackson, 1996].

Arg-specific and uORF-sequence-dependent regulation is observed *in vitro* in addition to sequence-independent uORF effects. Arg-specific regulation in wild-type cells mediated through the *arg-2* uORF is approximately 2.5-fold *in vivo*, based on

measurements of accumulated polypeptide products [Luo et al., 1995; Freitag et al., 1996; Luo and Sachs, 1996]; a similar level of regulation is observed *in vitro*, based on measurements of accumulated luciferase product (Fig. 2.8). Measurements of relative rates of ARG2 polypeptide synthesis *in vivo* immediately after switching cells from minimal medium to fresh minimal medium or to Arg-containing medium indicate a 2.5-fold reduction in the rate of polypeptide synthesis upon exposure to Arg [Luo et al., 1995]; a similar reduction in the rate of luciferase synthesis from uORF-containing RNA is observed *in vitro* when translation reactions contain excess Arg (Fig. 2.9), based on comparing the rates of synthesis of reactions containing 10 μM or 150 μM Arg.

Our combined data show that Arg-specific translational regulation can be reconstituted in an amino acid-dependent *N. crassa* cell-free translation system. Our findings show that the level of regulation observed *in vitro* is very similar to that observed *in vivo* and that regulation is highly specific for L-arginine. The mechanism of Arg-specific translational control remains to be elucidated; the amino acid-dependent *in vitro* system, in which translational effects can be monitored independently of transcriptional effects, should provide an invaluable tool for combining biochemical and genetic approaches to determining the details of this mechanism.

CHAPTER 3

RIBOSOME STALLING IS RESPONSIBLE FOR ARGININE-SPECIFIC TRANSLATIONAL ATTENUATION IN *NEUROSPORA CRASSA*[*]

3.1 Introduction

Upstream open reading frames (uORFs) are present in the 5′ leaders of a number of eukaryotic mRNAs, particularly those involved in growth and development [Geballe, 1996; Harigai et al., 1996; Hinnebusch, 1996; Lovett and Rogers, 1996; Reynolds et al., 1996]. Some of these are known to reduce translation from downstream initiation codons. In the best-understood example of eukaryotic uORF control, *Saccharomyces cerevisiae GCN4*, the predicted primary sequences of the uORFs appear relatively unimportant for their function. In other cases, the primary sequences of the uORFs are critical. The mechanistic basis for the action of these uORFs has been hypothesized to involve the sequence-dependent arrest of the ribosomes translating them. This creates a blockade to ribosomal scanning that reduces ribosome loading at the downstream initiation codon [Geballe and Morris, 1994]. Only for uORF2 of the cytomegalovirus *gp48* transcript has arrest of ribosomes at a eukaryotic uORF been directly demonstrated [Cao and Geballe, 1996a,b]. In this case, ribosomes appear to arrest by an unregulated mechanism at the uORF translation termination site. However, the mechanisms by which other sequence-specific eukaryotic uORFs act and how they might serve regulatory roles remain unknown.

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

Ribosome stalling can be detected by a primer extension inhibition ("toeprint") assay [Hartz et al., 1988, 1989]. Toeprinting showed ribosome arrest at the *gp48* uORF2 termination codon [Cao and Geballe, 1996a]. The toeprinting technique has also been applied to eukaryotic systems to detect ribosomes and translation factors at initiation codons [Anthony and Merrick, 1992; Kozak, 1995; Pestova et al., 1996a,b]. In these cases, ribosomes bound at an AUG initiator were found to cause reverse transcriptase to terminate primer extension at a site 15–17 nucleotides (nt) downstream from the A of this codon. A related technique has been used in other eukaryotic cell-free systems treated with cycloheximide to detect stalled, elongating ribosomes [Wolin and Walter, 1988] and ribosomes at initiation codons and termination codons [Doohan and Samuel, 1992, 1993].

The level of *Neurospora crassa* arginine-specific carbamoyl phosphate synthetase, which generally determines flux through the Arg biosynthetic pathway [Davis and Ristow, 1987], is determined by the level of the *arg-2*-encoded polypeptide subunit [Davis, 1986]. *arg-2* is the only gene encoding an *N. crassa* Arg biosynthetic enzyme that is negatively regulated by Arg [Davis, 1986]. The *arg-2* mRNA contains an uORF specifying a 24-residue peptide (Fig. 3.1A) [Orbach et al., 1990] that appears to be translated *in vivo* as determined using fusions of the uORF to *Escherichia coli lacZ* [Luo and Sachs, 1996]. The *arg-2* uORF sequence is important for negative translational regulation by Arg *in vivo*. For example, changing uORF Asp codon 12 to Asn (D12N) eliminates regulation by Arg [Freitag et al., 1996]. The number of ribosomes associated with RNA containing the wild-type uORF, but not the D12N uORF, is reduced in cells exposed to excess Arg [Freitag et al., 1996; Luo et al., 1995; Luo and Sachs, 1996].

The sequence of the *arg-2* uORF is evolutionarily conserved, which is consistent with its having functional significance. The homologous *arg-2* gene from the rice blast fungus *Magnaporthe grisea* contains an uORF specifying a nearly identical peptide [Shen and Ebbole, 1997]; the corresponding *CPA1* gene from *S. cerevisiae* contains an uORF specifying a closely related peptide (Fig. 3.1B). Arg-specific regulation of *CPA1* also requires the uORF; a mutation corresponding to the

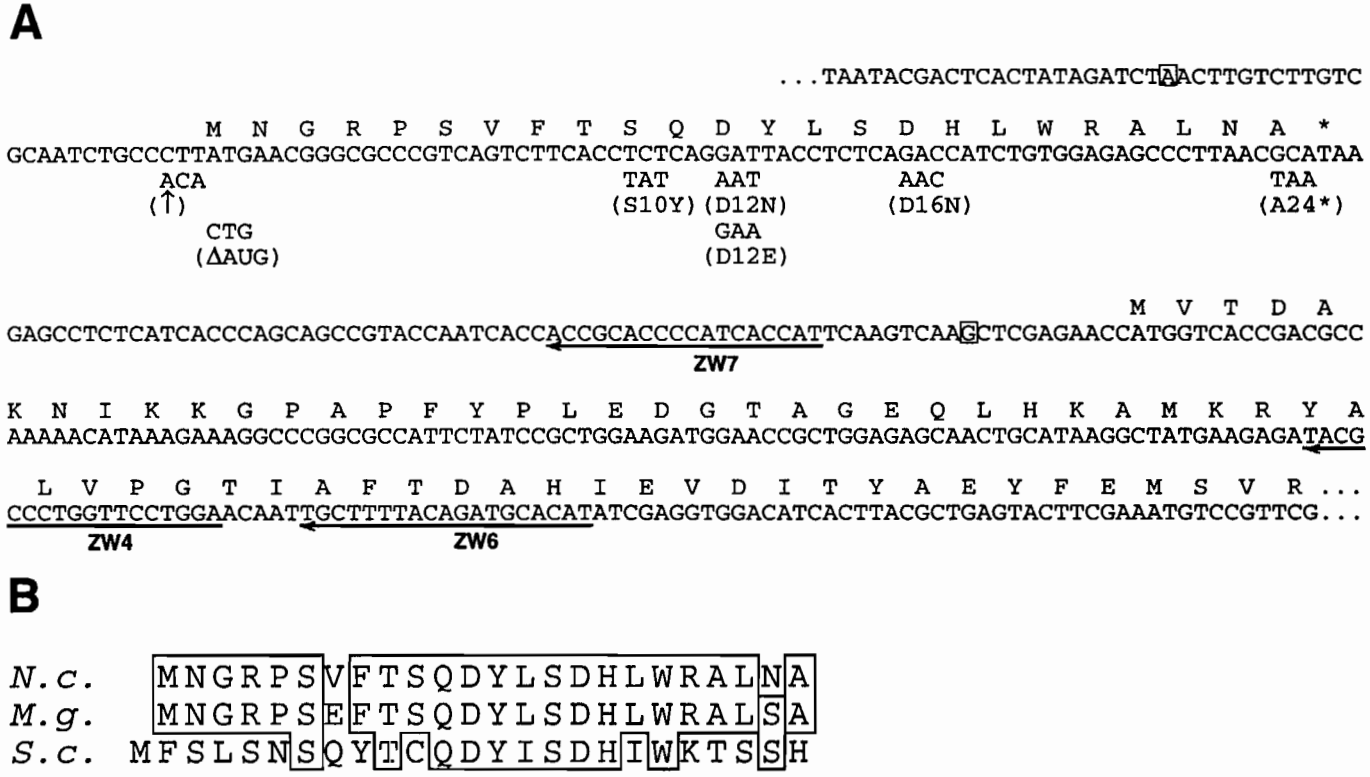

Figure 3.1 The 5′ region of the *arg-2–LUC* gene and comparison of *arg-2* uORF-related peptides. (A) Sequences of wild-type and mutant *arg-2–LUC* templates. The sequence shown begins with the T7 RNA polymerase-binding site and ends within the luciferase coding region. The 5′ and 3′ boundaries of the *arg-2* region are boxed. The amino acid sequences of the *arg-2* uORF and the amino terminus of luciferase are indicated. Specific mutations and their predicted consequences for uORF translation are shown below the wild-type sequence. The (↑) mutation improves the initiation context for uORF translation. The sequences for which the reverse complements were synthesized as primers ZW4, ZW6, and ZW7 are indicated by arrows. (B) Alignment of the peptide sequences encoded by the uORFs in the transcripts of the homologous genes *N. crassa arg-2* (*N.c.*) [Orbach et al., 1990], *M. grisea arg-2* (*M.g.*) [Shen and Ebbole, 1997], and *S. cerevisiae CPA1* (*S.c.*) [Werner et al., 1987].

arg-2 uORF D12N mutation eliminates Arg-specific regulation [Werner et al., 1987; Delbecq et al., 1994].

To gain a better understanding of the mechanism of Arg-specific translational control, we developed a cap-, poly(A)-, and amino acid-dependent cell-free *N. crassa* protein-synthesizing system [Wang and Sachs, 1997]. In this system, Arg-specific translational control by the *arg-2* uORF is reconstituted, as judged by translation of dicistronic *arg-2–LUC* RNA containing uORF and luciferase (*LUC*) coding regions [Wang and Sachs, 1997]. Arg-specific regulation, and the effects of uORF mutations on regulation appear similar *in vitro* and *in vivo*.

Here we present toeprint data indicating the presence of ribosomes on *arg-2–LUC* RNA during its translation *in vitro*, including ribosomes at the uORF initiation codon, the uORF termination codon, and the LUC initiation codon. We show that, in the presence of excess Arg, ribosome stalling at the uORF is increased. Stalling of ribosomes translating the uORF was accompanied by a decrease in the number of ribosomes associated with the downstream LUC initiation codon. The sequence of the uORF, and its capacity to be translated, were necessary for these effects. These data suggest that ribosome stalling accounts for Arg-specific negative regulation mediated by the *arg-2* uORF.

3.2 Materials and Methods

3.2.1 Preparation of Templates Containing Wild-type and Mutant *arg-2* Sequences

Megaprimer PCR [Sarkar and Sommer, 1990] was used to construct mutated *arg-2* sequences (Fig. 3.1A) flanked by 5′ *Bgl*II and 3′ *Xho*I sites. Mutagenic primers for megaprimer PCR included ZL20 (5′-ATCTGCCCTTCTGAACGGGC-3′), which eliminates the uORF AUG codon; OJC102 (5′-GTCAGTCTTCACCTATCA-GGA-3′), which introduces the S10Y mutation; OJC103 (5′-ACCTCTCAGGAATAC-CTCTCA-3′), which introduces the D12E mutation; and OJC104 (5′-TACCTCTC-AAACCATCTGTGG-3′), which introduces the D16N mutation; OJC108

(5′-GCCCTTAACTAATAAGAGCCTC-3′), which introduces the A24* mutation. *Bgl*II- and *Xho*I-digested PCR products were placed into the corresponding sites of pHLUC+NFS4, and the sequences of these constructs were confirmed as described previously [Wang and Sachs, 1997].

3.2.2 Preparation of Synthetic RNA Transcripts

Plasmid DNA templates were purified by equilibrium centrifugation and linearized with *Ppu*10I. Capped, polyadenylated RNA was synthesized using T7 RNA polymerase, and the yield of RNA was quantitated [Wang and Sachs, 1997].

3.2.3 Cell-Free Translation and Analyses of Translation Products

Amino acid-dependent *N. crassa* cell-free translation extracts were prepared, used, and assayed for luciferase enzyme activity as described previously [Wang and Sachs, 1997]. For reaction mixtures subjected to toeprint analyses, the concentration of *arg-2–LUC* transcript was increased to 6 ng/μl (which was near the maximal concentration of transcript for which luciferase production remained linearly proportional to the level of transcript added to translation reactions) and the concentration of RNasin RNA inhibitor was increased from 0.2 to 0.8 U/μl.

3.2.4 Preparation of 5′ ^{32}P-Labeled Primers for Toeprinting and Sequencing Reactions

Oligodeoxynucleotides ZW4, ZW6, and ZW7 (Fig. 3.1A) were labeled at their 5′ termini using T4 polynucleotide kinase (New England Biolabs) and [γ-^{32}P]ATP (>6000 Ci/mmol; Andotek Life Sciences, Irvine, CA). The reaction mixtures (100 μl) contained 50 pmol of oligodeoxynucleotide, 50 mM Tris-HCl (pH 8.0), 20 mM $MgCl_2$, 4 mM spermidine, 4 mM dithiothreitol, 400 μCi [γ-^{32}P]ATP, and 10 U of T4 kinase. The primer was first mixed with Tris-HCl, and the volume was adjusted to 34 μl; the primer was then heated at 90°C for 3 min and chilled on ice. Water, $MgCl_2$, spermidine, dithiothreitol, [γ-^{32}P]ATP, and kinase were added, and the mixture was incubated for 45 min at 37°C. EDTA was added to a final concentration of 50 mM, and the reaction mixture extracted with 120 μl of buffered phenol-

chloroform. The aqueous phase was transferred to a new tube; the phenol-chloroform phase was back-extracted with 80 μl Tris-EDTA, and this wash was combined with the original aqueous phase. This aqueous solution was extracted once more with an equal volume of chloroform and then chromatographed on a Sephadex G-25 superfine column (5-ml bed volume in a 5-ml disposable pipet) that was pre-equilibrated with 10 mM NH_4HCO_3 and developed in the same buffer. Fractions were collected, and portions (1 μl) were analyzed by polyethyleneimine thin-layer chromatography to check for the presence of inorganic phosphate and unreacted ATP. Fractions containing radiolabeled oligonucleotide were pooled and lyophilized. Oligonucleotides were dissolved in 250 μl of water (yielding a primer concentration of 0.1 μM if a recovery of 50% is assumed).

3.2.5 Primer Extension Inhibition (Toeprint) Assays

The primer extension inhibition (toeprint) assay was modified from previously described procedures [Hartz et al., 1988; Cao and Geballe, 1996a]. Toeprint assays were performed by adding 3 μl of translation reaction mixtures (or pure RNA) to 5.5 μl of reverse transcription buffer, which was already in a tube precooled on ice. This buffer contained components to bring the final 10-μl reverse transcription reaction to 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 10 mM $MgCl_2$, 0.01 M dithiothreitol, 0.25 mM each dATP, dCTP, dGTP, dTTP, and 1000 U of RNasin RNA inhibitor per ml (ignoring the contributions of components of the translation reaction mixture). The tubes were heated at 50°C for 2 min and then immediately placed on ice again. This heat step was essential to observe toeprinting. ^{32}P-labeled primer (1 μl; approximately 2×10^6 cpm Cerenkov) was added to each tube and annealed to the template by placing tubes in a 37°C water bath for 5 min. Then 0.5 μl (100 U) of Superscript II RNase H^- reverse transcriptase (Gibco BRL) was added, and reverse transcription reactions were incubated for 30 min at 37°C. The reactions were terminated by extraction with 10 μl of phenol-chloroform. The aqueous phase was removed and mixed with an equal volume of DNA sequencing stop solution (91% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF). Samples were heated at 85°C for 5 min, cooled on ice, and loaded on 6% DNA denaturation

sequencing polyacrylamide gels (4 μl of sample per lane). The gels were dried and exposed for at least 3 days to Kodak XAR-5 film without intensifying screens (screens decreased the sharpness of bands); photographs of these autoradiograms are presented here (see Figs. 3.4–3.7). Gels were also analyzed using a Molecular Dynamics PhosphorImager for quantitative data. All toeprint data presented are representative of multiple experiments.

Typically, translation reaction volumes for toeprint experiments were 20 μl. For time course experiments (see, e.g., Fig. 3.7), larger volumes were used. In time course studies, coordinated teamwork was required for precise, accurate handling of serial samples.

For studying the effects of chemical inhibitors on translation, compounds were added (0.5 μl of 40X stock solutions) to the indicated final concentrations: puromycin (640 μM), cycloheximide (320 μM), hygromycin (8 mM), and EDTA (5 mM). Additional $MgCl_2$ (5 mM) was added back to reaction mixtures incubated with EDTA prior to primer extension analyses.

DNA sequencing markers were obtained by using the same ^{32}P-labeled oligonucleotide primers used for toeprinting to sequence the plasmid template containing the wild-type uORF. Non-cycle sequencing was accomplished using the ΔTaq cycle sequencing kit (United States Biochemicals) by modifying the supplier's procedure; labeling reactions were omitted, and the termination reactions were incubated for 10 min at 67°C (primers ZW4 and ZW7) or 20 min at 57°C (primer ZW6).

3.3 Results

3.3.1 Translational Arrest Mediated by the *arg-2* uORF and Arg

Arg-specific translational control mediated by the *arg-2* uORF can be observed in a homologous cell-free translation system [Wang and Sachs, 1997]. When the concentration of Arg is increased from 10 μM (low Arg) to 500 μM (high Arg) in reaction mixtures programmed with dicistronic *arg-2–LUC* RNA containing the wild-type uORF in the 5′-leader sequence (Fig. 3.1A), synthesis of luciferase is reduced

(Fig. 3.2). Mutations (Fig. 3.1A) which alter the *arg-2* uORF initiation codon to eliminate uORF translation (ΔAUG) or which change a critical Asp residue at codon 12 of the uORF to Asn (D12N) eliminate this negative regulatory effect of Arg (Fig. 3.2) [Wang and Sachs, 1997].

Arg may exert its negative effect on translation of *arg-2* through stalling of ribosomes translating the uORF, thereby hindering access to the downstream luciferase translation initiation codon [Lovett and Rogers, 1996]. We tested this possibility by primer extension inhibition (toeprinting) [Hartz et al., 1988] assays, in which reverse transcriptase is used to extend a radiolabeled primer on an RNA template in the presence or absence of cellular factors (Fig. 3.3A). Toeprinting should reveal the positions of translational components, such as ribosomes, at sites where they accumulate on RNA (e.g., at sites of rate-limiting steps in translation). Below, we will use the term "ribosome" inclusively to refer to 80S ribosomes, translation initiation complexes, and termination complexes.

In the absence of extract, reverse transcription of synthetic *arg-2–LUC* RNAs with any of three radiolabeled primers (ZW4, ZW6, and ZW7 in Fig. 3.1A) yielded cDNA extension products predominantly corresponding to full-length transcripts (see, e.g., Fig. 3.4, lane 12, and Fig. 3.6, lane 19 [data not shown]). This was determined by comparing the positions of the primer-extended cDNA products to dideoxynucleotide-sequencing products obtained by using the same radiolabeled primers to sequence corresponding DNA templates. Several minor, shorter termination products were also reproducibly observed.

When *arg-2–LUC* RNA present in the cell-free translation reaction mixture was used for toeprint analyses, additional premature, site-specific termination products were observed (Fig. 3.4, lanes 1 and 2). Several modifications of previously described procedures for toeprinting RNA in eukaryotic translation-competent extracts [Anthony and Merrick, 1992; Cao and Geballe, 1996a] were important to obtain these results. Both heat treatment of the translation reaction prior to adding primer and maintenance of high magnesium concentrations during reverse transcription appeared necessary.

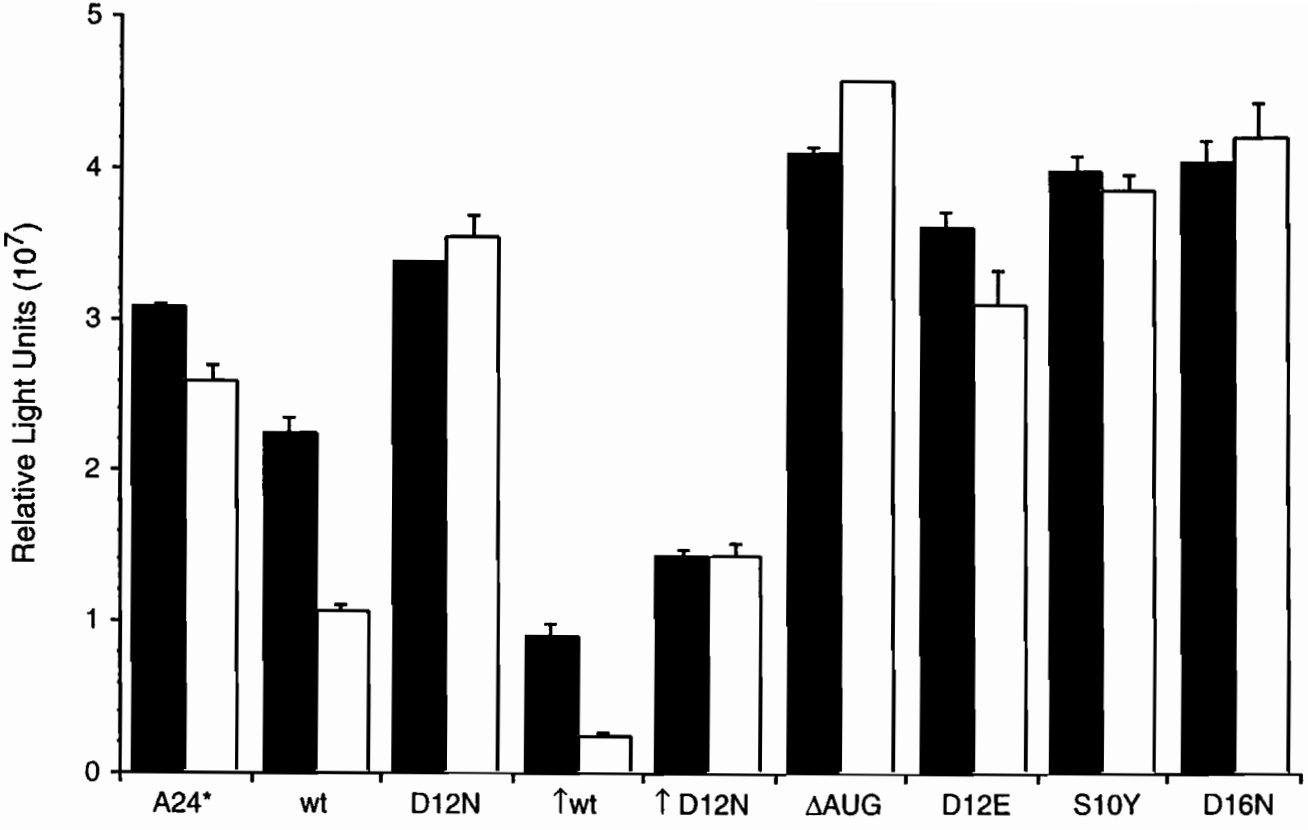

Figure 3.2 Effects of uORF mutations on Arg-specific translational control *in vitro*. Equal amounts (1.2 ng) of wild-type (wt) and mutant (Fig. 3.1A) RNA were translated in *Neurospora* extracts containing 10 μM (solid bars) or 500 μM (open bars) Arg and 10 μM of each of the other 19 amino acids [Wang and Sachs, 1997]. Mean values and standard deviations obtained from measuring luciferase enzyme activity in three independent translation reactions are given. The ↑wt RNA has the wild-type uORF in an improved initiation context; the ↑D12N RNA has the D12N uORF in this context.

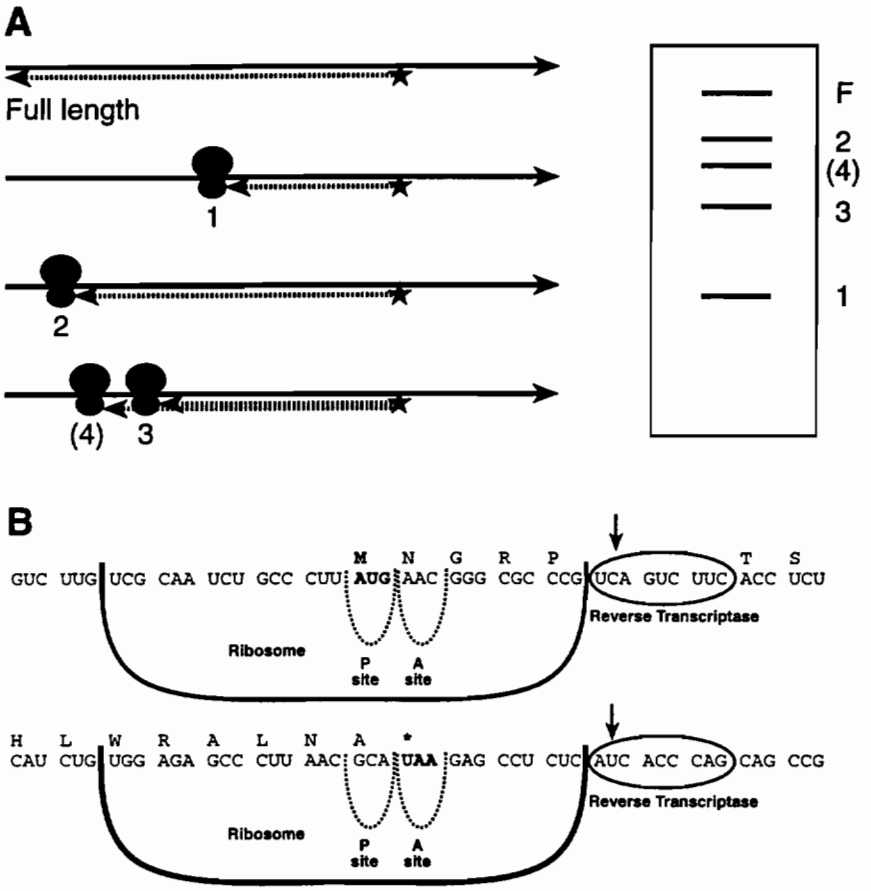

Figure 3.3 The primer extension inhibition (toeprint) assay. (A) Principle of the assay. Radiolabeled oligonucleotide primer (star) anneals to the RNA template (solid arrow) and is extended by reverse transcriptase (dotted arrow). On pure RNA, extension proceeds to the 5′ end of the template. Ribosomes accumulated at different discrete positions on the RNA will inhibit primer extension (e.g., ribosome 1). Analyses of primer extension (toeprint) products on denaturing sequencing gels (right) enable the determination of the sizes of primer extension products; the full length product (F) migrates most slowly. Ribosomes could also accumulate on RNA at multiple sites (e.g., ribosomes 2 and 3). When multiple complexes are associated with RNA, a toeprint analysis would be expected to yield only signals corresponding to the complex that is first encountered on an RNA template by the elongating reverse transcriptase, if this first complex were infinitely stable; however, dissociation of this complex would allow primer extension beyond this site. (B) The relationship between toeprint sites and the P and A sites of the ribosome. Eukaryotic ribosomes or 40S subunits bound to RNA with an initiation codon at their P site cause toeprints 15–17 nt downstream [Anthony and Merrick, 1992; Pestova et al., 1996a]. (*Top*) A ribosome with the *arg-2* uORF initiation codon in its P site blocks the movement of reverse transcriptase on the RNA template, causing premature termination (arrow) 16 nt downstream of the A of the AUG initiator. (*Bottom*) A ribosome with the uORF termination codon, UAA, at its A site causes premature termination 13 nt downstream of the U of the termination codon).

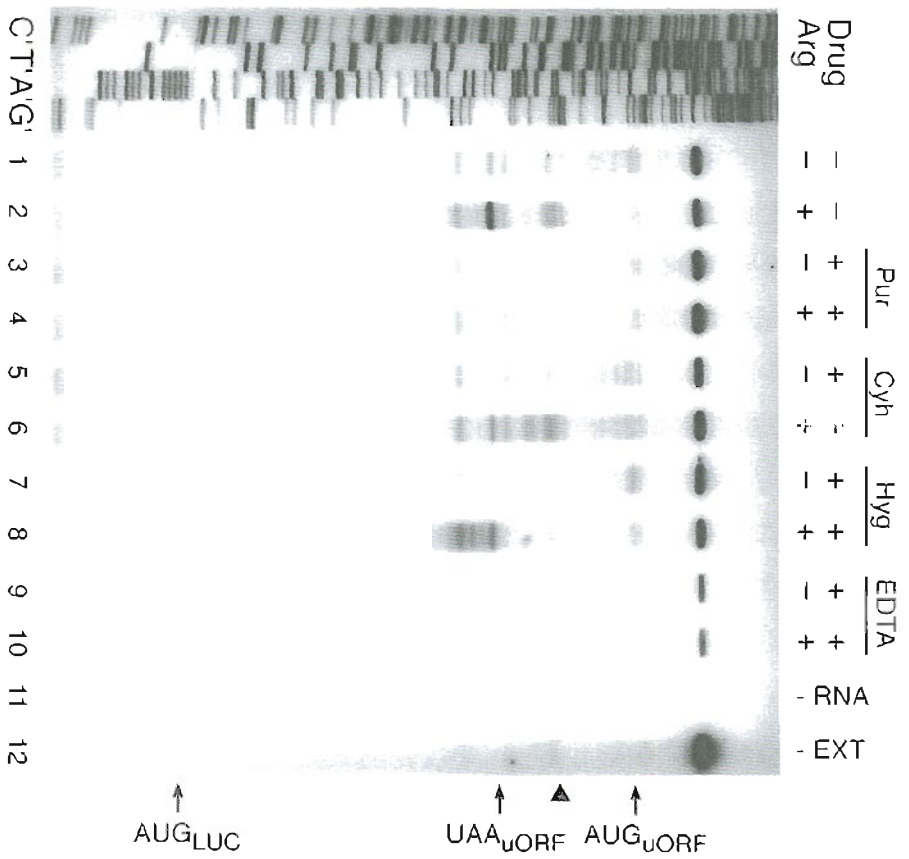

Figure 3.4 Effects of translational inhibitors on toeprinting. The RNA transcript containing the wild-type uORF (120 ng) was translated for 20 min in 20-μl reactions at 25°C. Reaction mixtures contained 10 μM (-) or 500 μM (+) Arg as indicated and 10 μM of each of the other 19 amino acids. Translation inhibitors were added as described in the text: puromycin (Pur), cycloheximide (Cyh), hygromycin (Hyg), and EDTA. Radiolabeled primer ZW6 was used for primer extension analyses (lanes 1–12) and for sequencing of the wild-type template (the four left-hand lanes). For the dideoxynucleotide sequencing of the wild-type template, the nucleotide complementary to the dideoxynucleotide added in each reaction mixture is indicated above the corresponding lane so that the sequence of the wild-type template (Fig. 3.1) can be directly deduced: the 5′-to-3′ sequence reads from top to bottom. The products obtained from primer extension of translation extract in the absence of input RNA [-RNA (lane 11)] and from a corresponding amount of pure RNA (18 ng) in the absence of translation extract [-EXT (lane 12)] are shown for comparison. Arrows indicate the positions of premature transcription termination products corresponding to ribosomes bound at AUG_{uORF}, UAA_{uORF}, and AUG_{LUC}; the arrowhead indicates the position of an additional toeprint site induced in 500 μM Arg.

Among the additional species observed in translation reaction mixtures containing low concentrations of Arg and wild-type *arg-2–LUC* RNA were toeprint sites ≈16 nt downstream of the A of the uORF AUG initiation codon, ≈13 nt downstream of the U of the uORF UAA termination codon, and ≈16 nt downstream of the A of the LUC AUG initiation codon (Fig. 3.4, lane 1; indicated by arrows as AUG_{uORF}, UAA_{uORF}, and AUG_{LUC}, respectively). The location of these products was determined by comparing series of alternating lanes of toeprinted translation reactions and sequencing reactions [data not shown]. While the migration of dideoxy-sequencing products and cDNA products differed slightly, the precision of these measurements was within a nucleotide.

The toeprint signals observed in the *Neurospora* extracts arose from interactions between the extract and the input RNA template; parallel primer extension analyses of extracts to which no *arg-2–LUC* RNA was added did not yield these products (see, e.g., Fig. 3.4, lane 11, and Fig. 3.6, lane 20 [data not shown]). Because eukaryotic translation initiation complexes and 80S ribosomes, when bound at initiation codons, cause toeprints at sites approximately 15–17 nt distal from the A of AUG initiation codons [Anthony and Merrick, 1992; Pestova et al., 1996a] and for additional reasons given below, these toeprint sites appear to correspond to ribosomes positioned on RNA with AUG_{uORF} and AUG_{LUC} initiation codons at their P sites and UAA_{uORF} termination codons at their A sites (Fig. 3.3B).

Addition of high instead of low Arg to translation reactions containing RNA with the wild-type uORF caused a substantial increase in the toeprint signal corresponding to the uORF termination codon (Fig. 3.4, compare lanes 2 and 1). In addition, high Arg levels caused the appearance of a cluster of strong toeprint signals 21–30 nt upstream of the termination codon toeprint. These effects of Arg on uORF toeprinting were observed with all three primers (Fig. 3.1A) used for primer extension. Addition of Arg also reduced the toeprint signal at the *LUC* initiation codon. Therefore, the translational response of this RNA to Arg, as measured by the two-fold reduction in luciferase synthesis (Fig. 3.2), was correlated, at the 20-min time point of the translation reaction, with increased toeprint signals at the uORF and a decreased toeprint signal at the *LUC* initiation codon. In some experiments (see,

e.g., Fig. 3.6), high Arg levels also caused a slightly increased signal at a site downstream of the uORF termination codon; a corresponding signal was present at a lower level in primer extension analyses of RNA in the absence of extract. This toeprint signal also increased when hygromycin was added to reaction mixtures as described below. The significance of this species is unknown. Finally, an additional strong toeprint was observed downstream of the LUC initiation codon at both low and high Arg concentrations. This toeprint might correspond to ribosomes with the rare *N. crassa* Lys codon, AAA [Edelmann and Staben, 1994], in their A sites or might arise from the binding of non-ribosomal factors to the RNA.

3.3.2 The Effects of Limiting Protein Synthesis

Several alternative explanations could be given for the additional toeprint signals observed on *arg-2-LUC* RNA when it is translated in cell-free extracts. These toeprints could arise by nucleolytic cleavage of the RNA at specific sites when the RNA is incubated in the cell-free translation extracts. Alternatively, they could arise from factors in the extract that complexed to RNA in a manner independent of translation or from secondary structures in the RNA that formed in the presence of extract independent of translation and that impede primer extension. To discriminate among these possibilities, we examined the effects of inhibiting translation by using chemical inhibitors and by limiting amino acids. Inhibiting translation in these ways should preferentially affect toeprint signals arising from the association of ribosomes with RNA.

Puromycin is an antibiotic analog of aminoacyl-tRNA that binds to the ribosomal A site and undergoes a transpeptidation reaction. The peptide-puromycin product that is formed cannot be extended further; translation terminates prematurely and ribosomes dissociate from the RNA. A second inhibitor, cycloheximide, specifically blocks the translocation of peptidyl-tRNA from A site to the P site in eukaryotic ribosomes and stabilizes polysomes. A third inhibitor, hygromycin B, affects ribosomes at the translocation step and is implicated in increased codon misreading; in prokaryotes, it also affects translation termination. EDTA dissociates Mg^{2+}-dependent complexes and thus would be expected to release ribosomes from

RNA. Pretreatment of translation reaction mixtures containing low or high Arg concentrations with these compounds prior to adding RNA resulted in the loss of luciferase translation, as measured by enzyme assay [data not shown]. In all cases, primer extension inhibition analyses of the transcripts in pretreated reaction mixtures showed the loss of the toeprint signals corresponding to ribosomes at the uORF stop codon [data not shown] and at the site 21–30 nt upstream. Therefore, these toeprint signals required translation.

To further examine the effects of chemical inhibitors of translation on toeprint signals, translation reaction mixtures containing low or high Arg concentrations and wild-type *arg-2–LUC* RNA were incubated for 20 min; then puromycin, cycloheximide, hygromycin, or EDTA was added; and the mixtures were incubated for a further 5 min (Fig. 3.4). Compared to the control, to which no translation inhibitors were added, puromycin or EDTA addition caused the loss of toeprints that corresponded to the uORF stop codon and to the site 21–30 nt upstream that occurred at high Arg concentrations (Fig. 3.4, lanes 1–4, 9, and 10). Puromycin did not reduce the toeprint signals corresponding to the uORF and *LUC* initiation codons but abrogated the effect of high Arg levels to reduce the toeprint signals corresponding to AUG_{LUC}. Thus, it appeared that, under these assay conditions, puromycin and EDTA preferentially released nascent peptides and their associated ribosomes from RNA but did not fully release initiation complexes from RNA. These data indicate that the Arg-induced toeprint signals arise from the continued association of ribosomes with RNA and not from cleavage of the RNA.

In contrast to the results obtained with puromycin, the effects of Arg on toeprint signals were still apparent in ongoing translation reaction mixtures that were treated with cycloheximide or hygromycin (Fig. 3.4, lanes 3–8). With cycloheximide, the toeprint signal corresponding to the uORF termination codon was substantially diminished, but the Arg-induced toeprint 21–30 nt upstream was not. Cycloheximide also caused a series of additional toeprints immediately downstream of the uORF and luciferase initiation codons. These additional toeprints may represent elongating ribosomes arrested by the drug. Thus, the decrease in the toeprint at the uORF termination site observed in these experiments might have occurred because

cycloheximide did not inhibit termination to the same extent as it inhibited elongation, and in the presence of the drug, ribosomes did not translocate to the termination site to replace those that had dissociated from this site.

Hygromycin reduced toeprint signals at sites corresponding to elongating ribosomes and increased toeprint signals at sites corresponding to translation initiation. Studies on the effects of hygromycin on bacteria indicate that hygromycin does not block elongating ribosomes except when the initiation codon is in the P site [Hausner et al., 1988], results which are consistent with our observations. The toeprint signal corresponding to ribosomes at the uORF termination codon was reduced, but an increase in the toeprint signal immediately downstream of this site was observed. This may reflect hygromycin-related effects on termination [Brown et al., 1993].

Experiments with chemical inhibitors indicated that the Arg-mediated differences in toeprint signals arose from the presence of translating ribosomes. Synthesis of luciferase in the *Neurospora* cell-free system requires exogenously supplied amino acids [Wang and Sachs, 1997]. Therefore, we also tested the effects of limiting the supply of amino acids in the toeprint assay. Toeprints corresponding to translating ribosomes would be expected to be influenced by charged-tRNA limitation. Representative results obtained with two independently derived extracts are shown in Fig. 3.5. Leaving out the amino acids caused a loss of the toeprint signal corresponding to the UAA_{uORF} (Fig. 3.5, lanes 1, 2, 10, and 11), as well as eliminating luciferase synthesis as determined by measurement of enzyme activity [data not shown]. The toeprint signals corresponding to AUG_{uORF} and AUG_{LUC} [data not shown] remained; in addition, several new toeprint sites were observed within the uORF coding region (Fig. 3.5, lanes 1, 2, 10, and 11).

Limitation for an aminoacyl-tRNA would be expected to cause a ribosome to stall with the corresponding codon in its A site; therefore, a toeprint would appear about 13 nt downstream. Mapping toeprints in this way revealed that two prominent new toeprint sites observed in the absence of added amino acids corresponded to ribosomes stalled with the A of Thr-9 codon ACC and the C of Gln-11 codon CAG at the A sites, respectively (Fig. 3.5). Addition of the amino acid Thr alone (Fig. 3.5,

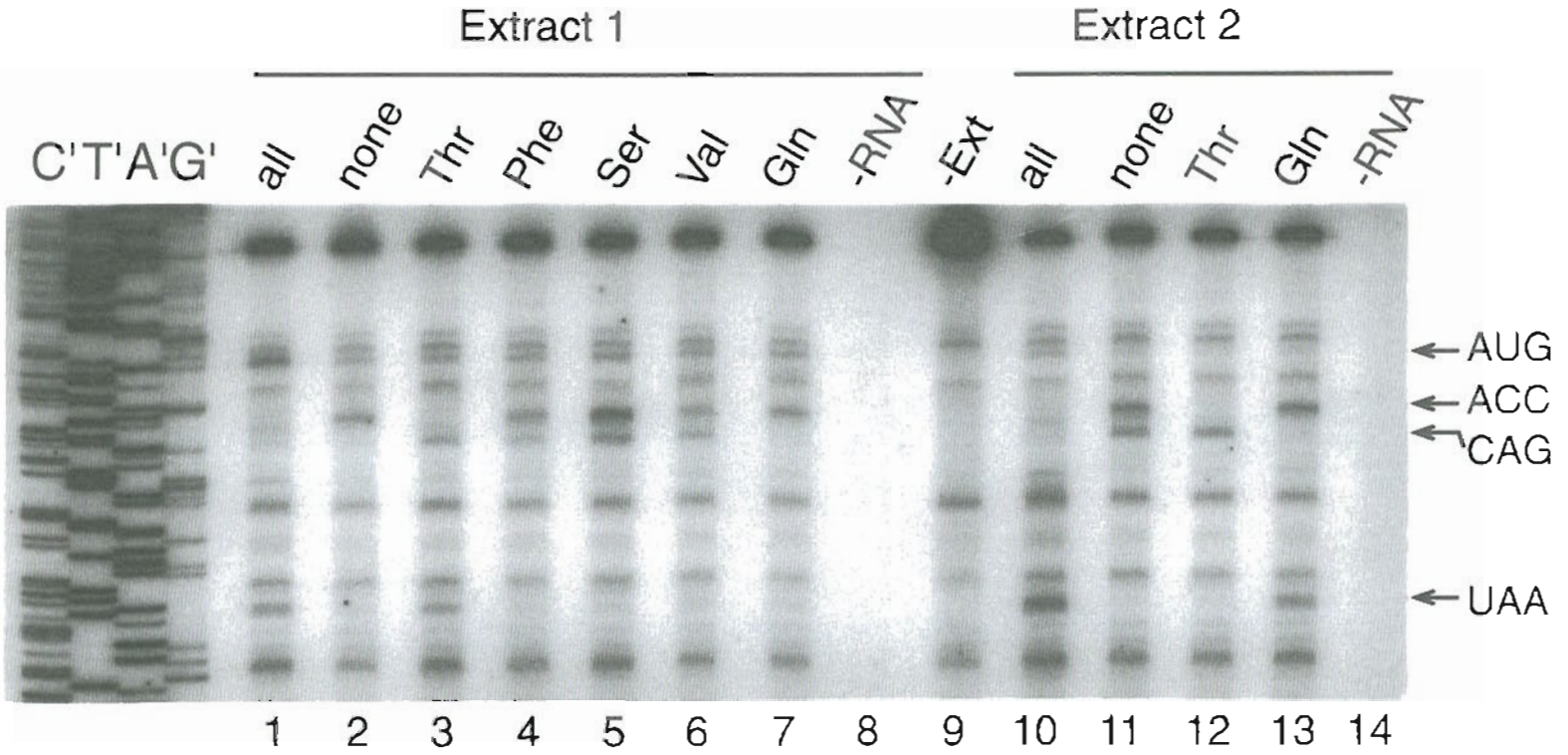

Figure 3.5 Effects of amino acid limitation on toeprinting. RNA containing the wild-type uORF in the improved initiation context (120 ng) was incubated in 20-μl translation reactions for 20 min. The reaction mixtures contained either of two independently derived *N. crassa* cell-free extracts (extract 1, lanes 1–8; extract 2, lanes 10–14) and were toeprinted and analyzed with controls as described in the legend to Fig. 3.4, except that primer ZW4 was used. The reaction mixtures contained different pools of amino acids. These extracts showed slight differences in their responses to added amino acids that presumably reflect differences in growth of the cultures or in the preparation of the extracts. Reactions marked "all" contained 10 μM (lane 1) or 50 μM (lane 10) each of all 20 amino acids. Reactions marked "none" (lanes 2 and 11) contained no exogenously supplied amino acids. For reactions marked Thr (lanes 3 and 12), Phe (lane 4), Ser (lane 5), Val (lane 6), and Gln (lanes 7 and 13), only the indicated amino acid was exogenously supplied, to 500 μM. Arrows indicate the toeprint products corresponding to ribosomes at AUG_{uORF}, ACC (uORF codon Thr-9), CAG (uORF codon Gln-11), and UAA_{uORF}.

compare lanes 2 and 3 and lanes 11 and 12) eliminated the toeprint corresponding to this site. In contrast, adding single amino acids corresponding to nearby codons (Phe, Ser and Val) did not eliminate this toeprint (Fig. 3.5, lanes 4, 5, and 6). While adding Ser alone increased the toeprint at the Thr-9 codon, adding both Ser and Thr together eliminated this toeprint [data not shown]. Addition of Gln alone eliminated the toeprint signal corresponding to this site (Fig. 3.5, compare lanes 11 and 13). Thus, the toeprint assay detected elongating ribosomes stalled at codons for which the corresponding amino acid was limiting.

The toeprint signals appeared in a manner consistent with their arising from translocating ribosomes (Fig. 3.5). In extract 1, Thr appears to be most limiting: when Thr was added, the toeprint at the uORF Thr codon disappeared and new toeprints appeared at the downstream Gln codon and at the termination codon. In extract 2, Gln is also highly limiting: when Gln was added, the toeprint at the upstream Thr codon was not affected but the toeprint at the Gln codon disappeared and a new toeprint appeared at the termination codon. Thus, the signal corresponding to the UAA_{uORF} termination codon appears to require ribosomes to translocate to that site.

3.3.3 The Effects of uORF Mutations on the Distribution of Ribosomes

Analyses of toeprint data obtained using a construct lacking the uORF initiation codon (ΔAUG) showed that the signals corresponding to AUG_{uORF} and UAA_{uORF} were missing (Fig. 3.6, lanes 3 and 4 and lanes 11 and 12). Thus, consistent with these toeprint sites arising as a consequence of uORF translation, they were absent when RNA without a translatable uORF was toeprinted. Furthermore, the RNA lacking an uORF initiation codon showed unregulated luciferase synthesis (Fig. 3.2). However, in contrast to results obtained with constructs containing the wild-type uORF, in which the intensity of the toeprint at the luciferase initiation codon was always reduced in high Arg compared to low Arg concentrations, in the ΔAUG construct, the intensity of the toeprint at the luciferase initiation codon sometimes appeared unaffected by high Arg levels [data not shown] or was sometimes reduced by high Arg levels (Fig. 3.6, lanes 11 and 12). The reason for this

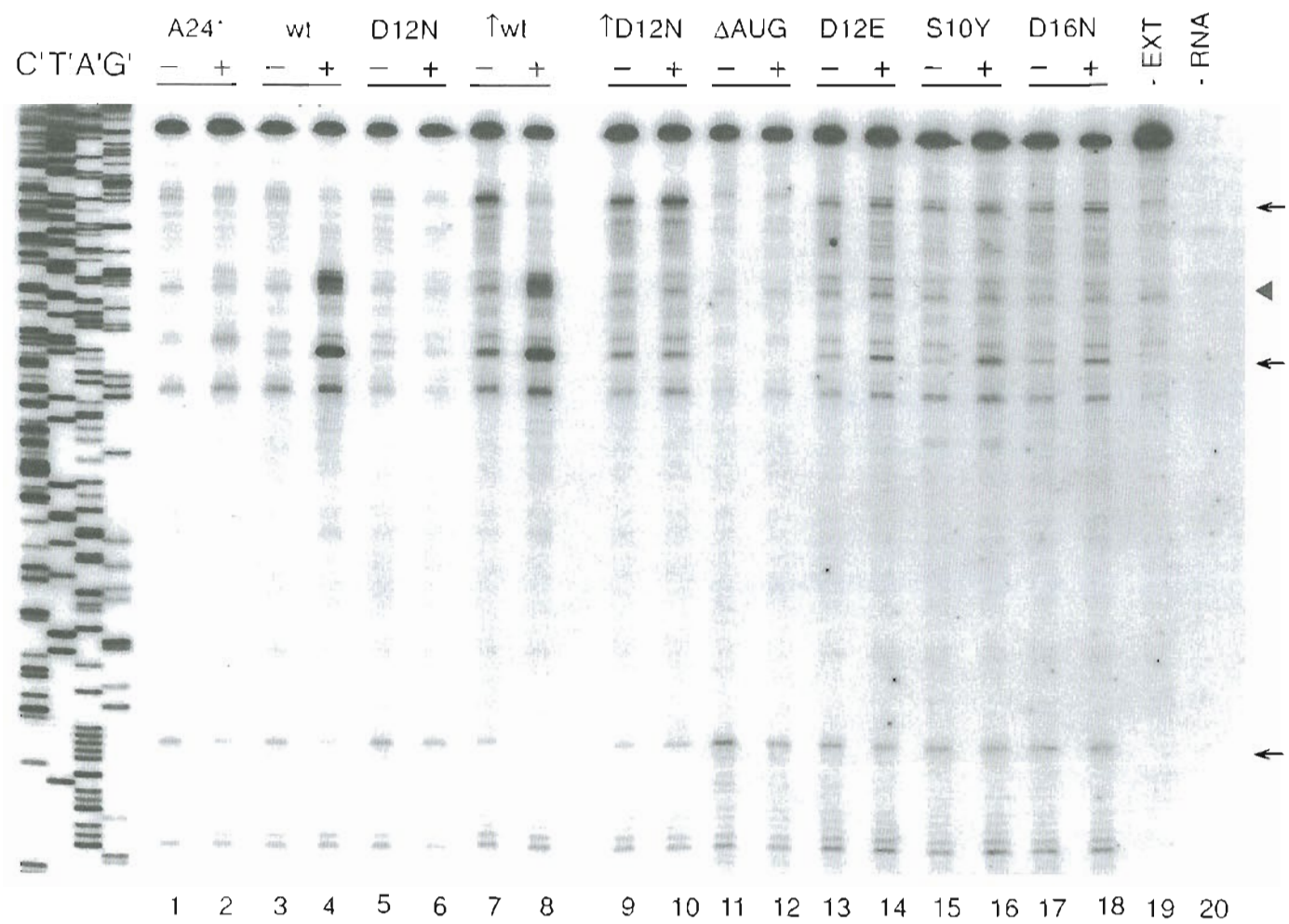

Figure 3.6
Toeprinting reveals Arg-specific, sequence-specific effects. Equal amounts of RNA transcripts (120 ng) were translated in 20-μl reactions containing 10 μM (–) or 500 μM (+) Arg and 10 μM each of the other 19 amino acids. The transcripts examined are indicated at the top and correspond to those assayed in Fig. 3.2. After 20 min of translation, the translation mixtures were toeprinted with primer ZW4. Slightly less sample is present in lane 6.

discrepancy is not known but might reflect effects of Arg not requiring uORF translation (e.g., effects on RNA structure).

The uORF D12N mutation eliminates Arg-specific translational regulation *in vivo* and *in vitro* [Freitag et al., 1996; Wang and Sachs, 1997]. The results of toeprinting RNA containing the D12N uORF revealed that, while toeprints corresponding to ribosomes at the uORF initiation and termination codons were present, Arg-specific differences in toeprint signals were lost (Fig. 3.6, lanes 5 and 6 compared to lanes 3 and 4). This corresponded to the loss of regulation of luciferase synthesis for this RNA (Fig. 3.2). In contrast, a conservative mutation at this codon, from Asp to Glu (D12E), reduced regulation but did not eliminate it, as judged by either assay (Fig. 3.2; Fig. 3.6, lanes 13 and 14).

Both *arg-2* and *CPA1* uORFs contain a second, conserved Asp codon (Fig. 3.1). Mutation of this Asp codon to Asn (D16N) also strongly reduced Arg-specific effects on toeprints (Fig. 3.6, lanes 17 and 18) and eliminated Arg-specific regulation (Fig. 3.2).

Placing the wild-type uORF in a predicted improved initiation context decreases synthesis of luciferase and slightly increases the magnitude of Arg-specific regulation. In contrast, placing the D12N uORF in this improved initiation context decreased synthesis of luciferase, but did not confer Arg-specific regulation (Fig. 3.2) [Wang and Sachs, 1997]. At low Arg levels, improving the translation initiation context for either the wild-type or D12N uORF increased the toeprint at the uORF initiation codon and decreased the toeprint at the *LUC* initiation codon (Fig. 3.6, lanes 7 and 9). At high Arg levels, the wild-type uORF in an improved initiation context showed increased toeprint signals corresponding to ribosomes at the uORF termination site and at the site 21–30 nt upstream of this site and a decreased toeprint signal corresponding to ribosomes at the *LUC* initiation codon (Fig. 3.6, lanes 8 and 7 compared to lanes 4 and 3). These Arg-specific changes were not observed for the D12N uORF in an improved initiation context (Fig. 3.6, lanes 10 and 9). Finally, at high Arg levels, the signal from the uORF initiation codon was reduced for the wild-type uORF in an improved initiation context but not for the D12N uORF in this context (Fig. 3.6, lanes 8 and 10). This may reflect reduced primer extension to this

site arising from increased occupancy by ribosomes of downstream sites in the wild-type uORF at high Arg levels (see Fig. 3.3A) and not diminished occupancy of this site by ribosomes.

The effect of shortening the uORF by one codon, by replacement of the GCA (Ala) codon with a UAA (stop) codon (A24*, Fig. 3.1A), was examined. When compared to the wild-type RNA, the UAA_{uORF} ribosome arrest site in the A24* mutant RNA was shifted exactly 3 nt in the 5′ direction, consistent with the one-codon shortening of the uORF (Fig. 3.6, lanes 1 and 2 compared to 3 and 4). Translation of the A24* RNA at high Arg levels caused only a slight increase in the toeprint signal at the uORF termination codon, in contrast to the large increase observed for the wild-type uORF. Also, the strong signal 21–30 nt upstream of the uORF termination codon observed with wild-type RNA translated at high Arg levels appeared correspondingly diminished, and possibly shifted, in the A24* mutant. Consistent with the reduction in Arg-mediated effects on uORF translation observed in toeprint assays, Arg-specific translational regulation was also reduced by the A24* mutation as determined by luciferase assay (Fig. 3.2).

The *N. crassa* and *M. grisea arg-2* uORFs encode Ser at codon 10; the corresponding codon of the *S. cerevisiae CPA1* uORF is conservatively substituted with Cys (Fig. 3.1B). Mutation of the *CPA1* Cys codon to Tyr eliminates Arg-specific regulation [Werner et al., 1987]; therefore, we examined the effect of the corresponding mutation in the *arg-2* uORF, S10Y (Fig. 3.1A). This mutation eliminated Arg-specific regulation (Fig. 3.2) and largely reduced Arg-specific effects on toeprint signals (Fig. 3.6, lanes 15 and 16). An additional primer extension product obtained with this RNA downstream of the uORF (Fig. 3.6, lanes 15 and 16) was also observed in primer extension reaction mixtures with pure S10Y RNA [data not shown] and does not represent novel interactions between this RNA and extract. Thus, a variety of different mutations that changed the predicted and evolutionarily conserved primary amino acid sequence of the *arg-2* uORF affected Arg-specific regulation.

3.3.4 The Effect of Arg is Rapid

Translation reactions containing wild-type *arg-2–LUC* RNA were initiated with low or high Arg concentrations and samples were examined by toeprint analyses at intervals thereafter (Fig. 3.7A). At either low or high Arg levels, a toeprint corresponding to the uORF termination codon became visible after 1 min of translation and continued to increase in intensity for the next several minutes. The effect of high Arg levels on ribosome stalling at the uORF termination codon was detectable after 3 min and readily discernible after 5 min; it increased with time. The appearance at high Arg levels of the broad toeprint that corresponded to the additional stalled ribosomes translating the uORF paralleled the appearance of the toeprint at the uORF termination codon. The toeprint corresponding to the LUC initiation codon was faint in the autoradiogram shown in Fig. 3.7A but was readily detectable after 30 s of translation through PhosphorImage analysis of this and other gels [data not shown]. The effect of Arg on reducing the toeprint at this site was observed after 5 min of translation, later than when its initial effect on the uORF toeprints was observed. The toeprint corresponding to the uORF initiation codon became detectable after 7 min of translation at low Arg levels and was not detectable at high Arg levels.

The effect of pre-incubating extracts with high Arg concentrations in the absence of *arg-2–LUC* RNA was also examined [data not shown]. Complete reaction mixtures lacking exogenous RNA were incubated for 10 min at 25°C with low or high Arg concentrations; then, at time zero, high Arg concentrations and RNA were added to the reaction mixture preincubated in low Arg concentrations, and RNA was added to the reaction mixture preincubated with high Arg concentrations. The progress of the reactions was monitored as in the experiments in Fig. 3.7A. Prewarming the translation reactions shortened the time required to observe ribosomes at the uORF termination codon to 30 s. However, preincubation with high Arg concentration but without RNA increased neither the rapidity nor the magnitude of the translational response to Arg, relative to preincubation with low Arg concentrations, as measured both by toeprint and luciferase enzyme assays [data not shown]. This suggests but does not prove that the *arg-2* RNA must be present for Arg to exert its effect on the translational machinery.

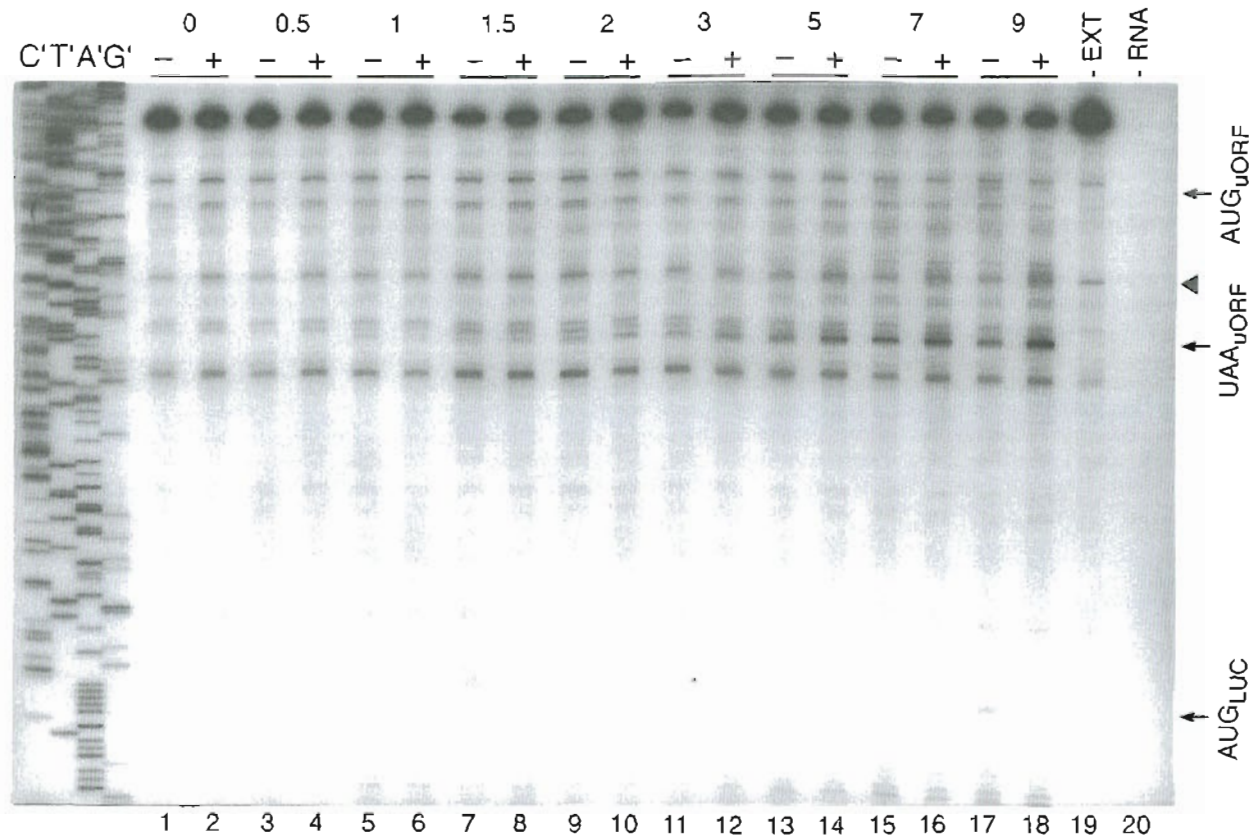

Figure 3.7A Time course analysis of the effect of Arg by toeprinting. Time course of toeprinting in translation reactions initiated at low or high Arg concentrations. RNA transcripts containing the wild-type uORF were added to translation reactions containing 10 μM (–) or 500 μM (+) Arg to final concentrations of 6 ng of transcript/μl. At the time points (0–9 min) indicated, aliquots (3 μl) of reaction mixtures were removed, added to precooled tubes containing reverse transcription buffer, and toeprinted, as described in Section 3.2 and the legend to Fig. 3.4 with primer ZW4.

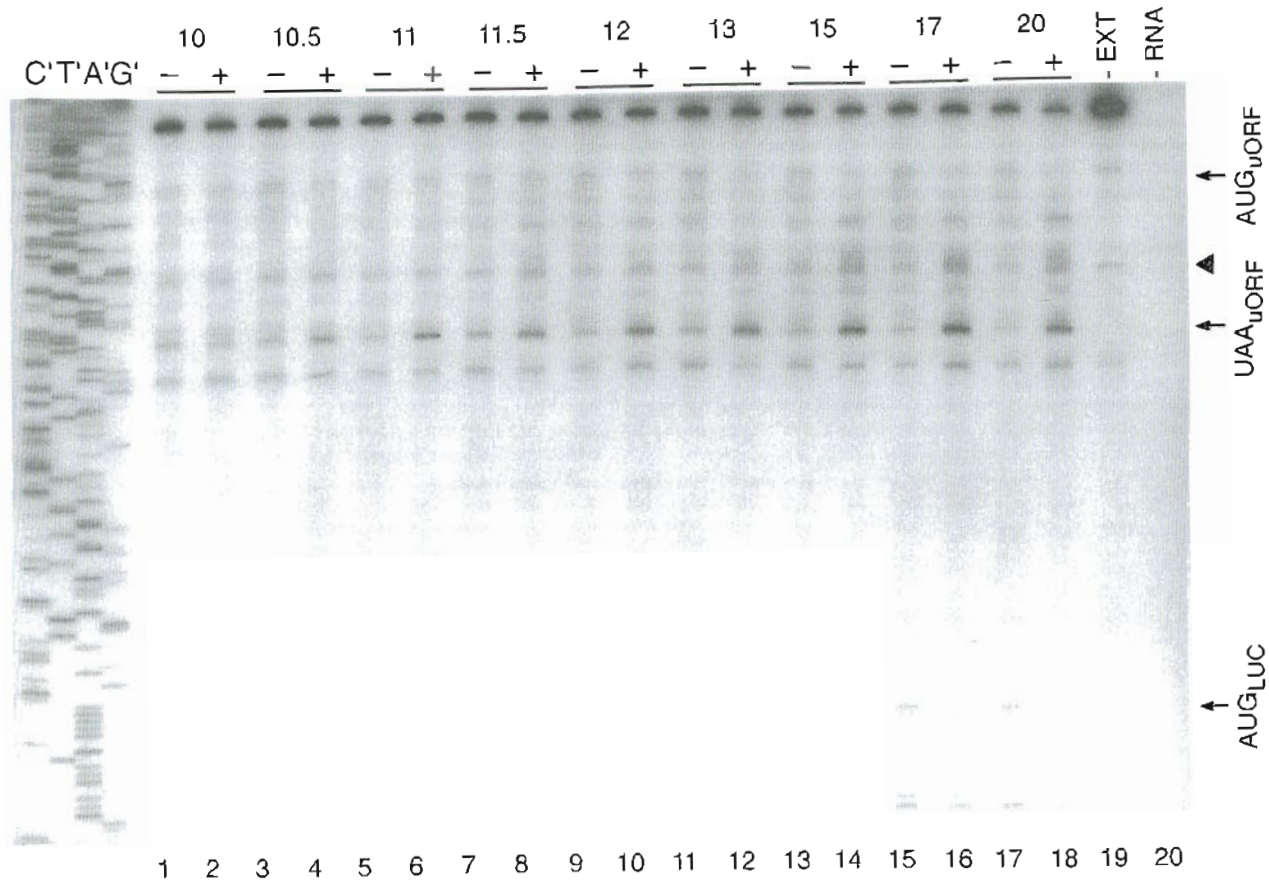

Figure 3.7B Time course analysis of the effect of Arg by toeprinting. Time course analysis of toeprinting on RNA switched from translation at low Arg levels to translation at high Arg levels. RNA transcript containing the wild-type uORF was added to a translation reaction mixtures containing 10 μM Arg (–) and incubated for 10 min as described in for panel A. Then the reaction was split into two tubes, one of which was supplemented with Arg to a final concentration of 500 μM (+). At the time points (10–20 min) indicated, aliquots (3 μl) of the reaction mixtures were removed, added to precooled tubes containing reverse transcription buffer, and toeprinted as described using primer ZW4. The 10-min time point represents the time at which the reaction mixture was divided and extra Arg was added to one portion.

Finally, the effect of high Arg levels on translation reaction mixtures containing low Arg levels that were already translating *arg-2–LUC* RNA for 10 min was examined (Fig. 3.7B). Under these conditions, the effect of adding high Arg levels on ribosome stalling at the uORF termination codon was detectable after 30 s, the earliest time analyzed. Within 1.5 min of adding high Arg, toeprints at both the uORF termination codon and the site 21–30 nt upstream were apparent, but the toeprint signal corresponding to ribosomes at the downstream *LUC* initiation codon was not discernibly reduced until 3 min after addition of high Arg levels. The magnitude of this effect then increased with time. Thus, the effect of Arg on causing ribosomes to stall while translating the *arg-2* uORF appeared to precede its effect on luciferase synthesis.

3.4 Discussion

We used primer extension inhibition (toeprinting) to examine the role of the *N. crassa arg-2* uORF in negative, Arg-specific translational regulation of *arg-2* RNA. Data obtained with an *N. crassa* cell-free system can be interpreted as revealing the presence of ribosomes at a variety of positions on an actively translated RNA. These positions include translation initiation and termination codons and codons for which the cognate amino acid is limiting for translation. This assay presumably resolves ribosomes that have specific codons in their P sites and ribosomes with codons in their A sites (illustrated in Fig. 3.3B). Ribosomes at initiation codons cause toeprints to appear 16 nt downstream from the start codons, whereas ribosomes at a termination codon, or stalled at a codon due to limitation for an amino acid, caused a toeprint to appear 13 nt downstream from that codon. Such differences in the extent of shielding of the RNA may be a consequence of physical differences between the P and A sites in the ribosome [Stark et al., 1997]. The toeprint assay revealed that Arg had substantial effects on translation of the *arg-2* uORF and that the primary sequence of the uORF was critical for this Arg-specific translational control.

3.4.1 Possible Mechanisms for Arg-Specific Translational Control

The translation of leader peptides in amino acid biosynthetic operons of bacteria is essential for regulation by amino acid availability through coupled processes of translation and transcription, now classically known as transcription attenuation [Landick et al., 1996]. In eukaryotes, transcription and translation are not intimately coupled, and eukaryotic ribosomes generally do not bind directly at translation initiation sites but reach these sites by scanning from the 5′ end of the RNA. These differences have ramifications for how the eukaryotic *arg-2* uORF, which in a formal genetic sense is similar to short bacterial leader peptides, must act to attenuate translation from a downstream initiation codon in the presence of high Arg. Toeprinting data obtained using the *Neurospora in vitro* system, in which a high Arg level changes the distribution of ribosomes on *arg-2–LUC* RNA, provides clues into this mechanism.

Do ribosomes reach the initiation codon downstream of the *arg-2* uORF primarily by leaky scanning of the 40S subunit past the uORF or by translation reinitiation following uORF translation? The scanning model posits that 40S ribosomal subunits scan from the 5′ cap of the RNA and initiate translation at the first AUG codon in an mRNA. Leaky scanning, in which the second as well as the first initiation codon in an RNA serves to initiate translation, occurs more frequently when the initiation context for the first AUG is poor [Kozak, 1995]. These conditions hold for *N. crassa arg-2*.

The *in vitro* data presented here indicate that ribosomes do not efficiently reinitiate at the downstream initiation codon following termination of *arg-2* uORF translation. At low Arg concentrations, improving the uORF initiation context increased the association of ribosomes with AUG_{uORF}, decreased their association with AUG_{LUC}, and reduced luciferase translation (Figs. 3.2 and 3.6). Were reinitiation (or internal initiation) important, increased translation of the uORF gained by improving its initiation context should not have been accompanied by decreased translation from the downstream initiation codon. Thus, it appears that most ribosomes reach the downstream initiation codon by leaky scanning. Consistent with this interpretation, analyses of the *in vivo* expression of an *arg-2–lacZ* gene in which the uORF and *lacZ*

coding regions were overlapping led to the conclusion that reinitiation of ribosomes at a downstream start codon was not essential for Arg-specific translational control [Luo and Sachs, 1996]. In yeast, reinitiation following uORF translation also appears to be the exception.

High concentrations of Arg caused an increase in the association of ribosomes with the uORF and a reduction in their association with AUG_{LUC}. High Arg concentrations might directly or indirectly increase initiation at AUG_{uORF}, which would correspondingly decrease the number of ribosomes reaching AUG_{LUC} by leaky scanning. Improving the initiation context of the uORF start codon caused increased loading of ribosomes at that site and decreased luciferase translation, indicating that such a mechanism would be functional. However, the increased association of ribosomes with the uORF at high Arg concentrations did not appear to arise from increased initiation of uORF translation as judged from toeprinting. Rather, Arg caused ribosomes translating the uORF to stall.

Ribosome stalling at the uORF termination codon is measurable within 30 s of adding high Arg concentrations to translation reaction mixtures in which the RNA is actively translating (Fig. 3.7B). This is soon accompanied by the appearance of additional stalled ribosomes in the uORF, and followed within minutes by a reduction in ribosomes at the downstream initiation codon. The relative rate of *in vivo* synthesis of ARG2 is also reduced within minutes of switching cells from minimal to Arg-containing medium [Luo et al., 1995].

Our data suggest the following model for Arg-specific translational control mediated by the *arg-2* uORF. At low Arg concentrations, the movement of scanning preinitiation complexes or translating ribosomes through the uORF is not hindered, and translation initiation at the downstream start codon is relatively high (Fig. 3.8A). At high Arg concentrations, ribosomes stall while translating the uORF. This hinders the movement of scanning preinitiation complexes or other ribosomes translating the uORF, reducing initiation at the downstream start codon (Fig. 3.8B).

An important remaining question concerns whether the ribosomal stall site 21–30 nt upstream of the ribosomal stall at the uORF termination codon represents an independent, Arg-mediated stalling event or occurs as a consequence of Arg-mediated

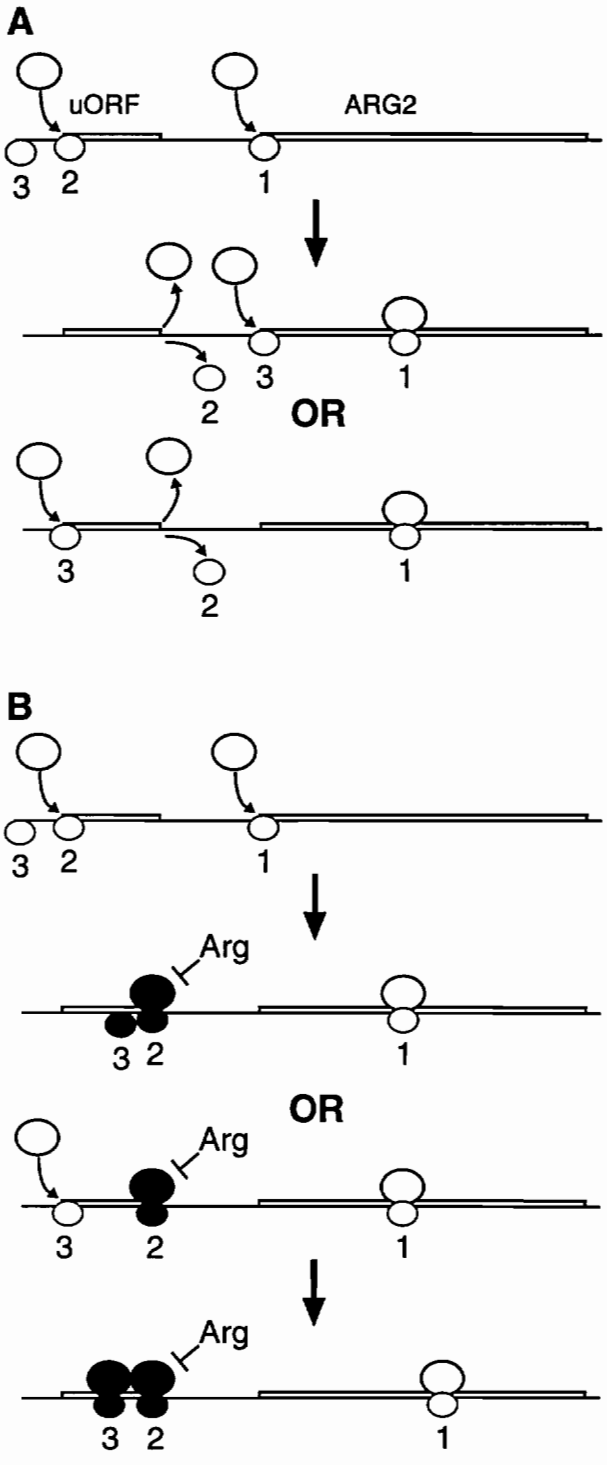

Figure 3.8 A method in which ribosome stalling could mediate Arg-specific attenuation of translation from a downstream start codon. In this model, all ribosomes scan from the 5′ end of the RNA. (A) At low Arg concentrations, ribosomes do not stall at the uORF termination site. 40S ribosomal subunits (ribosomes 1–3) loaded from the 5′ end scan for initiation codons. Ribosome 1 joins a large subunit and initiates translation at the ARG2 start codon; ribosome 2 similarly initiates translation at the uORF start codon; ribosome 3 begins scanning from the 5′ end. As time elapses (thick arrow), ribosome 1 elongates ARG2, ribosome 2 terminates uORF translation and dissociates, and ribosome 3 either scans past the uORF and initiates translation at the downstream start codon or initiates translation at the uORF (shown) and then terminates translation and dissociates (not shown). (B) At high Arg levels, ribosomes stall at the uORF termination site. 40S ribosomal subunits (ribosomes 1–3) loaded from the 5′ end scan for initiation codons as in panel A. As time elapses, ribosome 1 elongates ARG2, ribosome 2 reaches the uORF termination codon but Arg blocks termination and/or dissociation, and ribosme 3 either scans past the uORF initiation codon but its further progress is arrested by stalled ribosome 2 or it initiates translation at the uORF start codon and stalls behind ribosome 2. Hindered movement is indicated by the use of solid symbols. This model predicts that the reduced ability of ribosomes to complete uORF translation at high Arg concentrations prevents ribosomes from loading at the ARG2 initiation codon.

increased stalling at the termination codon. Stacked ribosomes have their centers 27–29 nt apart [Wolin and Walter, 1988]. The position and the broadness of the upstream toeprint cluster (21–30 nt upstream of the termination codon toeprint) is consistent with it arising from ribosomes or scanning 40S ribosomal subunits that stack behind ribosomes stalled at the termination codon. The upstream toeprint might be broad because these complexes would begin to translocate when ribosomes stalled at the termination codon release [as they must for toeprints of upstream ribosomes to be observed by primer extension inhibition (Fig. 3.3A)].

The A24* data (Fig. 3.2 and 3.6) also appear inconsistent with the upstream toeprint representing an independent stalling site. The upstream toeprint corresponds to ribosomes positioned 7–10 codons upstream of the termination codon. Shortening the uORF by one codon at the carboxyl-terminus of the predicted peptide should not affect ribosomes that have not reached this last codon. The effect of the A24* mutation (and all other mutations tested) to alter the toeprints at the upstream and termination sites concomitantly thus suggests that they are not independent. The obvious caveat to this interpretation is that effects on RNA secondary structure might occur in mutant RNAs that fortuitously eliminate the independent upstream stall site. That the stalled upstream ribosome "reins in" the downstream ribosome is also possible although unprecedented. Direct positive evidence to support the linkage of the stall sites, for example by moving the uORF termination codon to a new position so that regulation is retained and determining whether both stall sites move correspondingly, is not available at present.

Toeprints corresponding to hypothetical ribosomes stalled at the two Arg codons in the *arg-2* uORF were not observed. This argues against a regulatory mechanism in which ribosome stalling at these codons at low Arg levelsis important, although such codon-specific stalling is critical in transcription attenuation of bacterial amino acid biosynthetic operons. The absence of Arg codons in the homologous yeast *CPA1* uORF (Fig. 3.1B), whose predicted peptide sequence but not nucleotide sequence appears important for Arg-specific negative regulation [Werner et al., 1987; Delbecq et al., 1994], also implies that uORF Arg codons are not required for Arg-specific regulation.

3.4.2 Relation of *arg-2* uORF Regulation to Other Regulatory Phenomena

uORFs that arrest ribosomes at the stage of translation termination are found in prokaryotes and eukaryotes. A prokaryotic uORF peptide involved in regulating chloramphenicol acetyltransferase inhibits translation termination, possibly by blocking peptidyltransferase [Rogers and Lovett, 1994; Lovett and Rogers, 1996]. Synthesis of the bacterial TnaC leader peptide also regulates gene expression, and events at its termination codon appear important for regulation [Konan and Yanofsky, 1997]. The cytomegalovirus mRNA encoding gp48 contains an uORF whose sequence is critical for translational control [Geballe and Morris, 1994; Geballe, 1996]; biochemical studies indicate that ribosomes stall at the uORF termination codon [Cao and Geballe, 1996a,b]. Control at the termination step of uORF translation also is critical for regulation of *S. cerevisiae GCN4* [Hinnebusch, 1996].

How Arg controls translation of *arg-2* is an important, unanswered question. Control may be exerted directly by the level of the free amino acid (or a closely related metabolite). Arg can interact with the ribosomal peptidyltransferase center and inhibit transpeptidation in a puromycin-based assay [Palacián and Vazquez, 1979]. Conceivably, the interaction of Arg with this center might affect *arg-2* uORF translation. Arg might bind to polypeptides to mediate regulation. The *E. coli* arginine repressor is a DNA-binding protein whose conformation changes when it is bound to Arg corepressor [Maas, 1994]. The crystal structure of the Arg-binding domain complexed to Arg reveals that Arg binds to the protein through Asp residues in the repressor [Van Duyne et al., 1996]. This is interesting because two Asp residues that are conserved in the *arg-2* and *CPA1* uORF peptides are critical for Arg-specific attenuation (Figs. 3.1B, 3.2, and 3.6).

Alternatively, levels of Arg-tRNA^{Arg} charging might effect Arg-regulation. tRNA charging affects uORF-control in bacteria [Landick et al., 1996] and yeast [Hinnebusch, 1996]. If so, effects of tRNA charging must be rapid or require the mRNA, because preincubation of extracts with high Arg concentrations but without *arg-2* uORF-containing RNA did not increase the rapidity or magnitude of its effect. Initial efforts to examine the regulatory effects of depleting extracts of tRNA and/or

of adding exogenous tRNA in the *Neurospora* system have been inconclusive [data not shown].

The negative, Arg-specific regulation conferred by the *arg-2* uORF represents one of the few demonstrated examples in which a eukaryotic uORF modulates translation in response to a specific signal [Hinnebusch, 1996; Lovett and Rogers, 1996; Ruan et al., 1996]. Translation of the *N. crassa arg-2* uORF, whose sequence and function is evolutionarily conserved, appears to be a choke point to control the synthesis of the ARG2 polypeptide and therefore to control flux through the Arg biosynthetic pathway. The common occurrence of uORFs in mRNAs specifying polypeptides important in growth control and development [Geballe, 1996; Harigai et al., 1996] and the recent demonstration that uORFs are critical in tissue-specific regulation of retinoic acid receptor expression [Reynolds et al., 1996] indicate that eukaryotic uORF function will prove to be of general significance.

CHAPTER 4

THE EVOLUTIONARILY CONSERVED EUKARYOTIC ARGININE ATTENUATOR PEPTIDE REGULATES THE MOVEMENT OF RIBOSOMES THAT HAVE TRANSLATED IT*

4.1 Introduction

Short peptide coding regions in the 5′ leaders of prokaryotic mRNAs (leader peptides) and eukaryotic mRNAs [upstream open reading frames (uORFs)] can serve critical regulatory functions. For example, analyses of evolutionarily conserved mechanisms regulating bacterial amino acid biosynthetic gene expression revealed the phenomenon of transcription attenuation, in which the movement of ribosomes over specific leader peptide coding sequences regulates operon expression [Landick et al., 1996]. Studies on fungal genes involved in multiple-pathway control of amino acid biosynthesis, particularly those of *Saccharomyces cerevisiae GCN4*, have revealed the importance of multiple, *cis*-acting uORFs in the gene's mRNA 5′ leader and of modulating the activity of *trans*-acting translation factors in regulation [Hinnebusch, 1997].

The *Neurospora crassa arg-2* gene was one of the first amino acid biosynthetic genes to be identified [Srb and Horowitz, 1944]. It encodes the small subunit of arginine-specific carbamoyl phosphate synthetase. It is unique among *N. crassa* Arg biosynthetic genes in that it is negatively regulated by the concentration of Arg in the

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

cell [Davis, 1986]. The *arg-2* mRNA contains an uORF specifying a 24-residue peptide [Orbach et al., 1990]. The uORF encoding this peptide, henceforth called the *arg-2* arginine attenuator peptide (AAP) because of its involvement in Arg-specific translational regulation, is evolutionarily conserved; similar AAP sequences are encoded by uORFs in all of the other fungal genes specifying this enzyme that have been characterized so far [Werner et al., 1987; Shen and Ebbole, 1997; Baek and Kenerley, 1998].

Elimination of the uORF initiation codon in the *N. crassa arg-2* or the homologous *S. cerevisiae CPA1* transcripts shows that uORF translation is crucial for Arg-specific regulation *in vivo* [Werner et al., 1987; Freitag et al., 1996; Luo and Sachs, 1996]. In *N. crassa*, the *arg-2* uORF reduces translation of ARG2 *in vivo* by reducing the average number of ribosomes associated with the *arg-2* mRNA when Arg is plentiful in the growth medium [Luo et al., 1995]. Arg-specific translational regulation mediated by the *arg-2* uORF has been reconstituted *in vitro* using a homologous cell-free translation system [Wang and Sachs, 1997a]. A primer extension inhibition assay that allowed mapping of ribosomes on RNA at positions corresponding to rate-limiting steps in translation has been developed [Wang and Sachs, 1997b]. Through high-resolution mapping of primer extension products, this toeprint assay enabled the localization of ribosomes on RNAs that are engaged in initiation with AUG codons in their P sites and ribosomes engaged in termination with termination codons in their A sites. Ribosome movement that was slowed during elongation by limitation for specific amino acids became stalled with codons for the limiting amino acid in their A sites [Wang and Sachs, 1997b].

Toeprint assays indicate that a high level of Arg causes ribosomes translating the *arg-2* uORF to stall with its termination codon in the ribosomal A site [Wang and Sachs, 1997b]. This primary event of Arg-regulated stalling of ribosomes is hypothesized to result in Arg-specific negative regulation because the stalled ribosomes block ribosomal scanning from the 5′ end of the mRNA and therefore block trailing ribosomes from reaching the downstream initiation codon. Whether

termination is required for Arg-regulated ribosome stalling remains an important question that is not answered by these studies.

Amino acid residues in the *arg-2* uORF peptide critical for its function in Arg-specific regulation have been identified. For example, changing Asp-12 of the uORF coding region product to Asn (D12N) eliminates Arg-specific translational control *in vivo* [Freitag et al., 1996] and *in vitro* [Wang and Sachs, 1997a,b]. In parallel fashion, mutation of the corresponding Asp residue in the *S. cerevisiae CPA1* uORF eliminates Arg-specific regulation *in vivo* [Werner et al., 1987; Delbecq et al., 1994]. Other mutations in the *N. crassa* and *S. cerevisiae* uORFs that change the predicted amino acid sequence in the evolutionarily conserved region of the uORF peptide also reduce or eliminate Arg-specific translational control [Werner et al., 1987; Luo and Sachs, 1996; Wang and Sachs, 1997b]. When tested in the *N. crassa* cell-free system, loss of regulation is associated with reduced stalling at the uORF termination codon [Wang and Sachs, 1997b].

In this study, we investigated the requirements for *N. crassa arg-2* uORF function in translational regulation by using the *N. crassa* cell-free translation system. We first examined the effects of shortening the distance between the uORF termination codon and the downstream initiation codon. Subsequently, we changed the uORF termination codon, which is normally UAA, to UAG and UGA, to determine whether Arg-specific stalling at the termination codon is codon specific. Finally, to test whether negative translational regulation by Arg requires the uORF termination codon and a downstream initiation codon, we fused functional wild-type AAP and nonfunctional mutant AAP peptides directly to the luciferase (LUC) reporter at the LUC N terminus. The results obtained indicate that AAP-mediated, Arg-specific stalling of ribosomes occurs immediately following AAP translation. Translation of the AAP results in a situation in which the movement of ribosomes involved in either termination or elongation is stalled in response to Arg. While stalling requires a specific nascent peptide sequence, it does not appear to require specific RNA sequences distal to the AAP coding region. The results indicate that the nascent peptide encoded by the *arg-2* uORF modulates the expression of a

downstream gene product by affecting ribosome movement. The regulation of movement of ribosomes involved in either termination or elongation reveals a novel form of eukaryotic translational control.

4.2 Materials and Methods

4.2.1 Construction of Templates for RNA Synthesis

Plasmids were designed to produce capped and polyadenylated synthetic RNA encoding firefly LUC with wild-type or mutant *arg-2* sequences in the RNA 5′ leader region (Fig. 4.1 and Table 4.1). Mutations were introduced into this region by PCR with mutagenic primers (Table 4.1) by using procedures described previously [Freitag et al., 1996]. PCR products were ligated into the pHLUC+NFS4 vector (Table 4.1) [Wang and Sachs, 1997a]. A plasmid designed to produce capped and polyadenylated synthetic RNA which lacked *arg-2* sequences and that encoded sea pansy LUC was constructed by replacing the firefly LUC coding region of pHLUC+NFS4 with the sea pansy LUC coding region of pRL-CMV (Promega).

4.2.2 Cell-Free Translation of RNA and Analyses of Translation Products

Plasmid DNA templates were purified by equilibrium centrifugation and linearized with *Ppu*10I. Capped, polyadenylated RNA was synthesized with T7 RNA polymerase from linearized plasmid DNA templates, and the yield of RNA was quantified as described previously [Wang and Sachs, 1997a]. The preparation of cell translation extracts from *N. crassa* and the reaction conditions for *in vitro* translation were as described previously [Wang and Sachs, 1997a,b].

Translation was halted by freezing reaction mixtures in liquid nitrogen, and aliquots of the ice-thawed mixtures (5 μl) were used for luciferase assays. Luminometric measurements of enzyme activity were performed for 10 s after a programmed 2-s delay. For measurement of firefly LUC enzyme production only, the luciferase assay system (Promega) was used. For measurement of firefly and sea pansy LUC enzymes produced in the same reaction mixture (dual assay), an aliquot of translation mixture added to 50 μl of luciferase assay reagent II (Promega) and the

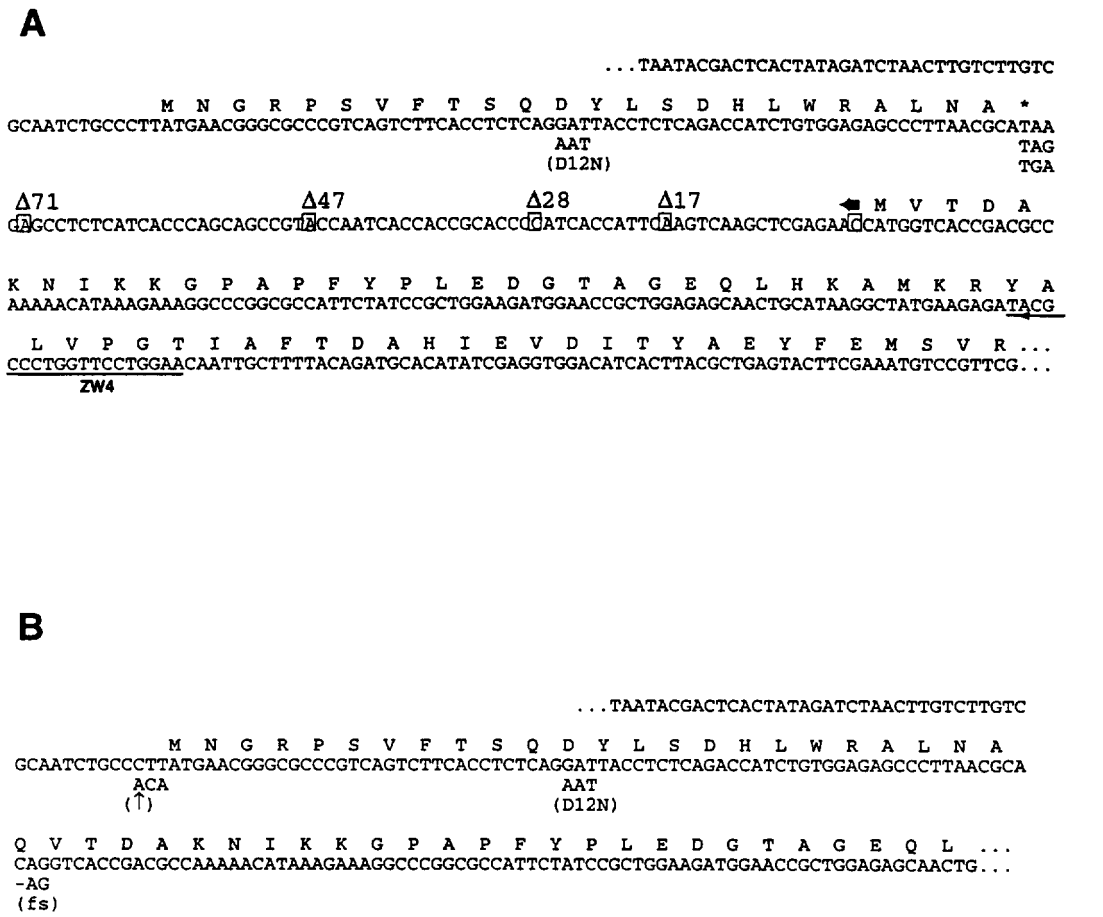

Figure 4.1. The 5′ leader regions of *arg-2-LUC* genes used in this study (see also Table 4.1). (**A**) Sequences of wild-type and mutant templates in which the AAP is encoded by a uORF. The sequence shown begins with the T7 RNA polymerase-binding site and ends within the LUC coding region [Wang and Sachs, 1997b]. The amino acid sequences of the *arg-2* AAP and the N terminus of LUC are indicated. Point mutations are shown below the wild-type sequence. The endpoints of deletion mutations that shorten the intercistronic region are indicated by the boxed nucleotides. All deletions share the 3′ endpoint, which is indicated by a horizontal arrow above the sequence. The extent of each deletion is indicated (e.g., Δ71 removes the greatest number of nucleotides, leaving 5′-CC-3′ between the uORF termination and LUC initiation codons). 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 indicated by ↑ improves the initiation context for uORF translation. The fs mutation is a −1 frameshift in which the first nucleotide of the Gln codon bridging the AAP and LUC coding sequences is deleted.

TABLE 4.1

Firefly Luciferase Constructs Used in This Study

Construct	5′ leader structure[a]	Mutagenic primer[b]	Template	Luciferase activity (−Arg/+Arg)[c]
pPR101	wild-type	-	-	2.13 ±0.14 (4)
pPS101	D12N	-	-	0.98 ±0.03 (3)
pPR105	Δ17	FP6 (CACGC CATGG AATGG TGATG GGGTG CG)	pPR101	2.11 ±0.18 (3)
pPR104	Δ28	FP5 (CACGC CATGG GGTGC GGTGG TGATT GGT)	pPR101	1.97 ±0.11 (3)
pPR103	Δ47	FP4 (CACGC CATGG CGGCT GCTGG GTGAT GAG)	pPR101	1.83 ±0.06 (3)
pPR102	Δ71	FP2 (CACGC CATGG TTATG CGTTA AGGGC TCTCC A)	pPR101	2.29 ±0.11 (3)
pSF103	D12N Δ71	FP2	pPS101	1.02 ±0.10 (2)
pRF102	Δ71-TGA	FP15 (CACGC CATGG TCATG CGTTA AGGGC TCTCC A)	pPR101	2.24 ±0.05 (2)
pSF101	D12N Δ71 TGA	FP15	pPS101	0.95 ±0.01 (2)
pRF103	Δ71-TAG	FP16 (CACGC CATGG CTATG CGTTA AGGGC TCTCC A)	pPR101	2.19 ±0.13 (2)
pSF102	D12N Δ71-TAG	FP16	pPS101	1.02 ±0.04 (2)
pRF107	AAP-LUC	FP3 (CACGG TGACC TGTGC GTTAA GGGCT CTCCA)	pPR101	n.d.
pSF104	D12N AAP-LUC	FP3	pPS101	n.d.
pT1011	↑AAP-LUC		pT101[d]	n.d.
pJC1081	D12N ↑AAP-LUC	FP3	pJC108[e]	n.d.
pPR1071	fs AAP-LUC	FP17 (CACGG TGACC TTGCG TTAAG GGCTC TCCA)	pPR101	n.d.

[a] For details, see legend to Fig. 4.1.

[b] The oligonucleotide sequences are shown in the 5′ to 3′ direction. In all cases, mutagenic primers were paired with the forward primer FP1 (5′-CCGCAAGGAATGGTGCAT-3′), which binds to the vector immediately upstream of the sequence shown in Fig. 4.1, to obtain PCR products from the indicated templates. PCR products were digested with *Bgl*II and *Nco*I (uORF-containing constructs) or *Bgl*II and *Bst*EII (AAP-LUC fusion constructs) for ligation into correspondingly digested pHLUC+NFS4 vector [Wang and Sachs, 1997a]. The *Nco*I site is at codon 1 and the *Bst*EII site is at codon 2 of the LUC coding region in this vector.

[c] The ratio of LUC enzyme activity produced after 30 min in reaction mixtures containing 10 μM Arg to that with 500 μM Arg. Values are the means $\pm$ standard deviations; the numbers of independent experiments analyzed are shown in parentheses. Values for regulation of enzyme synthesis by Arg are given for RNAs containing a uORF specifying the AAP. Analyses of the regulation of AAP-LUC fusion constructs were not done in this way (n.d.); they were analyzed as described in the text.

[d] The *arg-2–LUC* plasmid with the wild-type uORF in the improved initiation context [Wang and Sachs, 1997a].

[e] The *arg-2–LUC* plasmid with the mutant D12N uORF in an improved initiation context [Wang and Sachs, 1997a].

firefly LUC enzyme was measured. Then, 50 μl of Stop & Glo reagent (Promega) was added manually and the sea pansy enzyme was measured.

For [^{35}S]-Met labeling of polypeptides, *N. crassa* extracts were treated with micrococcal nuclease [Wang and Sachs, 1997a]. Quantitation of radiolabeled polypeptides was accomplished by using IPLab Gel and a Molecular Dynamics Phosphorimager to analyze the translation products that were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

4.2.3 Primer Extension Inhibition (Toeprint) Assays

The primer extension assays were accomplished as described by using primer ZW4 [Wang and Sachs, 1997b]; 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 h. All toeprint data shown are representative of multiple experiments.

4.3 Results

4.3.1 Effects of Reducing the Distance Between the uORF Termination Codon and the Downstream Initiation Codon

Wild-type *arg-2* mRNA contains 63 nucleotides (nt) between the uORF stop codon and the ARG2 initiation codon. The *arg-2* uORF coding sequence and the intercistronic region were placed upstream of the sequence coding for firefly LUC (another 10 nt of intergenic vector sequences are also present) (Fig. 4.1). This construct was used to produce capped and polyadenylated synthetic RNA. *In vitro* translation of this RNA in the presence of a high concentration of Arg (500 μM), in contrast to a low concentration of Arg (10 μM), results in an approximately two-fold reduction in LUC polypeptide synthesis (Table 4.1) [see Wang and Sachs, 1997a,b]. The D12N mutation of the AAP coding sequence (Fig. 4.1) eliminates the regulatory effect of the AAP (Table 4.1) [Wang and Sachs, 1997a,b].

Primer extension inhibition analyses of RNA containing the wild-type *arg-2* uORF translated under these conditions have been described previously in detail [Wang and Sachs, 1997b]. The longest primer extension products (Fig. 4.2) represent cDNA extended from the primer to the 5′ end of the synthetic RNA. One set of shorter extension products corresponds to the inhibition of reverse transcription of the RNA template by ribosomes with initiation codons in their P sites (ribosomes at the uORF initiation codon and ribosomes at the LUC initiation codon are indicated in Fig. 4.2). Other shorter extension products correspond to ribosomes with the uORF termination codon in their A sites (Fig. 4.2). A comparison of the translation of the *arg-2–LUC* RNA containing the full intergenic region in reaction mixtures containing a low (10 μM) or high (500 μM) concentration of Arg shows that a high Arg concentration caused a substantial change in the distribution of ribosomes on the RNA (Fig. 4.2, lanes 1 and 2). These Arg-induced changes did not occur when the D12N mutation was present in the AAP (Fig. 4.2, lanes 3 and 4). With the wild-type RNA, Arg increased the signal corresponding to ribosomes at the uORF termination codon and caused a cluster of strong toeprints to appear 21 to 30 nt upstream of the termination codon toeprint. This latter cluster of toeprints appears to represent

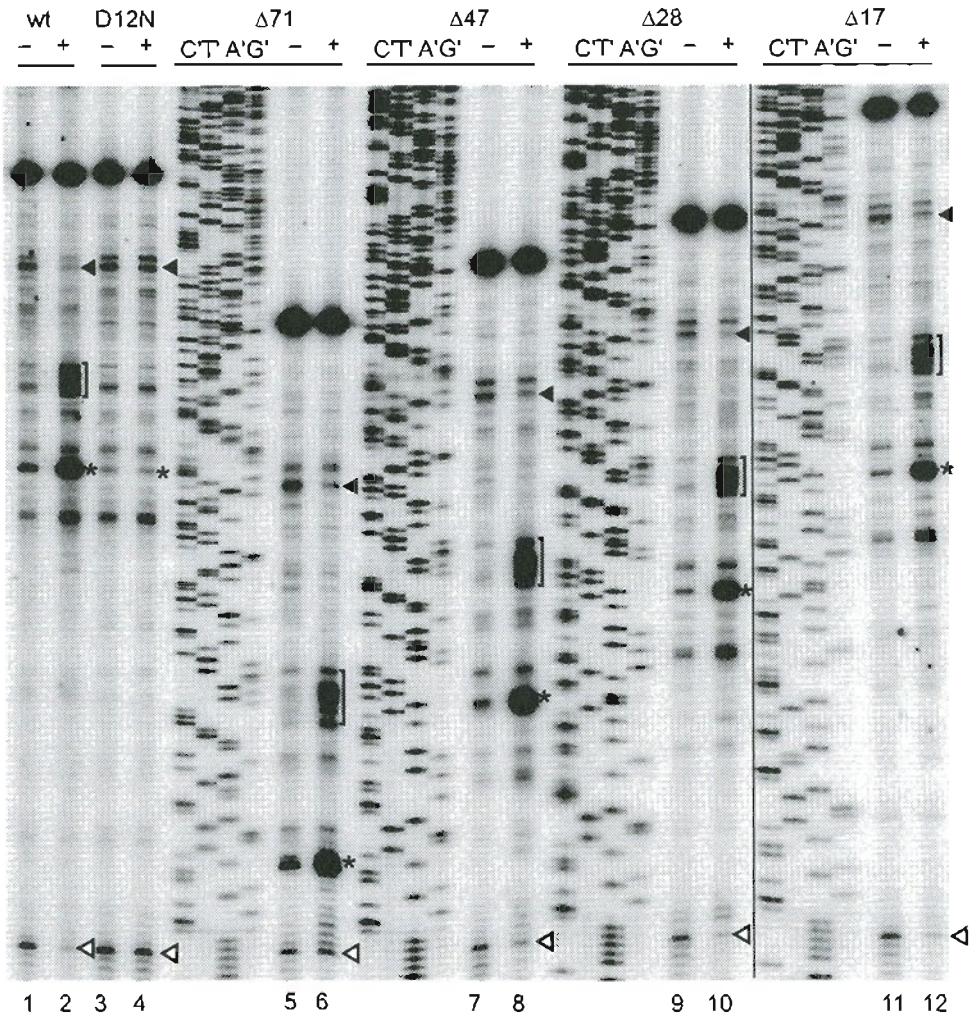

Figure 4.2. Effects of shortening the distance between the uORF termination codon and the downstream LUC initiation codon on Arg-specific regulation. Equal amounts of synthetic RNA transcripts (120 ng) were translated in 20-μl reaction mixtures for 20 min at 25°C. Reaction mixtures contained 10 μM (–) or 500 μM (+) Arg and 10 μM concentration of each of the other 19 amino acids. The transcripts examined are indicated at the top of the lanes. After 20 min of translation, the translation mixtures were toeprinted with primer ZW4 and analyzed next to dideoxynucleotide sequences of the corresponding DNA template. The nucleotide complementary to the dideoxynucleotide added to each reaction mixture is indicated above the corresponding lane so that the sequence of each template can be directly deduced; the 5′-to-3′ sequence reads from top to bottom. The asterisks indicate the positions of premature transcription termination products corresponding to ribosomes at the uORF termination codon; brackets indicate ribosomes stalled behind those at the termination codon. The closed arrowheads indicate ribosomes at the uORF initiation codon; the open arrowheads indicate ribosomes at the LUC initiation codon. wt, wild-type.

ribosomes whose movement is blocked by ribosomes that are stalled at the termination codon [Wang and Sachs, 1997b]. They are likely visible in the toeprint assay because of the dissociation of ribosomes stalled at the termination codon [Wang and Sachs, 1997b].

The addition of Arg reduced the toeprint at the LUC initiation codon, consistent with the reduced translation of LUC as determined by luciferase assay and with the model in which increased stalling of ribosomes at the uORF termination codon decreases ribosomal access to the downstream initiation codon (Fig. 4.2, lanes 1 and 2). The addition of Arg also reduced the toeprint corresponding to ribosomes at the uORF initiation codon. This decreased signal likely arises as a consequence of the primer extension assay and does not represent reduced binding at this site, for reasons discussed previously [Wang and Sachs, 1997b].

To examine whether the sequence or the distance between the uORF and the downstream start codon were important for regulation, a series of constructs (Fig. 4.1) were made in which there were progressive deletions of the region between the uORF termination codon and the downstream LUC initiation codon. Translation of RNA containing deletions of 17, 28, 47, or 71 nt of the 73-nt intercistronic region present in the original construct were examined by luciferase and toeprint assays. Progressive deletion of the intercistronic region had negligible effects on Arg-specific regulation as determined by luciferase activity and toeprint analyses. Translation of LUC in all cases was reduced approximately two-fold by a high concentration of Arg under standard assay conditions, similar to the regulation observed with the full-length intercistronic region (Table 4.1). Toeprint analyses showed that the Arg-specific effects on ribosomes translating the uORF were similar in every case (Fig. 4.2). For all of the mutant mRNAs tested, the addition of Arg increased the toeprint signals corresponding to the uORF termination codon (asterisks), and the appearance of the cluster of toeprints 21 to 30 nt upstream of the toeprint corresponding to the termination codon (brackets), as is observed for the wild-type mRNA. There were few qualitative differences in these signals of each mutant, except for the shortened distance between these signals, as predicted for the size of the deletion.

Consistent with reduced synthesis of LUC, a decrease in the signal corresponding to ribosomes at the LUC initiation codon was observed for most constructs. However, RNA constructs containing the largest deletion, in which only 2 nt separate the uORF termination codon and the LUC initiation codon, did not show a reduced toeprint signal at the LUC initiation codon under high-Arg-concentration conditions (Fig. 4.2, lanes 5 and 6). The reason for this difference is unknown, but it might arise because ribosomes at the nearby termination codon were contributing to the signal at this position.

4.3.2 Effects of Altering the uORF Termination Codon

The uORF termination codon, UAA, appears to be the most frequently used in *N. crassa* [Edelmann and Staben, 1994]. To test whether this specific termination codon was important for Arg-specific regulation, plasmids were constructed to produce synthetic RNA in which the wild-type or D12N mutant uORFs were terminated with UAA, UAG, or UGA codons. With the wild-type uORF, all three termination codons showed similar levels of Arg-specific regulation as determined by luciferase activity assays (Table 4.1, constructs pPR102, pRF102, and pRF103). Comparisons of toeprint assays of each RNA translated in reaction mixtures containing either low or high Arg concentrations indicated that the behaviors of ribosomes at each of these termination codons was indistinguishable (Fig. 4.3). Regardless of which uORF termination codon was present, the D12N mutant uORF conferred no Arg-specific regulation (Table 4.1 and Fig. 4.3). Therefore, while the sequence of the uORF coding region is important for regulation, the type of termination codon used to stop uORF translation did not appear to be important.

4.3.3 Effects of Fusing the *arg-2* uORF Peptide Directly to Luciferase

The experiments described above indicate that the function of the *arg-2* uORF in regulation was retained regardless of the distance between its termination codon and the downstream initiation codon and regardless of which termination codon was used to terminate uORF translation. Therefore, we next investigated whether the termination of uORF peptide synthesis followed by the initiation of new polypeptide

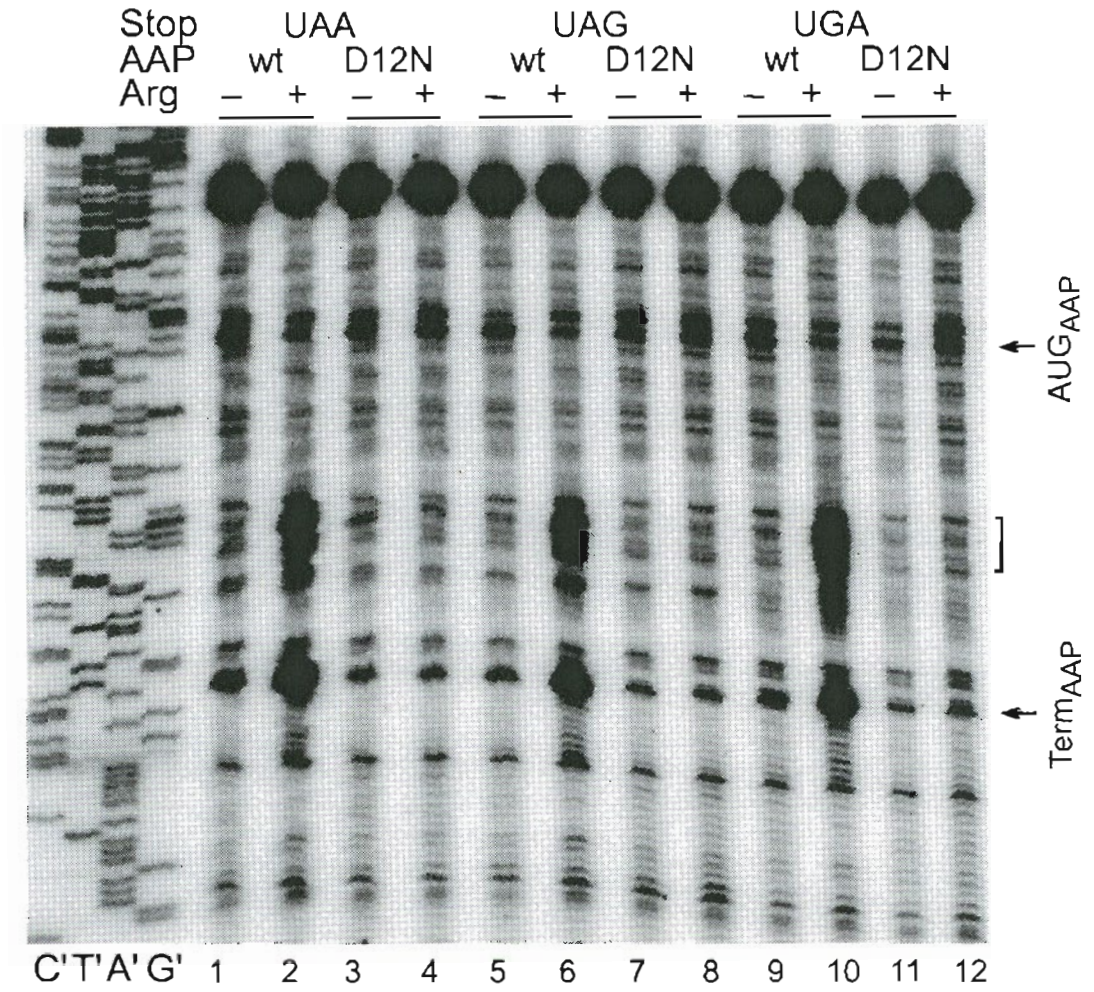

Figure 4.3. Effects of terminating uORF peptide synthesis with each of the three termination codons, UAA (lanes 1 to 4), UAG (lanes 5 to 8), and UGA (lanes 9 to 12), on Arg-specific regulation. Equal amounts of synthetic RNA transcripts (120 ng) were translated in 20-μl reaction mixtures for 20 min at 25°C. Reaction mixtures contained 10 μM (–) or 500 μM (+) Arg and a 10 μM concentration of each of the other 19 amino acids; they were analyzed by toeprinting as described in the legend to Fig. 4.2. The RNAs are from Δ71 deletion constructs containing either the wild-type (wt) or D12N mutant uORFs terminated with UAA, UAG, or UGA codons (Fig. 4.1 and Table 4.1) as indicated at the top of the lanes. Dideoxynucleotide sequencing reactions for the pPR102 template containing the wild-type uORF terminated with UAA are shown on the left (lanes C′, T′, A′, and G′). Arrows indicate the positions of premature transcription termination products corresponding to ribosomes bound at the uORF initiation codon (AUG_{AAP}) and the uORF termination codon ($Term_{AAP}$); brackets indicate the positions of ribosomes stalled behind those at the termination codon.

synthesis at a downstream start codon was necessary for AAP-mediated translational regulation. Constructs were made in which the normal LUC initiation codon was eliminated and the wild-type AAP coding region was fused directly to the LUC coding region. In such constructs, the AAP initiation codon would be responsible for initiating translation of an AAP-LUC fusion polypeptide.

Three different AAP-LUC fusion constructs containing the wild-type AAP sequence were examined (Fig. 4.1B). The first contained the AAP initiation codon in its wild-type context, which is relatively inefficient at capturing ribosomes to initiate translation. The second contained the AAP initiation codon in an improved initiation context that is relatively efficient at capturing ribosomes to initiate translation [Wang and Sachs, 1997a,b]. The final construct contained AAP and LUC coding regions deliberately fused out-of-frame with respect to each other, so that, in contrast to the first two constructs, the LUC polypeptide will not be produced by initiation at the AAP initiation codon.

Evidence that these constructs produced fusion polypeptides as predicted was obtained by programming micrococcal nuclease-treated *N. crassa* translation reaction mixtures with equal amounts of each of the RNAs encoding the fusions and examining [^{35}S]methionine incorporation into translation products (Fig. 4.4). RNA encoding normal firefly LUC polypeptide produced a major translation product whose migration in SDS-PAGE was consistent with its predicted size of 551 residues (Fig. 4.4, lane 2). RNA encoding the AAP-LUC fusion polypeptide in its normal initiation context produced a major translation product whose migration in SDS-PAGE was consistent with its predicted size of 574 residues (Fig. 4.4, lane 3). Improving the AAP-LUC translation initiation context increased the level of the fusion polypeptide (Fig. 4.4, lane 4). RNA in which the AAP coding region was fused out-of-frame with the LUC coding region did not produce this large fusion polypeptide (Fig. 4.4, lane 5), as expected. Consistent with these results, measurements of LUC production by enzyme assay showed that, after 30 min of translation, improving the AAP initiation codon increased the level of LUC translation approximately 4-fold and frameshifting reduced the level of LUC translation more than 50-fold [data not shown]. These results indicated that, in RNAs encoding AAP-LUC fusion polypeptides, the AAP initiation

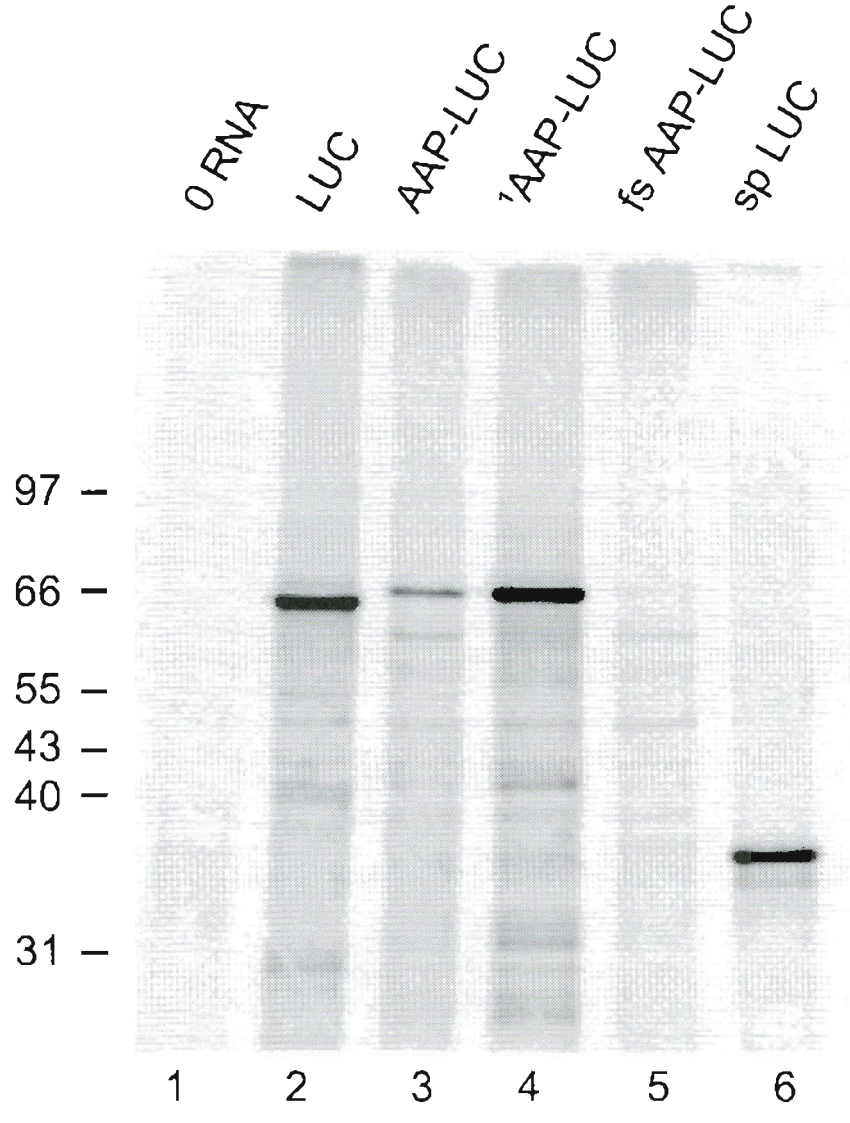

Figure 4.4. Analyses of [^{35}S]methionine-labeled polypeptides produced by translation of synthetic RNA transcripts in *N. crassa* cell extracts. Micrococcal nuclease-treated *N. crassa* extracts (20 μl containing 2 μCi of [^{35}S]methionine) were programmed with 120 ng of the indicated RNAs and incubated for 30 min at 25°C. Reactions were stopped by immersing the tube in liquid nitrogen and examined by SDS-PAGE in 10% polyacrylamide gels. Radiolabeled translation products were visualized by phosphorimaging; the positions of molecular mass markers (in kilodaltons) visualized by staining with Coomassie blue are indicated on the left. Lane: 1, reaction mixture with no added RNA; 2, with RNA encoding firefly LUC; 3, with RNA encoding the AAP-LUC fusion polypeptide in the wild-type initiation context; 4, with RNA encoding the AAP-LUC fusion polypeptide in the improved initiation context; 5, with RNA encoding the AAP coding region frameshifted (fs) with respect to the LUC coding region; 6, with RNA encoding sea pansy (sp) LUC.

codon was primarily responsible for initiating translation of active luciferase enzyme *in vitro*.

We examined the effect of adding 10 or 500 μM Arg to translation reaction mixtures on the synthesis of wild-type AAP-LUC or mutant D12N AAP-LUC fusion polypeptides. Each reaction mixture contained a second capped and polyadenylated RNA specifying sea pansy LUC as an internal control. This RNA lacked *arg-2* regulatory sequences. Sea pansy LUC is smaller (311 amino acids) (Fig. 4.4, lane 6) than the firefly enzyme, and it uses a different substrate to produce light.

The rates of production of sea pansy LUC were similar in all reaction mixtures (Fig. 4.5). This indicated that translation of this RNA was unaffected by these levels of Arg. Analyses of the time course of LUC production (Fig. 4.5) revealed that, in mixtures containing low or high Arg concentrations, the appearance of completed functional sea pansy enzyme synthesis preceded the appearance of firefly enzyme synthesis, consistent with its smaller size. Therefore, excluding protein folding considerations, on the basis of the differences in the sizes of the sea pansy and firefly LUC enzymes and the rates of first appearance of functional enzymes, the elongation rate in the reaction mixtures under these conditions can be estimated to be approximately 1 amino acid per s, comparable to that of other eukaryotic cell-free systems [Federov and Baldwin, 1998]. In contrast, translation of LUC from RNA encoding the wild-type AAP fused to LUC but not the D12N mutant AAP fused to LUC was reduced under high concentrations of Arg (Fig. 4.5). This indicated that the wild-type AAP at the N terminus of a fusion polypeptide functioned to reduce translation and therefore that it functioned in the absence of a termination codon and a downstream initiation codon. In addition, this Arg-responsive AAP activity was *cis*-acting: while it affected translation of RNA encoding the firefly enzyme, it did not affect translation of RNA encoding sea pansy LUC in the same reaction mixtures.

The effect of Arg on the translation of RNA specifying the AAP-LUC fusion was qualitatively different from its effect when the RNA specified the AAP as a uORF product and LUC as a separate, downstream coding region product with its own translation initiation site (Fig. 4.6). Arg substantially delayed the first appearance of enzymatically active AAP-LUC fusion polypeptide and subsequently

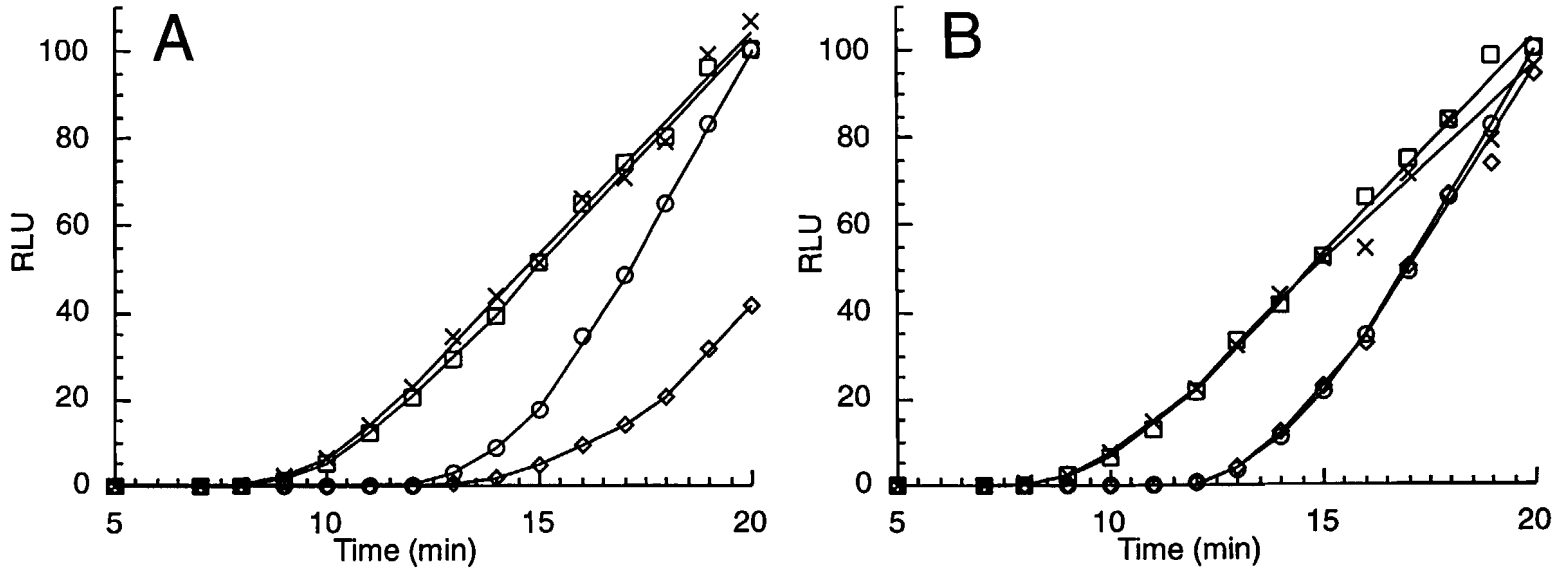

Figure 4.5 Time courses of translation from RNAs encoding AAP-LUC, D12N AAP-LUC, and sea pansy LUC enzymes in reaction mixtures containing 10 or 500 μM Arg. Translation in reaction mixtures (120 μl) was initiated by using a mixture of RNAs: 150 ng of RNA encoding the wild-type AAP-LUC fusion and 36 ng of RNA encoding sea pansy LUC (**A**) or 150 ng of RNA encoding the D12N AAP-LUC fusion and 36 ng of RNA encoding sea pansy LUC (**B**). Reaction mixtures were incubated at 25°C and contained either 10 or 500 μM Arg and a 10 μM concentration of each of the other 19 amino acids. Firefly LUC production [in reaction mixtures containing 10 (○) or 500 (◇) μM Arg] and sea pansy LUC production [in reaction mixtures containing 10 (□) or 500 (×) μM Arg] were determined by using the dual luciferase assay as described in Section 4.2. RW, relative light units.

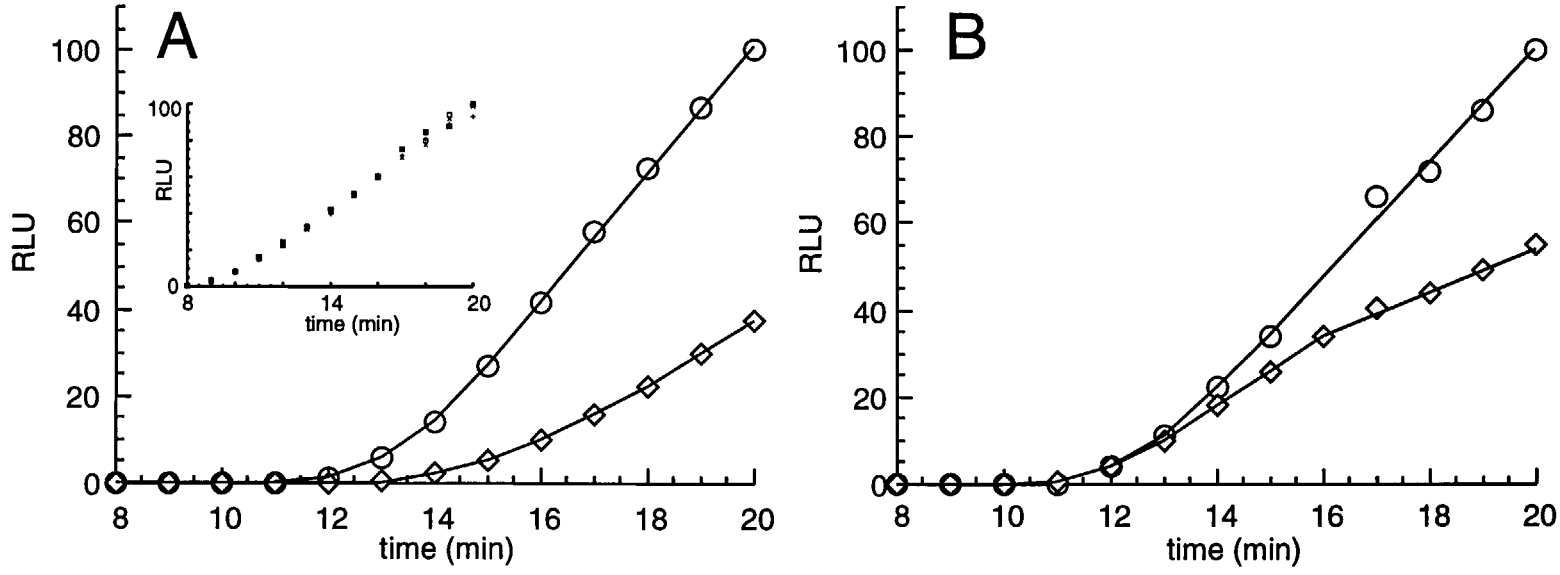

Figure 4.6 Time course of translation of RNAs encoding the wild-type AAP as an N-terminal domain and as a uORF product. Reaction conditions were as described in the legend to Fig. 4.5. (**A**) Results with 150 ng of RNA encoding the wild-type AAP-LUC fusion and 36 ng of RNA encoding sea pansy LUC; (**B**) results with 150 ng of RNA encoding the wild-type AAP as a uORF region and LUC as a separate downstream coding region with its own initiation codon and 36 ng of RNA encoding sea pansy LUC. The inset in panel A shows the combined data for translation of the internal control sea pansy LUC RNA in the four reaction mixtures plotted in panels A and B. Firefly luciferase production [in reaction mixtures containing 10 (○) or 500 (◇) μM Arg] and sea pansy LUC production were determined using the dual luciferase assay as described in Section 4.2. RLU, relative light units.

lowered the rate of accumulation of LUC (Fig. 4.6A). In parallel reactions, Arg did not delay the first appearance of enzymatically active LUC when the AAP was present as a uORF product, but subsequently lowered the rate of accumulation of LUC (Fig. 4.6B). This delay in the synthesis of LUC in translation reactions containing a high instead of a low concentration of Arg and programmed with RNA specifying the AAP at the N terminus of LUC but not RNA specifying the AAP as a separate reading frame product upstream of LUC was highly reproducible, and the length of this delay was extended when translation reaction mixtures were incubated at lower temperatures [data not shown]. These differences were also apparent when fusion and uORF constructs containing the AAP in an improved initiation context were compared [data not shown]. In these cases, the magnitude of the regulatory effect of Arg increased [data not shown], consistent with the model for regulation in which Arg-stalled ribosomes impede trailing ribosome traffic.

Toeprint analyses were used to examine the effects of Arg on the translation of RNAs specifying the wild-type AAP-LUC and the D12N AAP-LUC polypeptides following initiation at AUG codons in improved and wild-type initiation contexts (Fig. 4.7). The RNA coding for AAP-LUC in the wild-type initiation context was toeprinted in the absence of extract (Fig. 4.7, lane 9) as a control. As expected, the primer extension product was predominantly cDNA fully extended to the 5′ end of the RNA template. Also as expected, when toeprint signals from an extract lacking RNA coding for AAP-LUC were examined, near-negligible quantities of primer extension products were observed (Fig. 4.7, lane 10). Analyses of the wild-type AAP-LUC-specifying RNA translated in translation mixtures containing a low rather than a high Arg concentration (Fig. 4.7, compare lanes 5 and 6) revealed that a high concentration of Arg substantially increased the intensity of a series of toeprints. One of these corresponded to ribosomes translating the first codon following the AAP coding sequence (as borne out by experiments with the translation inhibitor puromycin as described below). Thus, this stall site in the fusion polypeptide corresponded in position, relative to the AAP coding sequence, to the uORF termination codon. This toeprint site was followed closely by another Arg-induced cluster of toeprints corresponding to ribosomes stalled in the nearby, downstream LUC coding region.

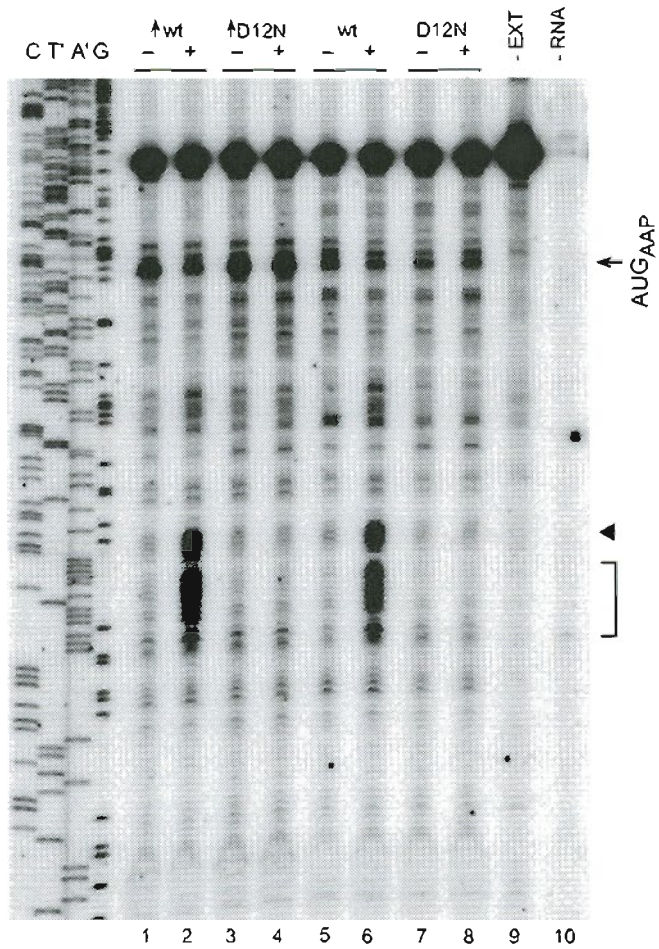

Figure 4.7 Effects of mutations on Arg-specific regulation of AAP-LUC fusion constructs. Equal amounts of synthetic RNA transcripts (120 ng) were translated in reaction mixtures and analyzed by toeprinting as described in the legend to Fig. 4.2. The transcripts encoded the wild-type (wt) AAP-LUC fusion or the D12N mutant AAP-LUC fusion as indicated in either the wild-type or improved (↑) initiation contexts. Dideoxynucleotide sequencing reactions for the template containing the wild-type AAP-LUC fusion are on the left (lanes C′, T′, A′, and G′). The products obtained from primer extension of pure AAP-LUC RNA (18 ng) in the absence of translation reaction mixture (-EXT; lane 9) and from a translation reaction mixture not programmed with RNA (-RNA; lane 10) are shown for comparison. The arrow indicates the position of the premature transcription termination products corresponding to ribosomes bound at the AAP initiation codon (AUG_{AAP}). The arrowhead indicates the position of premature termination products corresponding to ribosomes stalled in the presence of a high level of Arg at the codon immediately following the 24 codons of the AAP. The bracket indicates the position of premature termination products corresponding to ribosomes stalled in the presence of a high level of Arg in the LUC coding region.

The D12N mutation eliminated these Arg-specific effects on toeprints (Fig. 4.7, compare lanes 7 and 8). These data indicate that AAP-mediated stalling can occur at one or more sites immediately distal to the AAP coding region.

Improving the context of the initiation codon for AAP-LUC fusion polypeptide would be expected to increase translation by the more efficient capturing of scanning ribosomes. Consistent with this, we observed increased LUC translation from this RNA as determined by enzyme assay, as described above. Improving the initiation context also increased the toeprint signal corresponding to ribosomes initiating synthesis of the AAP-LUC polypeptide. This effect was observed for RNAs encoding both wild-type and D12N mutant fusions (Fig. 4.7, lanes 1 to 4). Addition of Arg reduced the toeprint corresponding to ribosomes at the wild-type AAP-LUC initiation codon in either context but not the D12N AAP-LUC initiation codon. This decreased signal in the wild-type cases may be a consequence of the assay procedure [Wang and Sachs, 1997b].

To verify that the Arg-specific toeprints corresponded to ribosomes, we examined the effect of puromycin, which releases ribosomes from mRNA, on these signals (Fig. 4.8). Translation reaction mixtures containing RNA encoding the wild-type AAP-LUC and either low or high concentrations of Arg were incubated for 20 min. Then, either water (negative control) or puromycin was added and the mixtures were incubated for an additional 5 min. Puromycin caused the loss of Arg-regulated toeprints (Fig. 4.8, lane 4), indicating that these signals corresponded to ribosomes. Puromycin did not release ribosomes from the AAP-LUC initiation codon as judged by toeprinting; this lack of an effect of puromycin on toeprints corresponding to initiation codons was observed previously [Wang and Sachs, 1997b]. The toeprint pattern obtained from RNA coding for wild-type AAP-LUC with puromycin resembled the pattern obtained from RNA coding for D12N AAP-LUC in the absence of drug in that no stalled ribosomes are detected in the region downstream of the AAP region. This indicates that the toeprint assay is detecting ribosomes stalling in response to Arg that is specific to ribosomes which have synthesized the wild-type AAP.

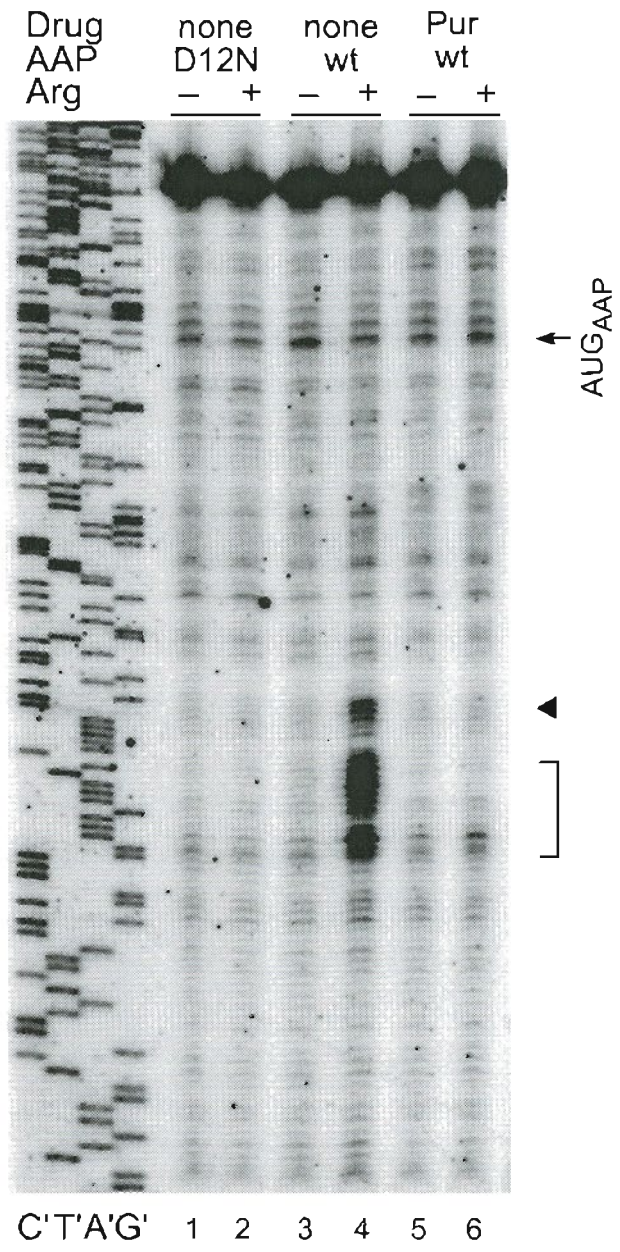

Figure 4.8 Effects of puromycin on Arg-specific regulation of AAP-LUC fusion constructs. Equal amounts of synthetic RNA transcripts (120 ng) were translated in reaction mixtures and analyzed by toeprinting as described in the legend to Fig. 4.2. The transcripts encoded either the D12N mutant AAP-LUC fusion or the wild-type (wt) AAP-LUC fusion as indicated. Puromycin (Pur) was added where indicated, as described in the text. Dideoxynucleotide sequencing reactions for the template containing the wild-type AAP-LUC fusion are on the left (lanes C′, T′, A′, and G′). The arrow indicates the position of the premature transcription termination products corresponding to ribosomes bound at the AAP initiation codon (AUG_{AAP}). The arrowhead indicates the position of premature termination products corresponding to ribosomes stalled in the presence of a high level of Arg at the codon immediately following the 24 codons of the AAP. The bracket indicates the position of premature termination products corresponding to ribosomes stalled in the presence of a high level of Arg in the LUC coding region.

4.4 Discussion

The leader region of the *N. crassa arg-2* mRNA contains a 24-residue, evolutionarily conserved uORF encoding the AAP. Translation of the AAP coding region appears necessary for Arg-regulated stalling of ribosomes. We observed that translational control through the AAP is *cis*-acting and does not appear to absolutely require specific downstream translational events to occur. Shortening the distance between the uORF termination codon and the downstream LUC initiation codon did not affect Arg-regulated stalling at the uORF termination codon or the extent of negative regulation conferred on translation initiated at the LUC initiation codon. Changing the uORF termination codon from UAA to UAG or UGA did not affect regulation. Indeed, negative translational regulation was observed when the AAP coding region was fused in-frame directly to the LUC coding region. The data suggest that the nascent AAP acts in *cis* within the ribosome that has translated it to cause stalling regardless of whether the ribosome is subsequently engaged in termination or elongation.

The regulatory function of the AAP to stall ribosomes was examined in two ways: by examining the kinetics of Arg-specific regulation in the *N. crassa* cell-free translation system and by mapping the positions of ribosomes on RNA in this system by using a primer extension inhibition (toeprint) assay. When the AAP is encoded by a uORF, the time of initial appearance of functional LUC enzyme (whose synthesis is initiated from a downstream start codon) is the same regardless of whether the reaction mixture contains a low or high concentration of Arg (Fig. 4.6B). The negative effect of Arg becomes apparent only later, when it reduces the rate of LUC enzyme accumulation. These data are consistent with the proposed model for regulation [Wang and Sachs, 1997b] mediated by the *arg-2* uORF in which some of the first scanning ribosomal subunits loaded on the mRNA leak past the codon initiating AAP and initiate instead at the LUC coding region regardless of whether the level of Arg is high or low. In this model, the time at which the first functional LUC polypeptide is translated from such an mRNA pool is not affected by Arg, but Arg reduces LUC polypeptide synthesis subsequently.

In contrast to the situation in which the AAP is encoded by a uORF, for RNA encoding the AAP-LUC fusion polypeptide, ribosomes initiating at the AAP codon are directly responsible for LUC synthesis, as judged by analysis of radiolabeled LUC polypeptide and LUC enzyme activity produced from such RNAs. Thus, all of the ribosomes that initiate LUC synthesis necessarily begin at the AAP start codon. Since each of these ribosomes must translate the AAP, they will be subject to AAP-mediated, Arg-specific ribosome stalling. Therefore, it would be predicted that the time of the first appearance of AAP-LUC fusion polypeptide would be delayed by Arg. This is what is observed (Fig. 4.6A).

The toeprint data (Figs. 4.2 and 4.3) indicate that translation of the wild-type AAP causes ribosomes engaged at termination codons to stall, which has parallels with other uORFs whose peptide coding sequences are important for controlling translation [Lovett and Rogers, 1996; Wang and Sachs, 1997b; Cao and Geballe, 1998]. In addition, the AAP also causes ribosomes engaged in elongation to stall (Figs. 4.7 and 4.8). The data indicate that stalling can occur in a short region of RNA following the AAP coding region. Previous considerations of how the movement of eukaryotic ribosomes involved in elongation is translationally controlled have focused on the physical structure of the RNA or on the limitation for charged tRNA. Secondary structures in the RNA have been implicated in the blockade of scanning 40S ribosomal subunits [Koloteva et al., 1997] and in the slowing of translating 80S ribosomes [Thanaraj and Argos, 1996]. The presence of pseudoknots or rare codons in the RNA are implicated in ribosome frameshifting [Farabaugh, 1996]. It has been hypothesized that rare codons affect ribosome stalling and allows time for the correct folding of nascent domains of a protein containing multiple domains [Komar and Jaenicke, 1995; Képès, 1996; Thanaraj and Argos, 1996].

The relative importance of different *cis*-acting sequences for *N. crassa arg-2* AAP-mediated regulation deduced by using a cell-free translation system from *Neurospora* correlates with observations on *CPA1* regulatory sequence effects on reporter gene expression *in vivo* in *S. cerevisiae* [Delbecq et al., 1994]. In these studies, the activity of the enzyme encoded by the reporter β-galactosidase gene was used to measure regulation; neither the distribution of ribosomes on RNA nor the

levels of RNA were measured. Therefore, it was not possible to assess the relative contributions of Arg-specific translational control and Arg-specific effects on *CPA1* transcription and *CPA1* transcript stability [Crabeel et al., 1990]. Nevertheless, these observations on regulation *in vivo* are entirely consistent with observations on regulation in the cell-free translation system. Changing the distance between the *CPA1* uORF termination codon and the downstream initiation codon did not alter Arg-specific regulation [Delbecq et al., 1994]. When the full-length *CPA1*-encoded AAP was fused directly to the β-galactosidase reporter gene product, Arg-specific regulation was retained [Delbecq et al., 1994]. These results are also consistent with the *in vitro* studies on *N. crassa arg-2*, although, in addition to the caveats listed above, a deliberately frameshifted construct was not tested *in vivo* to rule out the possibility that another initiation codon than that predicted was used for translation of the functional reporter.

The results presented here indicate that the movement of ribosomes involved in termination or elongation can be regulated by the nascent peptide being produced. The strong conservation of the AAP amino acid sequence among different fungi, with nucleotide differences in the coding region primarily in silent positions, and the demonstrated importance of the evolutionarily conserved peptide sequence for the regulatory function of stalling ribosomes in the *Neurospora* system indicate a primary role for the nascent peptide. Specific RNA sequences may also contribute to this regulatory process by other means than their capacity to encode polypeptide sequence. No evidence that identifies such sequences has as yet been obtained.

The observation that translation of the AAP can regulate the movement of ribosomes involved in elongation provides evidence for a novel form of eukaryotic translational control. In bacterial systems, there are precedents for the stalling of ribosomes involved in elongation mediated by nascent peptides [Lovett and Rogers, 1996]. Nascent peptides can also have other effects on ribosomes involved in elongation: ribosome jumping in the translation of T4 gene 60 requires a 16-residue region of the nascent peptide [Larsen et al., 1995]. In eukaryotes, N-terminal nascent peptide domains can interact with the signal recognition particle [Walter and Johnson, 1994], which halts ribosome movement until docking with membrane of the

endoplasmic reticulum. Interestingly, recent evidence shows that the nascent signal polypeptide interacts with the ribosome before it exits the ribosome [Liao et al., 1997].

Taken together, all of the available data indicate that translation of the AAP is an evolutionarily conserved event of primary importance for regulation. AAP-mediated translational regulation represents an unusual instance in which a nascent peptide appears to regulate the movement of ribosomes.

CHAPTER 5

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

5.1 Introduction

Upstream open reading frames (uORFs) in the 5′-leader regions of eukaryotic and prokaryotic transcripts can serve critical regulatory functions [Geballe, 1996; Lovett and Rogers, 1996; Jackson and Wickens, 1997; Konan and Yanofsky, 1999]. The fungal mRNAs specifying the small subunit of carbamoyl phosphate synthetase contain a uORF encoding an evolutionarily conserved peptide (Fig. 5.1A). In the cases of *Saccharomyces cerevisiae CPA1* and *Neurospora crassa arg-2*, the capacity to translate this uORF peptide is essential for establishing Arg-specific negative regulation of gene expression *in vivo* [Werner et al., 1987; Delbecq et al., 1994; Luo et al., 1995; Freitag et al., 1996; Luo and Sachs, 1996]. Evolutionarily conserved uORFs are also found in mammalian mRNAs including those specifying HER-2/NEU [see Child et al., 1999b, and references therein], BCL-2 [Harigai et al., 1996; Salomons et al., 1998], and CCAAT/enhancer-binding protein [Lincoln et al., 1998]. While these mammalian uORFs affect translation, their regulatory roles remain

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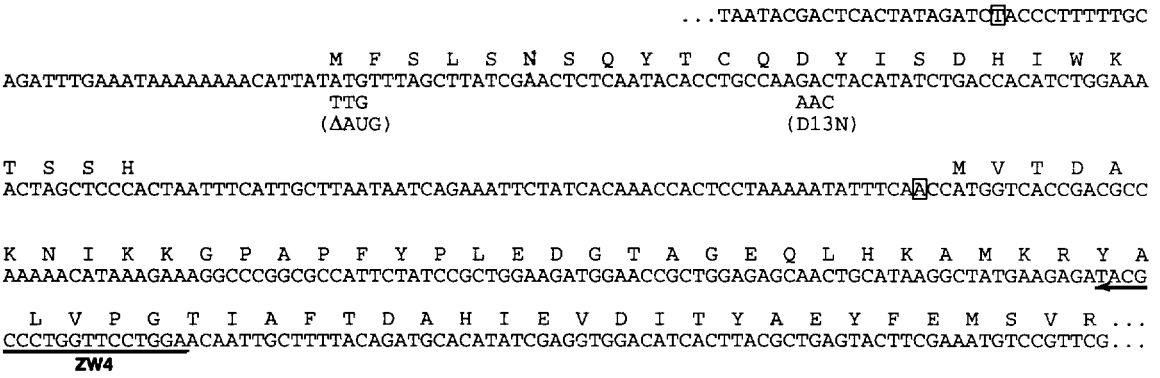
Wong, Z., Gaba, A., and Sachs, M. S. (1999) A highly conserved mechanism of regulated ribosome stalling mediated by fungal arginine attenuator peptides that appears independent of the charging status of arginyl-tRNAs. *J. Biol. Chem.* **274,** 37565–37574.

A


Sc MFSLSNSQYTCQDYISDHIWKTSSH
Nc MNGRPSVFTSQDYLSDHLWRALNA
Mg,Tv MNGRPSEFTSQDYLSDHLWRALSA
An MPAAPPSTFTSQDYISDHLWKADANI

B


...TAATACGACTCACTATAGATCTACCCTTTTTGC
M F S L S N S Q Y T C Q D Y I S D H I W K
AGATTTGAAATAAAAAAAACATTATATGTTTAGCTTATCGAACTCTCAATACACCTGCCAAGACTACATATCTGACCACATCTGGAAA
TTG
(ΔAUG)
AAC
(D13N)
T S S H
M V T D A
ACTAGCTCCCACTAATTTCATTGCTTAATAATCAGAAATTCTATCACAAACCACTCCTAAAAATATTTCAACCATGGTCACCGACGCC
K N I K K G P A P F Y P L E D G T A G E Q L H K A M K R Y A
AAAAACATAAAGAAAGGCCCGGCGCCATTCTATCCGCTGGAAGATGGAACCGCTGGAGAGCAACTGCATAAGGCTATGAAGAGATACG
L V P G T I A F T D A H I E V D I T Y A E Y F E M S V R...
CCCTGGTTCCTGGAACAATTGCTTTTACAGATGCACATATCGAGGTGGACATCACTTACGCTGAGTACTTCGAAATGTCCGTTCG...
ZW4

C

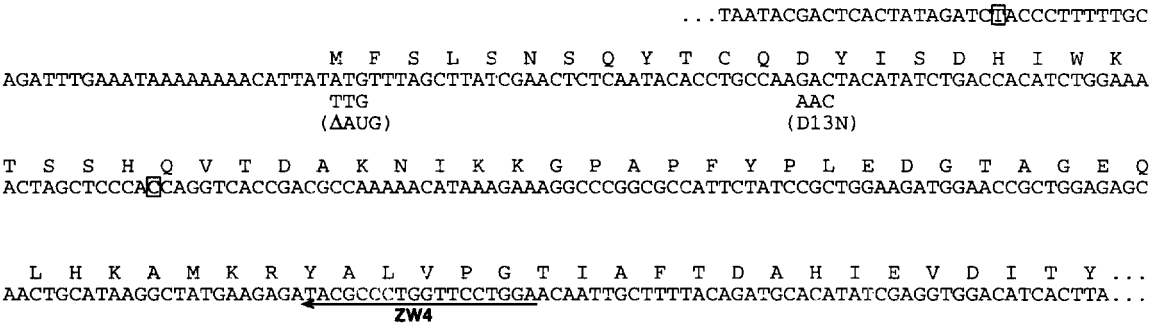

...TAATACGACTCACTATAGATCTACCCTTTTTGC
M F S L S N S Q Y T C Q D Y I S D H I W K
AGATTTGAAATAAAAAAAACATTATATGTTTAGCTTATCGAACTCTCAATACACCTGCCAAGACTACATATCTGACCACATCTGGAAA
TTG
(ΔAUG)
AAC
(D13N)
T S S H Q V T D A K N I K K G P A P F Y P L E D G T A G E Q
ACTAGCTCCCACCAGGTCACCGACGCCAAAAACATAAAGAAAGGCCCGGCGCCATTCTATCCGCTGGAAGATGGAACCGCTGGAGAGC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

Figure 5.1 Sequences of the fungal AAPs and the 5′ leader regions of *CPA1-LUC* genes used in this study (also see Table 5.1). (**A**) Comparisons of the AAPs from *S. cerevisiae* (*Sc*; Werner et al., 1987), *N. crassa* (*Nc*; Orbach et al., 1990), *Magnaporthe grisea* (*Mg*; Shen and Ebbole, 1997), *Trichoderma virens* (*Tv*; Baek and Kenerley, 1998), and *Aspergillus nidulans* (*An*; Genbank accession no. AJ224085). (**B**) Sequences of wild-type and mutant templates in which the *CPA1* AAP is encoded by a uORF. The sequence shown begins with the T7 RNA polymerase-binding site and ends within the LUC coding region [Wang and Sachs, 1997b]. The 5′ and 3′ boundaries of the *CPA1* region that was amplified by PCR are boxed. The amino acid sequences of the *CPA1* AAP and the N terminus of LUC are indicated. Point mutations are shown below the wild-type sequence. The sequence for which the reverse complement was synthesized and used as primer ZW4 for toeprint analysis is indicated by a horizontal arrow below the sequence. (**C**) Sequences of wild-type and mutant templates containing *CPA1* AAP-LUC fusion genes. The sequence shown begins with the T7 RNA polymerase-binding site and ends within the LUC coding region [Cao and Geballe, 1996b]. The 5′ and 3′ boundaries of the *CPA1* region that was amplified by PCR are boxed. The amino acid sequence of the N terminus of the AAP_{sc}-LUC fusion polypeptide is indicated. Point mutations are shown below the wild-type sequence. The sequence for which the reverse complement was synthesized and used as primer ZW4 for toeprint analysis is indicated by a horizontal arrow below the sequence.

unknown. Understanding how the *CPA1* and *arg-2* uORFs exert regulatory effects should provide insight into such uORF-mediated control mechanisms.

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

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

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

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

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

5.2 Experimental Procedures

5.2.1 Templates for RNA Synthesis

Linearized plasmid templates were designed to produce capped and polyadenylated synthetic RNAs encoding firefly LUC. The first type of RNA was designed to contain the entire *S. cerevisiae CPA1* uORF and intercistronic region in its 5′ leader (pAG101; Fig. 5.1B, Table 5.1). The second type of RNA was designed to have the *CPA1* uORF coding sequence fused directly in-frame with the LUC open

TABLE 5.1

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

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

[a] For details of how the *CPA1* constructs were made, see Fig. 5.1 legend and Section 5.2; for *arg-2* constructs, see Wang et al. [1998] and Wang and Sachs [1997a]. Wild-type represents the wild-type *CPA1* AAP or *arg-2* AAP as independent uORFs with wild-type initiation contexts. AAP-LUC represents the *CPA1* or *arg-2* AAPs as in-frame N-terminal fusions of the AAP to LUC. D13N and D12N represent amino acid substitutions in the AAPs that abolish regulation. ΔAUG represents an AUG to UUG mutation that eliminates the initiation codon for the uORF encoding the *CPA1* AAP. ↑AAP-LUC and D12N↑AAP-LUC represent wild-type and D12N *arg-2* AAP-LUC fusions in initiation contexts that yield greater translation in *N. crassa* extracts [Wang and Sachs, 1997a].

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

reading frame (pAG102; Fig. 5.1C, Table 5.1). Also constructed were mutant variants of each type containing either an Asp to Asn codon change at codon 13 of the *CPA1* uORF (pAG103 and pAG104) or a Met to Leu codon change (AUG→UUG) at the predicted *CPA1* uORF translation initiation codon (pAG105) by using PCR-based procedures [Freitag et al., 1996]. PCR products were placed into the pHLUC+NFS4 vector [Wang and Sachs, 1997a]. Primers for PCR reactions were: AG1 (5′-TGTTGAAGATCTACCCTTTTTGCAGATTTG-3′), which includes a 5′-*Bgl*II site, used for pAG101, pAG102, pAG103, and pAG104; AG3 (5′-ATCTGACCATGGTTGAAATATTTTTAGGAGTGGTT-3′), which includes a 3′-*Nco*I site, used for pAG101, pAG103, and pAG105; AG4 (5′-ATAGATGGTGACCTGGTGGGAGCTAGTTTTCCA-3′), which includes a 3′-*Bst*EII site, used for pAG102, pAG104, and pAG106; AG5 (5′-CAGATATGTAGTTTTGGCAGG-3′), which contains the Asp to Asn codon change at codon 13 of the *CPA1* uORF, used for pAG103 and pAG104; and AG6 (5′-TGTTGAAGATCTACCCTTTTTGCAGATTTGAAATAAAAAAAACATTATTTGT TTAGCTTAT-3′), which contains a 5′-*Bgl*II site and changes the predicted uORF translation initiation codon, used for pAG105 and pAG106. Corresponding templates for the synthesis of RNA containing the *N. crassa arg-2* AAP in the 5′-leader region (Table 5.1) were described previously [Wang et al., 1998; Wang and Sachs, 1997a,b], as was the template used to produce capped and adenylated synthetic mRNA encoding sea pansy LUC to serve as an internal control for translation reactions [Wang et al., 1998].

Plasmid DNA templates were purified by equilibrium centrifugation or by using a plasmid purification kit from Qiagen; capped, polyadenylated RNA was synthesized with T7 RNA polymerase from *Eco*RI-linearized plasmid DNA templates, and the yield of RNA was quantified [Wang and Sachs, 1997a].

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

The preparation of translation extracts was as described [Tarun and Sachs, 1995] from *S. cerevisiae* strain YAS1874 (*MAT***a** *MAK10::URA3 PEP4::HIS3 prb1 prc1 ade2 leu2 trp1 his3 ura3*) [Kessler and Sachs, 1998] with two modifications: buffer A was pH 7.6 instead of pH 7.4, and extracts were treated with micrococcal nuclease immediately after recovery from the Sephadex G-25 column, prior to freezing and storage. Nuclease-treated yeast extracts were used because nuclease treatment did not significantly affect amino acid-dependence or Arg-specific regulation under our assay conditions, but in initial comparative studies greatly increased the absolute level of reporter RNA translation and yielded superior toeprints [data not shown]. The preparation of translation extracts from *N. crassa* (with no nuclease treatment) was as described [Wang and Sachs, 1997a].

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

A wide range of conditions were examined to find those which were optimal for toeprinting in *S. cerevisiae*-derived reactions, including pH, heat pretreatment, reaction temperature, and Mg^{2+} concentration [Kozak, 1998]. These were similar to those earlier determined to be optimal for *N. crassa*-derived reactions [data not shown]. Therefore, the toeprint assays of both *S. cerevisiae*- and *N. crassa*-derived reaction mixtures were accomplished after incubation as described in the text using

primer ZW4 and the previously established method [Wang and Sachs, 1997b]. All toeprint data shown are representative of multiple experiments.

5.2.3 Measurement of tRNA Aminoacylation

The assays for tRNA aminoacylation were adapted from a previously described procedure [Varshney et al., 1991]. Translation reaction mixtures with total volumes of 100 μl (*S. cerevisiae* and *N. crassa*) or 60 μl (wheat germ) containing different concentrations of Arg were incubated for 10 min. Then aliquots of 90 μl (*S. cerevisiae* and *N. crassa*) or 54 μl (wheat germ) were removed (the remainder of translation reaction mixtures were incubated to the 30-min time point, then used for LUC assays) and immediately added to ice-cold tubes containing a mixture of 300 μl of phenol (pH 4.5) and 200 μl of sodium acetate (pH 4.5). Tubes were vortexed for 60 s and then centrifuged for 20 min. The aqueous layers were transferred to new tubes and mixed with 2.5 volumes of ethanol. Tubes were frozen at -80°C for at least 15 min, and then the total nucleic acids were recovered by centrifugation for 20 min. The nucleic acid pellet was dissolved in 20 μl of 10 mM sodium acetate, 1 mM EDTA (pH 4.5). An aliquot (2.0 μl) was used to measure A_{260}; immediately prior to gel electrophoresis, nucleic acids were adjusted to a final concentration of 2.5 μg/μl (assuming 40 μg nucleic acid/A_{260}) by the addition of acid gel loading buffer [Varshney et al., 1991].

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

mM sodium acetate, 1 mM EDTA (pH 4.5) prior to dilution with acid gel loading buffer.

The procedures for acid/urea gel electrophoresis, electrophoretic transfer, and northern blot hybridization to identify charged and uncharged tRNAs were essentially as described [Varshney et al., 1991], except that denatured salmon sperm DNA was not included in the prehybridization and hybridization solutions, and the membranes after hybridization were exposed to screens of a Molecular Dynamics PhosphorImager for approximately 4 h. DNA probes are the reverse complements of the these tRNA regions: JA11: 5′-TCGGTTTCGATCCGAGGACATCAGGGTTATGA-3′, complement to 32–63 of *S. cerevisiae* $tRNA_i^{Met}$ [Cigan and Donahue, 1986; Anderson et al., 1998; James Anderson, personal communication]; ZW32: 5′-ACGATGGGGGTCGAACCC-3′, complement to 50–67 of *S. cerevisiae* Arg-tRNA 3a and 3b [Keith and Dirheimer, 1980]; ZW33: 5′-TGGTTCGCAGCCAGACGC-3′, complement to 24–41 of *S. cerevisiae* Arg-tRNA 2 [Weissenbach et al., 1975]; ZW34: 5′-ATCTTCTGGTTCGCAGCC-3′, complement to 30–47 of *S. cerevisiae* Arg-tRNA 2; ZW38: 5′-ACCACGCTGGGAGTCGAACC-3′, complement to 52–71 of wheat germ tRNA-Arg (CCG) [Baum and Beier, 1998]; ZW39: 5′-ACTCCGCTGGGGATCGAACC-3′, complement to 52–71 of wheat germ tRNA-Arg (ICG) [Barciszewska et al., 1986]; ZW40: 5′-TGGGACCTGTGGGTTATGGG-3′, complement to 31–50 of wheat germ $tRNA_i^{Met}$ [Ghosh et al., 1982]; ZW41: 5′-TCGATCCTGGGACCTGTGG-3′, complement to 39–57 of wheat germ $tRNA_i^{Met}$; ZW42: 5′-ACCTCCGGGTTATGAGCCC-3′, complement to 28–46 of *N. crassa* $tRNA_i^{Met}$ [Gillum et al., 1977]; ZW43: 5′-TCGAGTGACCTCCGGGTT-3′, complement to 36–53 of *N. crassa* $tRNA_i^{Met}$; ZW44: 5′-CTTCAGTCTGACGCTCTCCC-3′, complement to 18–37 of *N. crassa* tRNA-Phe (GAA) [Alzner-DeWeerd et al., 1980]; ZW45: 5′-TGCGGTTTGTGTGGATCG-3′, complement to 56–73 of *N. crassa* tRNA-Phe (GAA).

5.3 Results

5.3.1 AAP-Mediated Arg-Specific Translational Attenuation in Three Cell-Free Translation Systems

We examined Arg-specific regulation mediated by the *S. cerevisiae* and *N. crassa* AAPs in translation extracts derived from *S. cerevisiae*, *N. crassa*, and wheat germ. Capped and polyadenylated synthetic RNAs were synthesized from templates in which the *S. cerevisiae CPA1* AAP or the *N. crassa arg-2* AAP were placed upstream of firefly LUC, either as uORFs or as in-frame N-terminal extensions (Fig. 5.1B and C, Table 5.1). Equal amounts of each RNA were used to program translation extracts. As an internal control, a second capped and polyadenylated synthetic RNA that encoded sea pansy LUC (which lacked fungal regulatory sequences) was also added to the extracts [Wang et al., 1998]. For each of the extracts used, the addition of 10 μM each of the 20 amino acids to reaction mixtures was sufficient for near maximal translation of LUC [data not shown]. Additional Arg could be added to translation extracts with relatively slight effects on protein synthesis from RNA templates lacking *arg-2* regulatory sequences (e.g., sea pansy LUC, Fig. 5.5B).

Comparisons of the translation of firefly LUC in reaction mixtures supplemented with low (10 μM) or high (2 mM) Arg showed that the wild-type *CPA1* AAP, when present as a uORF (Fig. 5.1B), reduced the translation of LUC when the concentration of Arg was high (Table 5.1). Translation of LUC from RNA containing the wild-type *N. crassa arg-2* AAP was also subject to Arg-specific negative regulation in each extract (Table 5.1). Introduction of the D13N mutation in the *S. cerevisiae* AAP coding region (Fig. 5.1B) or the corresponding D12N mutation into the *N. crassa* coding region eliminated this regulatory effect in all cases (Table 5.1).

The wild-type *CPA1* and *arg-2* AAPs, when fused directly to LUC as N-terminal extensions (Fig. 5.1C), also functioned to regulate translation in all three extracts (Table 5.1). The LUC polypeptide produced appeared to initiate at the AAP

start codon because changing this codon from AUG to UUG resulted in unregulated and substantially reduced (50-fold in *N. crassa*, 30-fold in *S. cerevisiae*) LUC synthesis [data not shown]. The *CPA1* AAP D13N mutation and the *arg-2* AAP D12N mutation eliminated the regulatory effect of Arg in all three systems, showing the strong dependence of Arg-regulation on the sequence of the AAP peptide and the lack of necessity of a uORF termination codon for regulation.

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

Toeprint assays, in which reverse transcriptase is used for primer extension in translation extracts, enables the mapping of the positions of *N. crassa* ribosomes on RNA [Wang and Sachs, 1997b; Wang et al., 1998]. Here we applied this technique to *S. cerevisiae* ribosomes. To our knowledge, this is the first use of toeprinting to examine the positions of *S. cerevisiae* ribosomes on RNA. Primer extension from RNA templates containing the *CPA1* uORF in the absence of extract yielded cDNA products predominantly corresponding to full-length extension of the primer as well as other shorter transcription products (Fig. 5.2, lanes 8 and 15). The shorter products are produced in relatively much lower quantities when the RNA is reverse-transcribed in buffer formulated for reverse transcription [data not shown] rather than the buffer formulated for *in vitro* translation necessary for these experiments. Extracts that were not programmed with RNA did not yield any of these signals (Fig. 5.2, lanes 7 and 16), as predicted if these represented the products obtained from priming on the synthetic RNA template. When RNA containing the *CPA1* uORF in its 5′-leader was used to program *S. cerevisiae* extracts containing high Arg (500 or 2000 μM, Fig. 5.2, lanes 2 and 3) but not low Arg (10 μM, Fig. 5.2, lanes 1), new signals were observed that corresponded to ribosomes stalled with the uORF termination codon in the ribosome A site (confirmed by high resolution mapping on other gels [data not shown]). The effect of Arg to stall ribosomes at the termination codon increased when the concentration of Arg was raised from 500 μM to 2000 μM. Arg also caused ribosome stalling at the *CPA1* uORF termination codon in *N. crassa* extracts

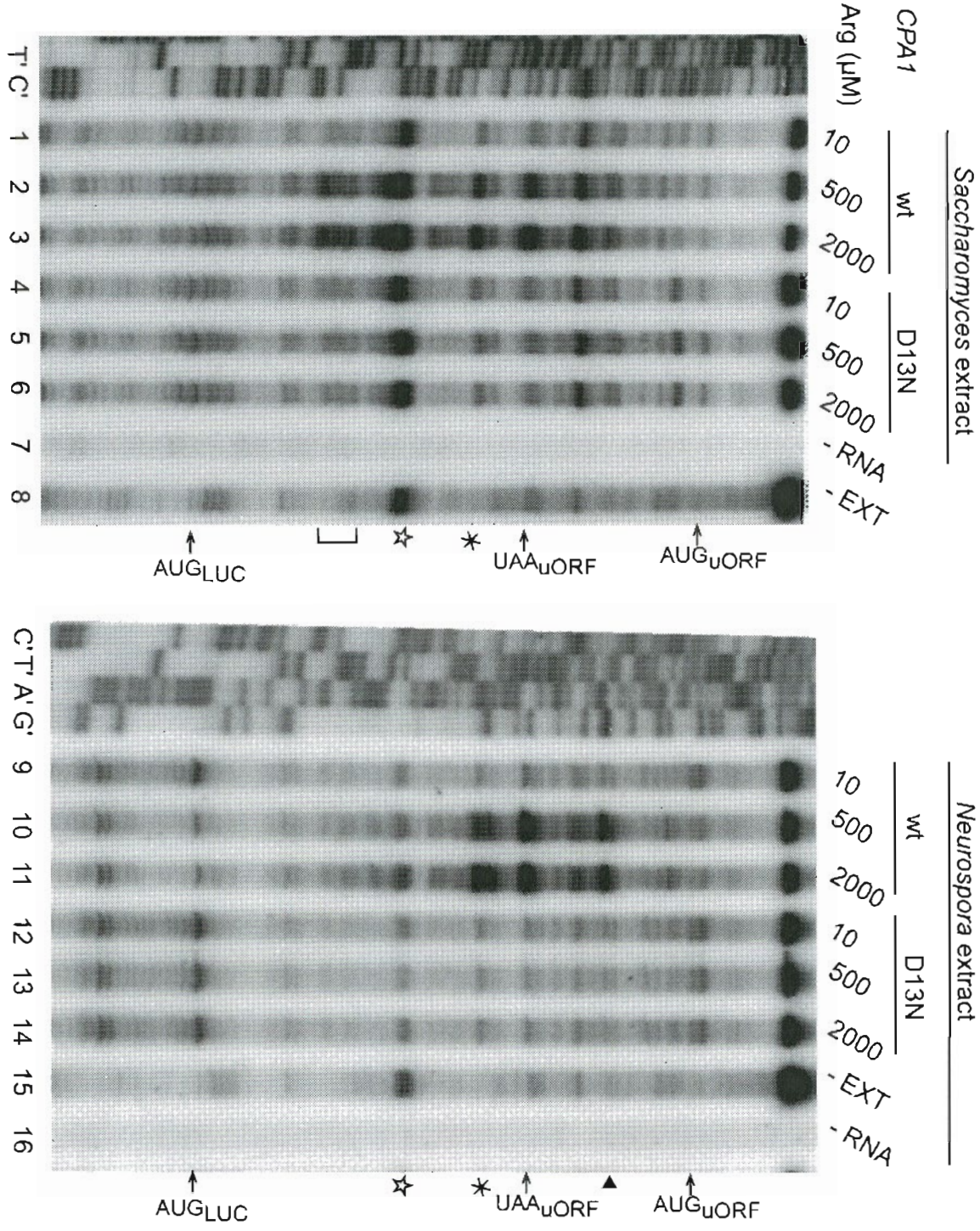

Saccharomyces extract
CPA1
wt
D13N
Arg (μM)
10
500
2000
10
500
2000
- RNA
- EXT
T' C'
1 2 3 4 5 6 7 8
AUG LUC
UAA uORF
AUG uORF
Neurospora extract
wt
D13N
10
500
2000
10
500
2000
- EXT
- RNA
C' T' A' G'
9 10 11 12 13 14 15 16
AUG LUC
UAA uORF
AUG uORF

Figure 5.2 Effects of the *CPA1* AAP encoded as a uORF on Arg-specific regulation in translation extracts derived from *S. cerevisiae* and *N. crassa*. Equal amounts (120 ng) of synthetic RNA transcripts were used to program translation mixtures derived from *S. cerevisiae* or *N. crassa*. The transcripts encoded either the wild-type (wt) or D13N mutant *CPA1* AAP as a uORF in the 5′ leader as indicated. The 20-μl reaction mixtures were supplemented with different concentrations of Arg (10, 500, or 2000 μM as indicated) and with 10 μM each of the other 19 amino acids. After 20 min of incubation at 25°C, the reaction mixtures were toeprinted with primer ZW4 as described [Wang and Sachs, 1997b]. The products obtained from primer extension of pure RNA (18 ng) in the absence of translation reaction mixture (–EXT) and from a translation reaction mixture not programmed with RNA (–RNA) are shown for comparison. The arrows indicate the positions of premature transcription termination products corresponding to ribosomes bound at AUG_{uORF}, UAA_{uORF}, or AUG_{LUC}. The arrowhead indicates the position of an additional toeprint site upstream of UAA_{uORF} observed in *N. crassa* extracts containing high Arg concentrations; asterisks indicate an additional toeprint site downstream of UAA_{uORF} observed in *S. cerevisiae* and *N. crassa* extracts containing high Arg concentrations. The bracket indicates additional toeprints observed downstream of UAA_{uORF} in *S. cerevisiae* extracts containing high Arg concentrations. The star indicates a strong signal observed from primer extension of RNA in the absence of extract (–EXT). Dideoxynucleotide sequencing reactions for the wild-type *CPA1* template are shown on the left; the nucleotide complementary to the dideoxynucleotide added to each sequencing reaction is indicated below the corresponding lane so that the sequence of the template can be directly deduced; the 5′-to-3′ sequence reads from top to bottom.

(Fig. 5.2, compare lanes 10 and 11 to lane 9). The *CPA1* AAP D13N mutation, which eliminates regulation *in vivo* [Werner et al., 1987], eliminated Arg-specific effects on toeprints in both extracts (Fig. 5.2, lanes 4–6 and lanes 12–14), consistent with the loss of regulation observed by LUC assay (Table 5.1).

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

In *N. crassa* extracts, signals corresponding to ribosomes with the uORF and LUC initiation codons in their P-sites were observed in extracts programmed with RNA. For constructs containing the wild-type but not the D13N uORF, these signals were reduced when Arg was added. Similar results are observed for the wild-type *arg-2* uORF in *N. crassa* extracts and are interpreted to arise as a consequence of ribosome stalling at the uORF termination codon, which decreases ribosome loading at the LUC AUG, and decreases the capacity to detect signal at the uORF AUG [Wang and Sachs, 1997b; Wang et al., 1998].

The wild-type *CPA1* AAP caused additional Arg-specific effects in each extract, some common and some system-specific (Figs. 5.2 and data not shown). In *N. crassa* extracts, an additional signal (arrowhead) approximately 30 nt upstream of the stop codon appeared in high Arg. These possibly represent ribosomes queued behind ribosomes that have stalled at the uORF termination codon [Wang and Sachs, 1997b; Wang et al., 1998]. In both systems, Arg caused a substantial increase in (puromycin-releasable) toeprints in the intercistronic region (asterisk, approximately 12 and 16 nt downstream of the stop codon in *N. crassa*, and 16 nt downstream of the stop codon in *S. cerevisiae*). In *S. cerevisiae*, additional Arg-regulated toeprints were

observed further downstream in the intercistronic region (star and bracket). One of these signals corresponds in position to a strong signal in the intercistronic region of the RNA, which was also present in primer extension products obtained from the RNA in the absence of translation extract (Fig. 5.2, lanes 8 and 15, star). The physical basis for these additional bands, which may arise for reasons similar or different than those responsible for the "echo band" phenomenon, in which a ribosome located at an initiation codon can cause a primary toeprint and an additional toeprint [Kozak, 1998], remain to be elucidated. Possibly, they could represent ribosomes or additional machinery recruited by ribosomes; alternatively, they could reflect increased secondary structure in the RNA arising as a consequence of ribosome binding.

In a manner highly similar to the wild-type *CPA1* AAP, the wild-type *arg-2* AAP (in these experiments placed in an improved initiation context [Wang and Sachs, 1997b]) caused ribosomes to stall at the uORF termination codon in response to Arg in both *S. cerevisiae* and *N. crassa* systems; the D12N mutation eliminated regulation (Fig. 5.3). Thus, the two fungal AAPs acted similarly when present as uORFs to stall ribosomes in *S. cerevisiae* and *N. crassa* systems.

While AAP-dependent regulation was observed using wheat germ extracts (Table 5.1), in primer extension experiments, no signals indicating Arg-specific stalling were apparent [data not shown]. All signals, including full length cDNA products, were weaker in primer extension analyses using wheat germ. This failure to achieve results in the wheat germ system that were comparable to those obtained with the fungal systems is possibly attributable to the presence of an RNase H activity in wheat germ extracts [Haeuptle et al., 1986].

The effect of Arg-specific, AAP-mediated regulation on ribosomes involved in elongation was tested using *CPA1* AAP-LUC and *arg-2* AAP-LUC fusions in *S. cerevisiae* and *N. crassa* extracts. A high concentration of Arg substantially increased the intensity of a series of toeprints on the *CPA1* AAP-LUC RNA in both extracts (Fig. 5.4, compare lanes 4 and 3, and lanes 12 and 11). The D13N mutation eliminated these Arg-specific effects on toeprints (Fig. 5.4, compare lanes 1 and 2, and lanes 9 and 10), as did treatment with puromycin after 15 min, as described

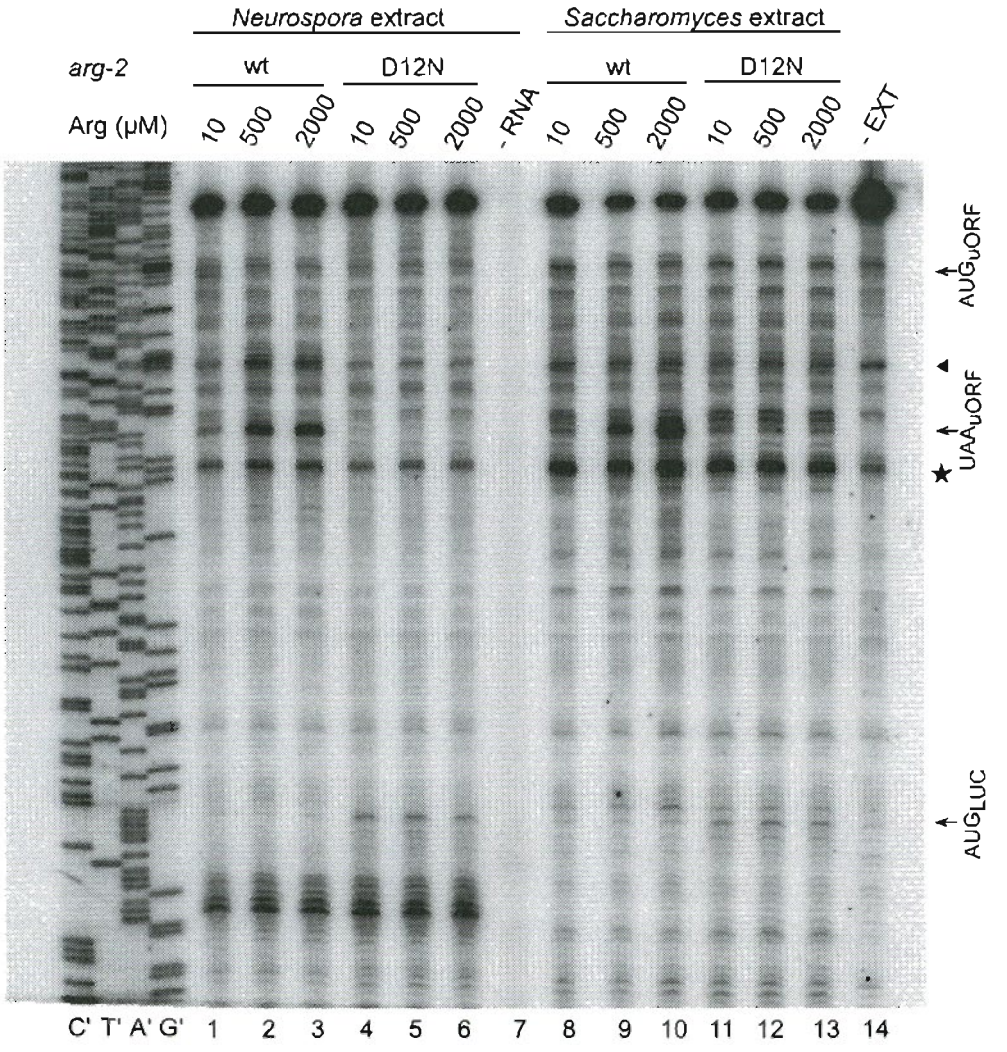


Figure 5.3 Effects of the *arg-2* AAP encoded as a uORF on Arg-specific regulation in translation extracts derived from *N. crassa* and *S. cerevisiae*. Equal amounts of synthetic RNA transcripts (120 ng) were translated in reaction mixtures and analyzed by toeprinting as described in the legend to Fig. 5.2. The transcripts encoded either the wild-type (wt) AAP in an improved initiation context or the D12N AAP [Wang and Sachs, 1997b]. Arrows indicate the positions of premature transcription termination products corresponding to ribosomes bound at AUG_{uORF}, UAA_{uORF}, or AUG_{LUC}. The arrowhead indicates the position of an additional toeprint site upstream of UAA_{uORF} observed in *N. crassa* extracts containing high Arg concentrations. The star indicates a signal observed from primer extension of RNA in the absence of extract (–EXT) that coincides with a toeprint signal that increases with high Arg [Wang and Sachs, 1997b]. Dideoxynucleotide sequencing reactions for the wild-type *arg-2* template are shown on the left; the nucleotide complementary to the dideoxynucleotide added to each sequencing reaction is indicated below the corresponding lane so that the sequence of the template can be directly deduced; the 5′-to-3′ sequence reads from top to bottom.

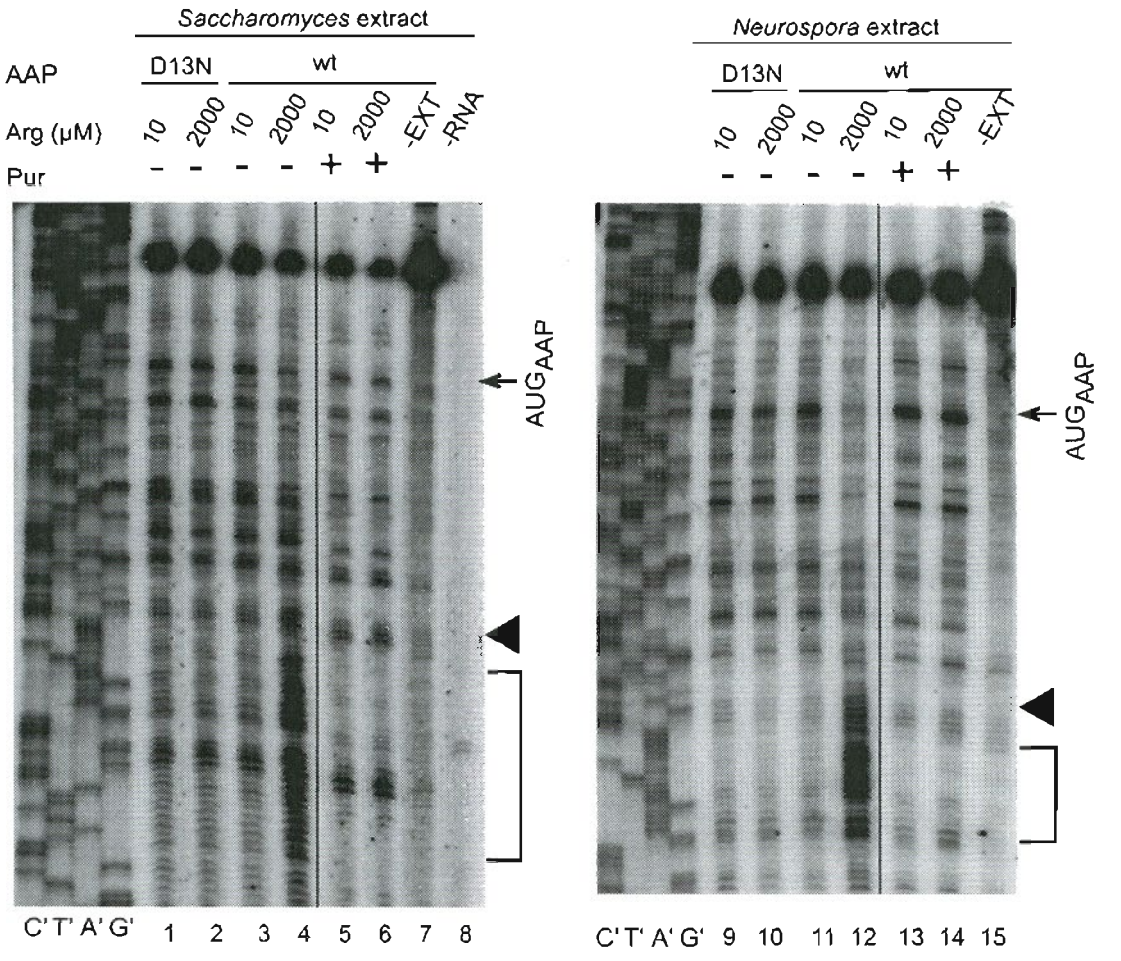


Figure 5.4 Effects of the *CPA1* AAP as an N-terminal fusion to LUC on Arg-specific regulation in translation extracts derived from *S. cerevisiae* and *N. crassa*. Equal amounts of synthetic RNA transcripts (120 ng) were translated in reaction mixtures and analyzed by toeprinting as described in Fig. 5.2. The transcripts encoded either the wild-type (wt) AAP-LUC fusion or the D13N mutant AAP-LUC fusion as indicated. Puromycin (Pur) was added where indicated (+), as described in the text. The arrow indicates the position of premature transcription termination products corresponding to ribosomes bound at the AAP initiation codon (AUG_{AAP}). The arrowhead indicates the positions of premature termination products corresponding to ribosomes stalled at the codon immediately following the last codon of the AAP in *S. cerevisiae* and *N. crassa* extracts containing a high Arg concentration. The bracket indicates the position of premature termination products corresponding to ribosomes stalled in the LUC coding region in *S. cerevisiae* and *N. crassa* extracts containing a high Arg concentration. Dideoxynucleotide sequencing reactions for the wild-type *CPA1* AAP-LUC fusion template are shown on the left; the nucleotide complementary to the dideoxynucleotide added to each sequencing reaction is indicated below the corresponding lane so that the sequence of the template can be directly deduced; the 5′-to-3′ sequence reads from top to bottom.

above (Fig. 5.4, lanes 5, 6, 13, and 14), indicating that they resulted from an interaction of ribosomes with the RNA. In both extracts, the most 5′-proximal of the Arg-specific toeprints corresponded to ribosomes translating the first codon following the AAP coding sequence (Fig. 5.4, arrowheads). This stall site in the fusion polypeptide corresponds to the position, relative to the *CPA1* AAP coding sequence, of the uORF termination codon. This toeprint site was followed by additional Arg-induced toeprints corresponding to ribosomes stalled in the downstream LUC coding region (indicated by brackets). These signals extend further downstream in *S. cerevisiae* extracts.

The length of the region in which ribosomes involved in elongation were stalled in response to Arg appeared to be determined by the source of the extract and not the source of the AAP. Both *CPA1* and *arg-2* AAPs yielded a more extended series of toeprint sites in *S. cerevisiae* than *N. crassa* (Fig. 5.4; data not shown). The reasons for these extract-dependent differences in toeprinting are not known but might reflect faster translation elongation rates in *S. cerevisiae*-derived extracts [data not shown].

5.3.3 Arg-Specific Regulation Appears Independent of the Charging Status of Arginyl-tRNAs

When the effect of Arg on ribosome stalling on transcripts encoding the wild-type *arg-2* AAP-LUC fusion was examined in *N. crassa* translation extracts, the amount of stalling increased as the concentration of Arg increased (Fig. 5.5A, lanes 1–5). Similar effects were observed with the *CPA1* AAP-LUC fusion in *N. crassa* extracts and with both fusions in *S. cerevisiae* extracts [data not shown]. The *S. cerevisiae* D13N and *N. crassa* D12N mutants did not show stalling at any Arg concentration in either extract (Fig. 5.4; Fig. 5.5A, lanes 6–8; data not shown). Consistent with the observed increase in stalling of ribosomes on RNA containing the wild-type AAP-LUC fusion, the magnitude of Arg-specific regulation increased in *S. cerevisiae*, *N. crassa*, and wheat germ extracts as the concentration of added Arg was increased from 10 μM to 5 mM (Fig. 5.5B) as determined by LUC assay. It should

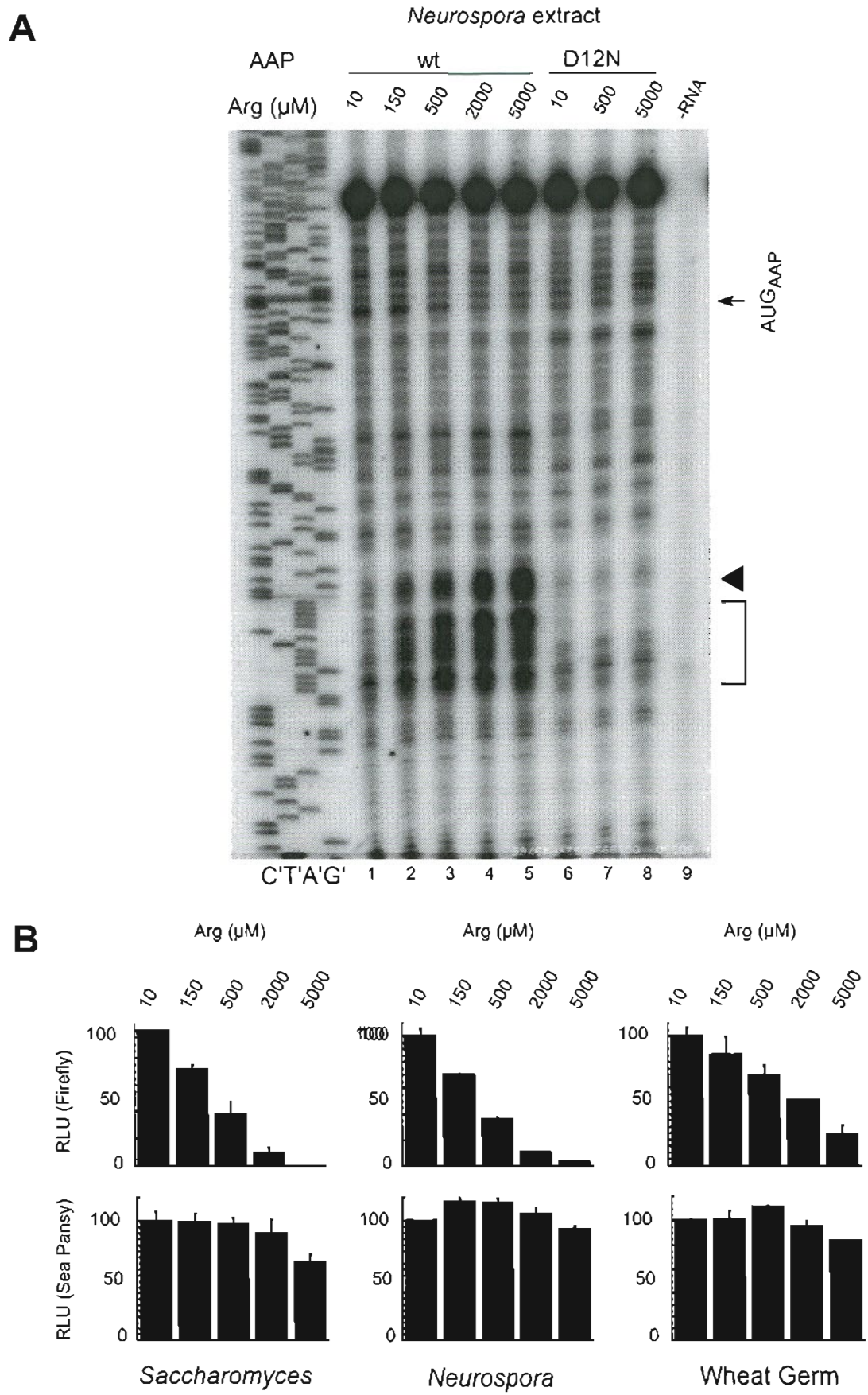

A
Neurospora extract
AAP
wt
D12N
Arg (μM)
10
150
500
2000
5000
10
500
5000
-RNA
AUG_AAP
C'T'A'G'
1
2
3
4
5
6
7
8
9
B
Arg (μM)
Arg (μM)
Arg (μM)
RLU (Firefly)
RLU (Sea Pansy)
100
50
0
Saccharomyces
Neurospora
Wheat Germ

Figure 5.5 Effects of Arg concentration on Arg-specific regulation. (**A**) Effects of *arg-2* AAP-LUC fusion in *N. crassa* translation extracts containing varying concentrations of Arg assayed by toeprinting. Equal amounts of synthetic RNA transcripts (120 ng) were translated in reaction mixtures that contained either 10, 150, 500, 2000, or 5000 μM Arg and a 10-μM concentration of each of the other 19 amino acids. Transcripts were analyzed by toeprinting as described in the legend to Fig. 5.2. The transcripts encoded either the wild-type (wt) or the D12N mutant *arg-2* AAP as a uORF in the 5′ leader. The arrow indicates the position of toeprint products corresponding to ribosomes bound at the AAP initiation codon (AUG_{AAP}). The arrowhead indicates the position of toeprint products corresponding to ribosomes stalled at the codon immediately following the last codon of the AAP in *N. crassa* translation extracts containing high Arg concentrations. The bracket indicates the positions of toeprint products corresponding to ribosomes stalled in the luciferase coding region in *N. crassa* translation extracts containing high Arg concentrations. Dideoxynucleotide sequencing reactions for the wild-type *arg-2* AAP-LUC fusion template are shown on the left; the nucleotide complementary to the dideoxynucleotide added to each sequencing reaction is indicated below the corresponding lane so that the sequence of the template can be directly deduced; the 5′-to-3′ sequence reads from top to bottom. (**B**) Effects of *arg-2* AAP-LUC fusion on Arg-specific regulation in translation extracts derived from *S. cerevisiae, N. crassa,* and wheat germ assayed by measuring luciferase enzyme activity. Equal amounts (12 ng) of *arg-2* AAP-LUC fusion RNA was translated in *S. cerevisiae, N. crassa,* and wheat germ extracts containing either 10, 150, 500, 2000, or 5000 μM Arg and a 10-μM concentration of each of the other 19 amino acids. Mean values and standard deviations from measuring the firefly luciferase enzyme activity in two independent translation reactions are given. Activities of sea pansy luciferase translated from a second RNA encoding this enzyme (an RNA lacking AAP regulatory sequences that was included as an internal control in each reaction mixture) are indicated below the corresponding firefly luciferase activities.

be noted that, while the precision of measurements in a given experiment is high, the absolute magnitude of regulation by Arg differs between extract preparations and experiments (e.g., the extracts used in the experiment shown in Table 5.1 showed a lower magnitude effect than those used in Fig. 5.5B). Nonetheless, in multiple experiments using any of the amino acid-dependent fungal extracts prepared in our laboratory to date (28 independently prepared *N. crassa* extracts and 12 independently prepared *S. cerevisiae* extracts), Arg-specific regulation is always observed.

The level of charged arginyl-tRNA might be a signal for Arg-specific regulation mediated by the AAP. Transcriptional attenuation of the amino acid biosynthetic operons in bacteria is modulated by the level of charged tRNA [Landick et al., 1996]. The charging status of tRNA controls the translation of Gcn4p in yeast [Hinnebusch, 1996]. To determine whether the levels of aminoacylation of arginyl-tRNAs change when different Arg concentrations are present in translation extracts, we adapted a method that has been successful in determining the levels of aminoacylation of tRNAs *in vivo* [Varshney et al., 1991]. Reaction mixtures were supplemented with increasing concentrations of Arg and incubated for 10 min. Then total nucleic acid was obtained under conditions in which tRNA charging is maintained and the tRNAs separated by polyacrylamide gel electrophoresis using conditions that resolve charged and uncharged tRNAs. The charging status of different tRNA species were detected by northern blot hybridization using ^{32}P-labeled oligonucleotide probes complementary to the specific tRNAs of interest.

We first checked the charging status of *S. cerevisiae* tRNAs in yeast extracts with a positive control $tRNA_i^{Met}$ probe. Predominantly uncharged tRNA is observed after alkali treatment of the tRNAs isolated from extracts (Fig. 5.6A, lane 1). tRNA is mostly uncharged in T_0 *S. cerevisiae* extracts, which have not been incubated and which have not been supplied with an energy regeneration system or additional amino acids (Fig. 5.6A, lane 2). In contrast, in complete translation extracts containing 10, 150, 500, or 2,000 μM of Arg and 10 μM each of the other 19 amino acids that have been incubated for 10 min, the $tRNA_i^{Met}$ is predominantly fully charged (Fig. 5.6A, lanes 3–6).

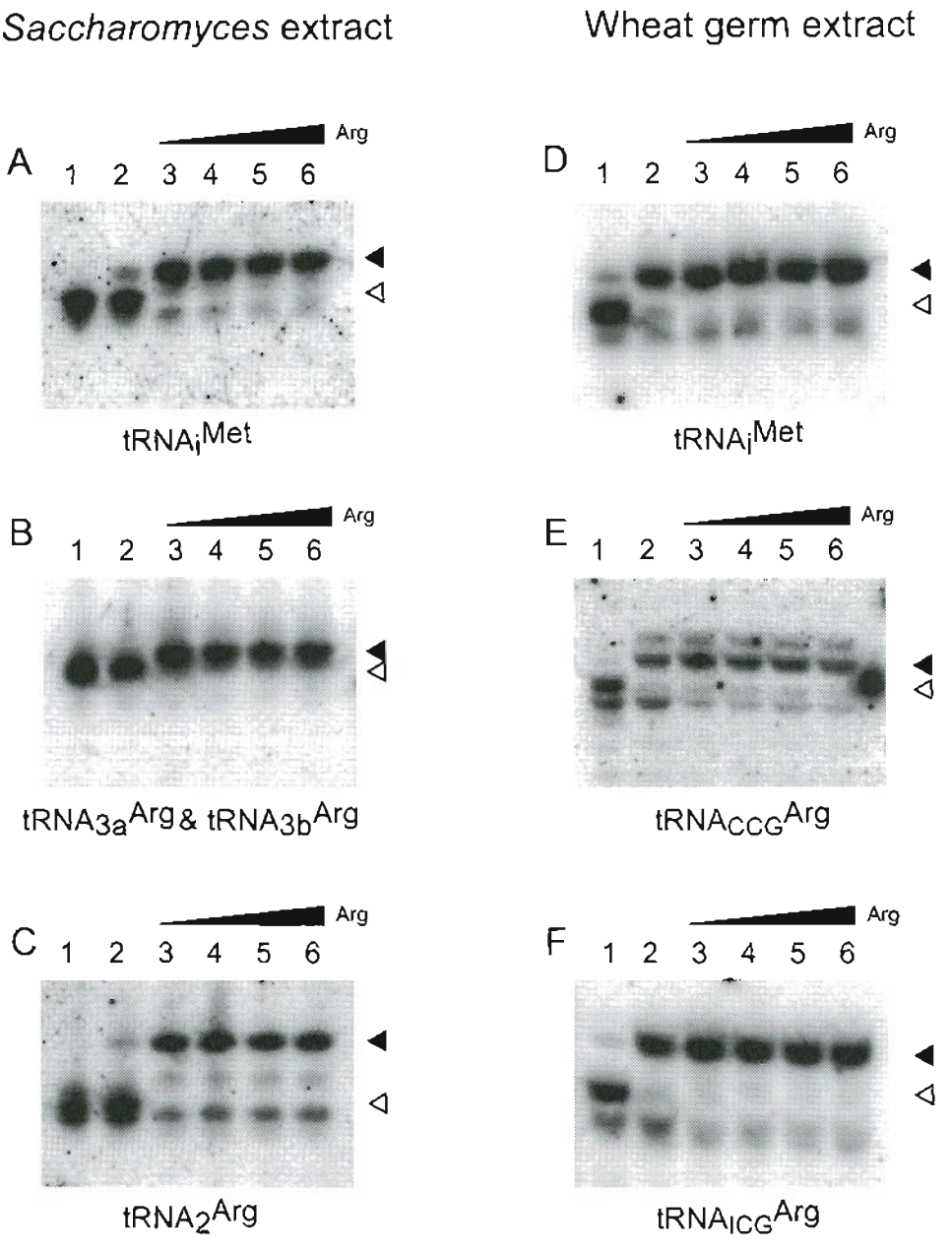


Figure 5.6 Charging status of tRNAs in *S. cerevisiae* and wheat germ extracts. Charged (aminoacylated) tRNAs (filled arrowheads) and uncharged (deacylated) tRNAs (open arrowheads) were separated on acid-urea-polyacrylamide gels followed by electrophoretic transfer to Nytran Plus membranes. Specific tRNA species were detected by northern blot hybridization [Varshney et al., 1991] using 5'-^{32}P-labeled oligonucleotides complementary to specific regions of the tRNA species that are indicated at the bottom of each panel. For each panel, lane 1 contains alkali-treated tRNAs (deacylated tRNAs) from the extracts indicated; lane 2 contains tRNAs in extracts at time 0 (T_0 extract); lanes 3-6 show tRNAs in translation reaction mixtures after 10 min of incubation; reaction mixtures contained 10, 150, 500, and 2000 μM of Arg, respectively, and 10 μM each of the other 19 amino acids.

The arginyl-tRNAs detected with probes that should recognize three different $tRNA^{Arg}$ species showed the same pattern of charging as the $tRNA_i^{Met}$ control (Fig. 5.6B and C). The arginyl-tRNAs were mainly uncharged in T_0 extracts but maximally charged at even the lowest concentration of Arg added to extracts (10 μM). Thus, in *S. cerevisiae* extracts, the charging status of arginyl-tRNAs did not appear to change in response to levels of Arg supplement that result in Arg-specific translational regulation.

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

Since AAP-mediated Arg-specific regulation was observed in wheat germ extracts (Fig. 5.5B), we analyzed the charging status of methionyl- and arginyl-tRNAs in these extracts (Fig. 5.6D–F). The results were similar to those obtained with yeast extracts except that wheat germ tRNAs were already charged in T_0 extracts. The reason for this difference between wheat germ and yeast extracts was not determined, but it might reflect the presence of a high level of ATP in T_0 wheat germ extracts, since ATP and an ATP regenerating system are present in T_0 wheat germ extracts but not T_0 yeast extracts.

5.4 Discussion

The *S. cerevisiae* Arg biosynthetic gene *CPA1* contains a *cis*-acting control region functionally analogous to a bacterial operator in repressing gene expression in response to Arg [Thuriaux et al., 1972]. Regulation is indicated to act at the level of translation because mutations causing constitutive expression affect the translation of a peptide encoded in the 5′-leader of the *CPA1* mRNA [Werner et al., 1987] that is evolutionarily conserved (Fig. 5.1A). We examined the role of this AAP in Arg-specific regulation by programming translation extracts from *S. cerevisiae*, *N. crassa*, and wheat germ with mRNA containing the AAP and firefly LUC reporter sequences.

Using fungal extracts, in which the movement of ribosomes could be examined by primer extension inhibition, the wild-type but not mutant *CPA1* and *arg-2* AAPs acted similarly to stall the movement of ribosomes immediately after AAP translation. Regulation did not appear to be a response to the level of charged Arg-tRNA. The observation that tRNAs were maximally charged in extracts provided with 10 μM exogenously supplied amino acids is consistent with the observation that this amount of supplement is sufficient for near maximal translational activity and consistent with the observed K_m of 1.5 μM for Arg of the purified *S. cerevisiae* arginyl-tRNA synthetase [Gangloff et al., 1976].

The absence of an apparent role for the level of charged tRNA in a case of translational regulation of an amino acid biosynthetic gene is unprecedented. tRNA charging is important for the transcriptional attenuation of amino acid biosynthetic genes in prokaryotes, in which lack of specific charged tRNAs causes critical stalls in the translation of upstream leader peptides [Landick et al., 1996], and in the translational regulation of *S. cerevisiae GCN4* through the *GCN2*-encoded kinase, which is activated by binding to uncharged tRNA [Hinnebusch, 1997]. However, these latter control mechanisms that respond to tRNA charging are designed to respond to amino acid limitation. *CPA1* in fact responds to Arg limitation through a *GCN4*-mediated process [Kinney and Lusty, 1989], and *N. crassa arg-2* responds to amino acid limitation through a *cpc-1* mediated process [Ebbole et al., 1991; Sachs and Yanofsky, 1991; Freitag et al., 1996]. *cpc-1* is the homolog of *GCN4* [Paluh et al., 1988; Sachs, 1996], and the translation of its mRNA is also regulated by amino acid limitation [Luo et al., 1995]. The available evidence concerning the signal for the CPC-1-mediated response to amino acid limitation in *N. crassa* indicates that it is uncharged tRNA [Sachs, 1996], as it is for the Gcn4p-mediated response in yeast. Strikingly, *N. crassa* contains a close homolog of *GCN2* known as *cpc-3*, and *cpc-3* mutants have phenotypes similar to *gcn2* mutants [Sattlegger et al., 1998]. Thus, it appears that *CPA1* and *arg-2* share both a conserved mechanism to respond to amino acid limitation (through *GCN4/GCN2* and *cpc-1/cpc-3*, respectively) and a conserved mechanism to respond to Arg surplus (through translation of the *cis*-acting AAP).

The response to limitation appears mediated by the level of tRNA; the response to surplus appears to be mediated differently. Because fungi store large amounts of Arg in the vacuole [Davis, 1986]—the concentration of Arg in the vacuole of *S. cerevisiae* grown in Arg-containing medium is 430 mM [Kitamoto et al., 1988] and a high concentration of Arg is also stored in the vacuole of *N. crassa* [Keenan and Weiss, 1997]—it would seem logical that they possess a regulatory mechanism to modulate Arg biosynthesis in response to cytosolic concentrations of Arg far exceeding those necessary for the charging of tRNA.

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

Second, Arg might not cause modification of a translational component, but instead might interact directly with the translational machinery, causing the machinery to become sensitive to AAP-mediated stalling. In addition to the well-established interaction of Arg with regulatory proteins such as the *E. coli* Arg repressor (e.g., Ni et al., 1999), Arg can also interact with RNA. In the case of the *Tetrahymena* rRNA self-splicing intron, Arg competes for GTP binding [Yarus, 1989]. The human immunodeficiency virus trans-acting responsive element RNA binds an Arg residue of Tat; it also binds the free amino acid, blocking the interaction of the RNA with Tat [Tan and Frankel, 1992]. RNA aptamers can also be selected on the basis of their Arg binding [Geiger et al., 1996]. There is already precedent for the direct inhibition of ribosomal peptidyl transferase activity by Arg [Palacián and Vazquez, 1979]. However, that inhibitory effect was elicited by either D-Arg or L-Arg, but D-Arg does

not elicit AAP-mediated control in *N. crassa* extracts [Wang and Sachs, 1997a] or *S. cerevisiae* extracts [data not shown].

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

That ribosomes which have translated the AAP are sensitive to stalling by Arg in extracts is clear. This effect could explain the translational response to Arg observed *in vivo* in *N. crassa*, in which Arg reduces the average number of ribosomes associated with *arg-2* mRNA [Luo et al., 1995]. But, in addition to reduced translation, the steady state level of *N. crassa arg-2* mRNA is also reduced by growth in Arg [Orbach et al., 1990; Sachs and Yanofsky, 1991; Luo et al., 1995; Freitag et al., 1996; Luo and Sachs, 1996]. Similarly, Arg affects the level of *CPA1* transcript [Crabeel et al., 1990]. Could there be a role for the uORF-encoded AAP in regulating the level of transcript in response to Arg in these systems, perhaps as a consequence of its function to modulate ribosome stalling? In *N. crassa* continuously grown in the presence of Arg, a reporter gene containing the wild-type *arg-2* uORF shows a reduction in both the level of translation and the level of mRNA, as does the endogenous *arg-2* gene. Introduction of the D12N mutation into the uORF of the reporter gene causes loss of regulation at both translation and mRNA levels *in vivo*, while the endogenous *arg-2* gene remains regulated [Freitag et al., 1996]. The wild-type *CPA1* mRNA is known to be destabilized by growth in Arg [Crabeel et al., 1990]. One hypothesis, which remains to be tested, that could link our observations on stalling *in vitro* in *S. cerevisiae* and *N. crassa* systems with observations *in vivo* in these fungi on regulation at the level of mRNA is that ribosome stalling at the wild-type AAP termination codon in response to Arg triggers RNA destabilization. Consistent with this possibility, links between uORF termination codons and RNA stability are observed in *S. cerevisiae* [Hilleren and Parker, 1999; Vilela et al., 1999].

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

CHAPTER 6
CONCLUSIONS AND FUTURE DIRECTIONS

6.1 Summary of Research

The long-term goal of this research has been to fully understand how *arg-2* and *CPA1* uORF encoded arginine attenuator peptides (AAPs) interact with the translational machinery in high concentrations of Arg to cause ribosomal stalling. Previous work done in the Sachs lab already demonstrated that, *in vivo*, translation of mRNA containing the wild-type *arg-2* uORF decreases in high Arg medium and that the decrease in translation is associated with a decrease in the number of ribosomes associated with the mRNA [Luo et al., 1995]. These and other results established that there is a uORF-mediated translational regulation for *arg-2* expression and that the coding sequence of the uORF is important to maintain the regulation [Luo et al., 1995; Freitag et al., 1996; Luo and Sachs, 1996]. Previous *in vivo* studies of *CPA1* in yeast also suggest a similar phenomenon [Werner et al., 1987; Delbecq et al., 1994]. The major focus of my Ph.D. research has been to elucidate the molecular mechanism of AAP-mediated, Arg-specific translational regulation.

6.1.1 Development of an Amino Acid-Dependent Cell-Free Translation System in Which the Arginine-Specific Regulation of *N. crassa arg-2* Is Fully Reconstituted [Wang and Sachs, 1997a]

In this *in vitro* translation system, the cap and poly(A) structures in a synthetic RNA worked synergistically to stimulate its translation. Synthetic RNA in which the wild-type *arg-2* uORF is placed in front of the firefly luciferase region, when added to this translation system, results in a substantial decrease in luciferase production in response to a high level of Arg. Mutation of uORF Asp codon 12 to Asn, which

eliminated regulation *in vivo*, eliminated regulation *in vitro*. Elimination of the uORF translation initiation codon also eliminated Arg-specific regulation. mRNA translation in this system is also initiation context-sensitive, because improving the uORF translation initiation context decreased the luciferase production and only slightly increased the magnitude of regulation. An Arg dilution time course experiment suggested that Arg had little effect on mRNA stability. Finally, analysis of Arg-related compounds indicated that this Arg-specific translational regulation was specific for L-arginine.

6.1.2 Introduction of a Sensitive Assay Called "Toeprinting" (Primer Extension Inhibition) into This Cell-Free Translation System [Wang and Sachs, 1997b]

In analyses of RNA containing uORF sequences, prematurely terminated reverse-transcription products were found ~16 nt distal from the uORF start codon, ~13 nt distal from the uORF termination codon, and ~16 nt distal from the LUC start codon. These toeprinting sites correspond to ribosomes positioned on RNA, with initiation codons at their P sites and termination codon or codons of the limiting amino acids at their A sites, respectively. The appearance of these toeprint signals depended on the coding sequence and coding capacity of the uORF. Their appearance also was affected by adding translational inhibitors such as puromycin or limiting amino acids in this cell-free translation system. When surplus Arg was added to translation reactions, a marked increase in the intensity of the toeprint signal at the wild-type uORF termination codon was observed, and an additional signal appeared 21 to 30 nt upstream of this codon. A decrease in the toeprint signal at the luciferase initiation codon was also observed. Mutants that change the predicted amino acid sequence in the evolutionarily conserved region of the uORF peptide abolished Arg-specific regulation by luciferase assay, also associated with reduced or loss of stalling at the uORF termination codon by toeprinting.

6.1.3 Investigation of the Requirements for *N. crassa arg-2* uORF Function in Arg-Specific Regulation [Wang et al., 1998]

With assistance from my fellow graduate student Peng Fang, we showed that neither the distance between the uORF stop codon and the downstream initiation codon nor the nature of the stop codon used to terminate translation of the AAP is important for regulation. Furthermore, in high arginine, elongating ribosomes are stalled just after they have translated the AAP sequence when this sequence is directly fused at the N terminus of luciferase. Thus, the AAP appears to function as a nascent peptide that acts in *cis* to cause regulated stalling of ribosomes. Recent work done in the Sachs lab [Fang et al., 2000] showed that parallel introduction of silent mutations at each possible codon where substitution was possible (in a functional, shortened AAP coding region; 26/63 nt changes) did not significantly affect AAP function, although single nucleotide changes altering the conserved peptide sequence eliminated function. These data clearly indicate that the sequence of the evolutionarily conserved nascent peptide, but not the sequence of the mRNA that encodes it, is responsible for arginine-specific translational attenuation.

6.1.4 Investigation of the Generality of This AAP-Mediated Regulation, Through a Collaborative Effort with Fellow Student Anthony Gaba [Wang et al., 2000]

We were able to show that the *N. crassa* AAP and the closely related AAP encoded by the uORF in the 5′-leader of the *S. cerevisiae CPA1* mRNA exert Arg-specific, negative translational regulation in *N. crassa*, *S. cerevisiae*, and wheat germ cell-free translation reactions. AAP-containing mRNAs were used to demonstrate that the extent of translational regulation by [Arg] was proportional to [Arg] at concentrations between 150 and 5,000 μM. Yet, in the *S. cerevisiae* and wheat germ systems, each of the arginyl-tRNAs examined appeared fully charged even at much lower Arg concentrations (10 μM). These experiments indicate that the level of charged Arg-tRNAs is not significant for AAP-mediated, Arg-specific translational control.

6.2 Future Directions

My work represents the demonstration of translational control in response to the availability of a single amino acid in a eukaryotic cell-free translation system. This system is one of a handful of instances in which we understand how the uORF-encoded peptides control protein synthesis. Furthermore, AAP-mediated ribosomal stalling is the strongest evidence to date for the primary importance of *cis*-acting translational control of the nascent peptide.

The major function of AAP is to negatively regulate the translation of the small subunit of carbamoyl phosphate synthetase, an enzyme involved in Arg synthesis, when Arg itself is plentiful. Cells have evolved regulatory pathways at different levels to respond to changes in physiological conditions. Some of the regulatory mechanisms are very complicated and involve numerous players. However, examples are also seen in which cells utilize simpler and more economical mechanisms to achieve regulation, as opposed to those that are more complicated and energy-consuming. We can appreciate such mechanisms from studies of transcriptional attenuation of the *E. coli* Trp operon. A recent study on the mechanism of translational induction of heat shock transcription factor σ^{32} in bacteria also supports this strategy, in which partial melting of the mRNA secondary structure at high temperature enhances ribosome entry and translational initiation without involvement of other cellular components [Morita et al., 1999]. Arg-specific regulation mediated by the AAP is a *cis*-acting regulatory mechanism. How does it work? This is the key question that needs to be answered. By answering questions such as those discussed below, we should be able to determine the detailed mechanism of Arg-specific regulation.

6.2.1 Do Arg and AAP Interact with Each Other to Cause Ribosomal Stalling?

Results provided in this demonstration demonstrated that Arg exerts its effect rapidly. AAP-LUC fusion polypeptide synthesis is inversely proportional to [Arg], while stalling is proportional to [Arg]. Furthermore, the level of arginyl-tRNA

charging appears invariant. These results favor the idea that Arg is directly involved in ribosomal stalling. To address the question whether Arg and AAP interact with each other, several biochemical experiments can be executed. (1) AAP can be fused to a reporter polypeptide (e.g., calmodulin binding protein, or maltose binding protein), expressed, purified to homogeneity, and then immobilized to a column. Radio-labeled Arg can be loaded onto the column. If under appropriate conditions, Arg is retained in the resin immobilized with the wild-type AAP but not the D12N mutant AAP, that would strongly support the direct interaction of Arg-AAP. (2) A size filtration column may show directly the existence of an AAP-Arg complex. After incubation of AAP with radio-labeled Arg, the mixture can be loaded onto the column. If AAP and Arg form a complex, a peak of radioactivity should appear ahead of free Arg. (3) NMR experiments will be extremely instructive if, in solution, Arg can cause a substantial conformational change for wild-type AAP compared to D12N AAP.

6.2.2 What Is the Target(s) for AAP and Arg?

For AAP to function in the presence of high Arg, it must to somehow interact with the translational machinery to stall the movement of ribosomes. How can we explore the targeting sites of the AAP-Arg complex?

The examination of mutants has been a powerful tool in *Neurospora* and yeast to understand gene regulation and function. By applying a similar strategy used in previous *in vivo* mutagenesis studies [Freitag et al., 1996], *trans*-acting mutants that affect AAP-mediated regulation can be obtained. Then, using available genomic or cDNA libraries in these systems, the particular genes involved will be identified. Factors encoded by the genes will be tested by knock-out and add-back experiments in wild-type and mutant backgrounds to confirm their effects.

Cross-linking experiments (e.g., UV cross-linking [Vagner et al., 1995b]) can be used to check the direct interaction of AAP with Arg in the stalling event. Antibodies against AAP could be used to probe the AAP or Arg's target site by checking the cross-linked complex including AAP. By purifying the cross-linked complex with AAP, we would expect to identify the RNA or protein sequences

involved in the complex by reverse transcription linked to PCR (RT-PCR) or by microsequencing of the polypeptide.

The ultimate elucidation of the mechanism may result from studies in a purified translation system. Purified systems available in bacteria and mammals for initiation studies [Hartz et al., 1989; Pestova et al., 1996a,b]. Progress has been made in a few yeast labs trying to develop a fully purified translation system (e.g., Mangiarotti and Chiaberge [1997]).

If a fully purified cell-free translation system is reconstituted, adding Arg to the system and checking its effects will tell if Arg exerts its effect by itself. In crude extract, Arg may be involved in a quick metabolic pathway that converts Arg to its immediate metabolite, which can exert the effect. In the purified system, the likelihood for Arg to be converted to other metabolites is very low.

By applying cross-linking experiments in the purified system, we would also have a good chance of demonstrating the direct interaction of AAP with Arg. Furthermore, it would be easier and more convincing to purify the nucleotide sequences or protein sequences involved in the complex by RT-PCR or microsequencing of peptides; therefore, we could find the target candidates for the AAP-Arg complex. If the same gene product(s) was discovered by cross-linking and *in vivo* mutagenesis, that would strongly indicate its involvement in AAP-mediated regulation. If the candidate is different from the ones identified by *in vivo* mutagenesis, then by applying *in vitro* mutagenesis and genetics, strains with the wild-type candidate gene disrupted and mutant forms reintroduced will be used to check if the Arg-specific regulation is abolished. Thus, a better idea of how this regulation is achieved can be obtained.

6.2.3 Applications of Toeprinting *in Vitro* for Studying Other Events of Translation

By applying toeprinting in our cell-free systems, we have been able to monitor the movements of ribosomes in initiation, elongation, and termination. We can see the effect of initiation context in the *Neurospora* cell-free system. We have been able to show that leaky scanning is the major initiation event when AAP functions as a

uORF. These data lead us to believe that we can apply the technique to study other related translational regulation phenomena.

There are four uORFs in *S. cerevisiae GCN4* which have important biological functions for the translation of *GCN4* [Chapter 1; Hinnebusch, 1996]. Its *Neurospora* homolog *cpc-1* has two uORFs [Ebbole et al., 1991; Luo et al., 1995]. For *GCN4*, it has been believed that all ribosomes loaded onto the mRNA initiate at uORF1. After that, about 50% of them will stay on the mRNA and keep scanning downstream to reinitiate at uORFs 2-4 or the *GCN4* ORF. uORF4 inhibits any reinitiation once it is translated. By applying a toeprinting assay, it should be possible to obtain physical evidence for the functions of the uORFs and the reinitiation mechanism. In fact, recent studies in Sachs lab have shown encouraging results [Wang, Z., Gaba, A., and Sachs, M. S., unpublished data].

FRQ is a central component for a circadian clock in *N. crassa* [Dunlap, 1999]. The mRNA of *frq* contains several uORFs in a secondary structure-rich region and three in-frame AUGs for its main ORF. Two isoforms of FRQ are believed to be synthesized by alternative translation initiating from AUG1 and AUG3 [Liu et al., 1997]. Previous results indicate that, at higher temperature, the initiation ratio at AUG1 increases compared to that of AUG3. The initiation is hypothesized to be through an internal initiation mechanism. Could this differential initiation resemble that of bacterial heat shock transcription factor σ^{32}? That is, could a higher temperature affect the structure of the mRNA, which would allow more ribosomes to load at AUG1? We have the tools to examine this possibility.

Many genes encoding proto-oncogenes, transcription factors, growth factors, and their receptors can have uORFs, but how these uORFs function remains largely unknown. The approaches and techniques developed in my thesis work can be used for the study of other uORFs. Furthermore, more detailed understanding of the AAP-mediated ribosomal stalling will expand our knowledge of translational control and benefit human health.

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BIOGRAPHICAL SKETCH

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