Translational regulation of arg-2 gene expression in Neurospora crassa

Zongli Luo

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Translational regulation of arg-2 gene expression in *Neurospora crassa*

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B.S. Nankai University, 1989

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DEDICATION

To my parents, Benyuan Luo and Chaoming Zhou,
and to my sister, Zhongzhi Luo
ACKNOWLEDGMENT

I especially want to thank Dr. Matthew S. Sachs, my supervisor, for his wonderfully dedicate guidance and instruction during my five-year Ph.D. studies. The guidance and instruction will certainly benefit me in the future to continue successfully as a scientist in the field of biology. I also want to thank Dr. Michael H. Gold and Dr. James M. Cregg for their numerous help on lab works, seminars, and scientific strategies. I want to thank Dr. Merille H. Hille from the University of Washington for her attention as a member for my thesis examination committee. I want to thank my co-worker Michael Freitag for help and cooperation with the lab work. Finally, I would like to thank Nancy Christie and Terrie Hadfield for their administrative help during the last five years.
# TABLE OF CONTENTS

DEDICATION ........................................................................................................................................ iii
ACKNOWLEDGMENTS ...................................................................................................................... iv
TABLE OF CONTENTS .................................................................................................................... v
LIST OF FIGURES .......................................................................................................................... viii
LIST OF TABLES .............................................................................................................................. ix
ABSTRACT .......................................................................................................................................... x

CHAPTER 1 INTRODUCTION ............................................................................................................. 1
  1.1 The molecular mechanism of translation ................................................................................. 2
    1.1.1 Initiation ......................................................................................................................... 2
    1.1.2 Elongation ....................................................................................................................... 9
    1.1.3 Termination ..................................................................................................................... 11
  1.2 Regulation at the level of translation ....................................................................................... 12
    1.2.1 The structure of mRNA ................................................................................................. 13
    1.2.2 Phosphorylation of protein factors in translational machinery .................................... 20
  1.3 mRNA stability and translation ............................................................................................... 25
  1.4 upstream open reading frame (uORF), a feature indicating translational regulation ................ 26
    1.4.1 uORFs which do not require amino acid coding information for regulation ................. 27
    1.4.2 uORFs which require amino acid coding information for regulation ............................. 30
  1.5 *Neurospora crassa* as a model system .................................................................................. 35
  1.6 *arg-2* and its regulation ........................................................................................................ 36
CHAPTER 2 MATERIALS AND METHODS ......................................................... 39
  2.1 Growth of *N. crassa* ................................................................. 39
  2.2 Preparation of anti-Arg2p serum .................................................. 39
  2.3 Immunoblot analyses ................................................................. 40
  2.4 Pulse-labeling with $^{35}$S-methionine
      and immunoprecipitation ....................................................... 41
  2.5 Preparation of RNA ................................................................. 42
  2.6 Northern blot analyses ............................................................. 43
  2.7 Polysome analysis ................................................................. 43
  2.8 Plasmid construction for *lacZ* expression studies ....................... 44
  2.9 Construction of *N. crassa* strains containing *arg-2-lacZ*
      integrated at the *his-3* locus .................................................. 46
  2.10 Southern blot analysis ............................................................ 46
  2.11 $\beta$-galactosidase assays ..................................................... 47
  2.12 Immunoprecipitation of LacZ from *N. crassa* crude cell extracts ...... 47
  2.13 Quantification ....................................................................... 48

CHAPTER 3 RESULTS ............................................................................. 49
  3.1 Prolonged exposure to Arg reduces CPS-A activity,
      the level of Arg2p, the rate of Arg2p synthesis, and
      the level of *arg-2* transcript .................................................... 49
  3.2 Short-term exposure to Arg reduces the rate of Arg2p
      synthesis but not the level of *arg-2* transcript ........................... 51
  3.3 Comparison of short-term changes in response to Arg
      or His availability ................................................................ 58
  3.4 Obtaining *N. crassa* strains containing *arg-2-lacZ* integrated
      at the *his-3* locus .................................................................. 61
  3.5 Effects of altering *arg-2* 5' sequences on the expression
      of $\beta$-galactosidase ................................................................ 63
  3.6 All constructs produced similar levels of *arg-2-lacZ* mRNA
      that were not subjected to negative regulation by Arg .................... 67
3.7 The arg-2 uORF is important for negative regulation of
argin-2-lacZ by Arg at the translational level ........................................ 69

CHAPTER 4 DISCUSSION ................................................................................. 72
4.1 Multiple levels of regulation for arg-2 and cpc-1 ............................. 72
4.2 Defining the role of arg-2 uORF in Arg-specific regulation ............. 74
4.3 Common features of the arg-2 uORF and other uORFs .................... 76
4.4 Ribosomal stalling could be involved in Arg-specific
regulation of arg-2 and CPAI through uORF ........................................... 79
4.5 Future work ......................................................................................... 80
4.6 Other aspects of translational and transcriptional regulation
in N. crassa ......................................................................................... 81

REFERENCES ......................................................................................... 83
BIOGRAPHICAL SKETCH ................................................................. 121
LIST OF FIGURES

Figure 1.1 The initiation phase of protein synthesis predicted by the scanning model .......................................................... 3
Figure 1.2 The elongation and termination phase of protein synthesis ................................................................. 4
Figure 3.1 The effect of prolonged exposure to Arg on the expression of arg-2 and cox-5 ................................................. 50
Figure 3.2 Analysis of mRNA distribution in polysomes prepared from cells grown in Min and Arg media ................. 52
Figure 3.3 The effect of short-term cell exposure to Arg on arg-2 and cox-5 ................................................................. 55
Figure 3.4 The effect of short-term cell exposure to Arg on the arg-2 and cox-5 poly(A) mRNA ........................................ 56
Figure 3.5 Polysome profile analysis of cells switched from minimal medium to minimal, Arg, AT, or AT + His media for 30 min ................................................................. 57
Figure 3.6 The effect of amino acid availability on arg-2, cox-5, leu-6, and cpc-1 ......................................................... 59
Figure 3.7 Homologous recombination of transforming plasmids at the his-3 locus ............................................................ 62
Figure 3.8 Effect of growth in Arg on the level of ß-galactosidase activity expressed from arg-2-lacZ reporter genes .......... 64
Figure 3.9 Effect of growth in Arg on the levels of arg-2-lacZ, arg-2 and cox-5 mRNA .................................................. 68
Figure 3.10 Effect of Arg on the distribution of arg-2-lacZ, arg-2, cox-5 and cpc-1 mRNA in polysomes ...................... 70
Figure 4.1 Some models for arg-2 uORF function ................................................................. 77
LIST OF TABLES

Table 3.1 Relative expression of *arg*-2 and *cox*-5 after short-term exposure to Arg ................................................................. 53
Table 3.2 Relative expression of *arg*-2 and *cox*-5 after short-term exposure to Arg, AT, AT + His, or His ........................................ 60
Table 3.3 β-galactosidase activities before or after immunoprecipitation with anti *E. coli* β-galactosidase antibody ........................................... 66
ABSTRACT

Translational regulation of arg-2 gene expression in Neurospora crassa

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Neurospora crassa arg-2 encodes the small subunit of arginine (Arg)-specific carbamoyl phosphate synthetase; it is subject to unique negative regulation by Arg and is positively regulated in response to limitation for many different amino acids through a mechanism known as cross-pathway control. Control of arg-2 expression regulates the flux of metabolites through the Arg biosynthetic pathway under most growth conditions.

Analyses of mRNA levels, polypeptide synthesis rates, and the distribution of mRNA in polysomes indicated that Arg-specific negative regulation of arg-2 affected the levels of both arg-2 mRNA and arg-2 mRNA translation. Negative translational effects on arg-2 and positive translational effects on cpc-1 were apparent soon after cells were provided with exogenous Arg. In cells limited for histidine, increased expression of arg-2 and cpc-1, and decreased expression of cox-5, also had translational and transcriptional components.

arg-2 contains a 24-codon upstream open reading frame (uORF) in the 5' region of its mRNA. Examination of arg-2-lacZ reporter genes containing wild-type or mutated arg-2 5' sequences revealed that none of them showed regulation by Arg at the mRNA level, indicating that their Arg-specific regulation were post-transcriptional. Constructs containing a wild type uORF or a frame-shifted uORF showed reduced gene expression
compared to a construct lacking an uORF, as determined by β-galactosidase measurements. However, only the wild type uORF conferred additional, Arg-specific regulation. Transcripts containing either uORF showed reduced association with polysomes relative to transcripts lacking an uORF, but only the transcript with the wild type uORF showed a reduction in the average number of ribosomes associated with it in response to Arg. Additional results indicated that the uORF is translated and that ribosome reinitiation is not an integral part of uORF-mediated regulation. These studies provide direct biochemical evidence for arg-2 uORF function in translational control.
CHAPTER 1
INTRODUCTION

Protein synthesis or translation, mediated by ribosomes using messenger RNA (mRNA) as the template, is one of the hallmarks of life on earth. It is an integral part of the pathway of gene expression and makes important contributions to modulation of the expression of specific genes. Protein synthesis represents the final process in the direct transfer of genetic information within the cell. The rate of protein synthesis will inevitably limit how quickly genetic events can be transmuted into cell growth and division. The process of translation is propelled by a complex translational machinery involving interactions among ribosomes, mRNA, aminoacyl-tRNAs, and other factors.

The process of translation is generally divided into three major steps: initiation, elongation, and termination. This is true for both prokaryotes and eukaryotes. Ribosomes, which are the major components of translational machinery, have the same overall structure from bacteria to mammals. The processes of aminoacyl-transfer RNA (tRNA) binding, peptide bond formation, and peptidyl-tRNA translocation are virtually identical in all organisms, and all ribosomes display the same division of labor between the small and large subunits. However, striking dissimilarities do exist between prokaryotes and eukaryotes in the detailed molecular mechanisms of translation, particularly during the stage of initiation. Whereas prokaryotic ribosomes interact directly with the AUG initiator codon or with the nearby Shine-Dalgarno sequence (Calogero, et al., 1988), most eukaryotic ribosomes apparently interact with the capped 5'-end of the mRNA and advance to the AUG codon by linear scanning (Kozak, 1989c). The prevalence of polycistronic mRNAs in prokaryotes versus monocistronic mRNAs in eukaryotes may follow from that basic difference in the mechanism of initiation. Whereas the initial contact between bacterial ribosomes and mRNA requires no initiation factors or cofactors, ATP and a considerable number of protein factors are needed for eukaryotic ribosomes to bind the mRNA. Another difference is that in prokaryotes the small ribosomal subunit can bind mRNA at the Shine-Dalgarno sequence before binding
initiator Met-tRNA, whereas in eukaryotes, the small ribosomal subunit binds stably to mRNA only after initiator Met-tRNA has bound.

Besides the mechanism of initiation of translation, other differences, such as the structure of mRNA and the overall cell structure, exist between prokaryotes and eukaryotes to dictate different approaches to regulating translation. Since *Neurospora crassa* is an eukaryotic organism, this introduction will concentrate on describing the mechanism and regulation of translation in eukaryotic cells.

1.1 The molecular mechanism of translation in eukaryotes

In order to understand how translation is regulated at the molecular level, knowledge of the molecular mechanism of protein synthesis is required. As described above, protein synthesis is driven by a complex translational machinery and the whole process is divided into three phases: initiation, elongation, and termination. The reactions in each phase are promoted by soluble protein factors that transiently interact with the ribosome, mRNA, and aminoacyl-tRNAs. A schematic diagram depicting the process of translation is shown in Figures 1.1 and 1.2.

1.1.1 Initiation

Initiation of protein synthesis (Figure 1.1) in eukaryotic cells requires two tasks to be achieved: (i) an mRNA among many available candidates is selected for translation by the initiating ribosome; (ii) the ribosome identifies the initiator codon and begins translation in an appropriate reading frame. It is a complex process in which many protein and RNA molecules interact with each other giving rise to an initiation complex which consists of an 80S ribosome, mRNA, and initiator methionyl-tRNA (Met-tRNA_\text{f}__). The Met-tRNA_\text{f}__ binds directly into the P site of the ribosome, thus leaving the A site available for the binding of a second aminoacyl-tRNA, whereafter the elongation cycle of protein synthesis starts.

At least ten initiation factors (abbreviated eIF) have been found to be involved in promoting initiation. These protein factors have been highly purified, and a number of their cDNAs or genes have been cloned and sequenced. The initiation pathway can be summarized as a four-step process:
Figure 1.1 The initiation phase of protein synthesis predicted by the scanning model (Redrawn from Hershey, 1991).
Figure 1.2 The elongation and termination phase of protein synthesis (Redrawn from Hershey, 1991).
(i) Dissociation of 80S ribosome into 40S and 60S ribosomal subunits. 80S ribosomes predominate in active equilibrium with dissociated subunits at Mg$^{2+}$ concentrations thought to be physiological (>1 mM). Two initiation factors, eIF-1A (eIF-4C) and eIF-3, shift the equilibrium towards dissociation by binding to 40S subunits and preventing their association with 60S subunits (Goumans, et al., 1980). eIF-1A has a pronounced stimulatory effect on initiation complex formation with native 80S ribosomes, but only a small effect when washed 40S subunits are used. eIF-1A is accessory to eIF-3 in dissociating 80S ribosomes. Another factor, eIF-3A (eIF-6), binds to 60S subunits and prevents subunit association \textit{in vitro} (Russell and Spremulli, 1979).

(ii) Formation of 43S preinitiation complex. Met-tRNA$_i$ binds to the native 40S ribosomal subunit by means of eIF-2 and GTP. The binding of Met-tRNA$_i$ to 40S ribosomal subunits is a step common to the translation of all eukaryotic mRNAs. eIF-2, in a binary complex with GTP, binds Met-tRNA$_i$ to form a ternary complex. The ternary complex is an obligatory intermediate that can be readily identified and isolated. Ternary complexes are formed in high yield with purified components at physiological concentrations of eIF-2 and tRNA (Benne, et al., 1979; Konieczny and Safer, 1983). At lower concentrations, stabilization by ancillary factors has been observed. One of these, eIF-2C, acts stoichiometrically to stabilize the complex and prevent its disruption when naked mRNA is added (Roy, et al., 1988). Another stabilizing ancillary factor is eIF-3, which also stimulates ternary complex formation at low concentrations of eIF-2 and Met-tRNA$_i$. However, disruption by mRNA is not prevented in the case of eIF-3.

The ternary complex eIF-2-GTP-Met-tRNA$_i$ binds to the 40S ribosomal subunit to form a 43S preinitiation complex that is sufficiently stable to be isolated by sucrose density gradient centrifugation. Such complexes are stabilized by eIF-3 and eIF-1A, but do not require the presence of mRNA (Benne and Hershey, 1978; Trachsel, et al., 1977). Furthermore, GTP hydrolysis does not occur and nonhydrolyzable GTP analog can function in these steps. Since stable mRNA-40S complexes are not detected in the absence of Met-tRNA$_i$, the proposed pathway of initiation places Met-tRNA$_i$ binding prior to mRNA binding. However, other pathways involving unstable intermediates are possible since initiation complexes are most frequently analyzed by sucrose density gradient centrifugation, which usually requires hours of centrifugation to separate ribosome-bound and free components. Many physiologically relevant complexes may
dissociate in seconds rather than hours. Thus failing to detect stable mRNA-40S complexes does not exclude the possibility that the initial binding of mRNA to 40S ribosomal subunits occurs as an unstable complex in the absence of eIF-2 and Met-tRNAi.

(iii) Binding of 43S preinitiation complex to mRNA and selection of the translation initiation site. Either of the following two modes of mRNA binding and translation initiation site selection are known to occur: (i) ribosome scanning, in which the ribosome binds to the 5'-terminus of the mRNA, then scans linearly along the mRNA until it recognizes the initiator codon; (ii) internal initiation, in which the ribosome binds to an internal region of the mRNA, either directly at the initiator codon, or upstream followed by a scanning process. In either case, binding of 43S preinitiation complex to mRNA is thought to be promoted by ATP hydrolysis and by a number of initiation factors. Initiation factors eIF-4A, eIF-4B, eIF-4E, and eIF-4F, all of which display an RNA-dependent ATPase activity, can help unwind the secondary structure of the 5' untranslated region (5'-UTR) of the mRNA and allow the 43S preinitiation complex to scan along the single-stranded RNA towards the AUG initiation codon. The selection of AUG codon is made possible by the presence of the Met-tRNAi on the 43S preinitiation complex. It is generally believed that the 43S preinitiation complex with Met-tRNAi, eIF-2, eIF-3, and eIF-1A is the active form of the ribosomal subunit (Benne and Hershey, 1978), but this view is not firmly established.

The scanning model seems to account for initiation of the large majority of cellular mRNAs (Kozak, 1989c). Important features of mRNA structure that affect translation initiation include the presence of a 7-methyl-guanylic acid "cap" (m7G cap) at the 5'-terminus, aspects of secondary and tertiary structure, the choice of initiator codon (usually AUG) and surrounding sequence context, and the poly(A) tail at the 3'-terminus (Kozak, 1991d; Sachs and Wahle, 1993).

The role of initiation factors in the scanning mechanism is beginning to be understood (Rhoads, 1988; Sonenberg, 1988). A working hypothesis is that eIF-4F binds to the cap through its α-subunit (eIF-4E), which is known as the cap-binding protein because it binds and can be crosslinked to the cap structure. eIF-4A and eIF-4B may then join the complex. Highly purified eIF-4A or eIF-4F in combination with eIF-4B exhibits an ATP-dependent RNA helicase activity (Lawson, et al., 1989; Ray, et al.,
eIF-4A is an RNA-dependent ATPase (Grifo, et al., 1984) and is homologous to other "D-E-A-D box" proteins with helicase activity (Linder, et al., 1989); eIF-4B contains a consensus RNA-binding domain found in RNA-binding proteins (Milburn, et al., 1990b). ATP hydrolysis is essential for the unwinding process but may or may not be directly involved in 40S ribosomal subunit migration. It is possible that higher-order initiation factor complexes are involved in cap recognition. Both eIF-4F and eIF-4B bind to eIF-3, and evidence for such super-factor complexes has been reported (Duncan, et al., 1987; Etchison and Smith, 1990). Since eIF-3 binds tightly to 40S ribosomal subunits and also to mRNA, it may be the link between the 40S ribosomal subunit and the mRNA-factor complexes.

Following recognition of the cap structure by the initiation factors and the melting of secondary structure near the 5'-terminus, the 43S preinitiation complex binds to the cap-proximal region of the mRNA. The 40S ribosomal subunit then begins the scanning process in search of the initiator codon. The scanning model predicts that the 5'-proximal initiator codon should be preferred, and this is true for more than 90% of cellular mRNAs (Kozak, 1987a). However, the sequence context of AUG affects the recognition. A strong AUG context includes purines at positions -3 and +4, where the A of AUG is +1 (Kozak, 1989c). When a scanning ribosome encounters an AUG with a weak context consensus sequence, it may pass over the AUG and continue to scan the mRNA. Genetic studies implicate eIF-2 in the recognition process; mutations in the α- and β-subunits of eIF-2 alter codon selection in yeast (Cigan, et al., 1989; Donahue, et al., 1988). The anticodon of Met-tRNA; interacts with the AUG, as has been shown in yeast by mutational analysis (Cigan, et al., 1988). The 40S initiation complex presumably pauses at AUGs with strong consensus sequences, thereby enabling the 60S ribosomal subunit joining reaction to occur. mRNA secondary structure properly placed downstream of the initiator AUG promotes initiation, presumably by enhancing pausing near the AUG and impeding further scanning (Kozak, 1990).

mRNA binding to ribosomes by the "internal initiation" mechanism has been demonstrated for poliovirus (Pelletier and Sonenberg, 1988) and encephalomyocarditis (EMC) virus RNAs (Jang, et al., 1989). The long 5'-UTRs and the presence of numerous upstream AUGs between the uncapped 5'-terminus and initiator codon raised the possibility that these mRNAs are probably not or difficult to be translated by a scanning
mechanism. Proof for an internal initiation mechanism has been accomplished by constructing di-cistronic genes with the ribosome entry region of the poliovirus or EMC virus 5'-UTRs inserted between the cistrons (Jang, et al., 1989; Pelletier and Sonenberg, 1988). Ribosomal entry for translation between the two cistrons is indicated by enhanced in vivo expression of the downstream cistron in transfected cells, even when translation of the upstream cistron is inhibited. Northern blot analyses of RNA on polysomes actively translating only the second cistron ruled out the possibility that translation of the second cistron is due to partial RNA cleavage. Although first evidence for internal initiation has been obtained with viral RNAs, the translation of a cellular mRNA encoding the immunoglobulin heavy chain binding protein in poliovirus-infected cells (Macejak and Sarnow, 1991) and alternative translation of human fibroblast growth factor 2 mRNA (Vagner, et al., 1995) have been shown to be initiated by the internal ribosome entry mechanism. The translation of *N. crassa* Albino-3 mRNA may also involve an internal translation initiation mechanism (Vittorioso, et al., 1994). Other eukaryotic cellular mRNAs with long 5'-UTRs that contain numerous AUGs, e.g., human c-abl mRNA (Bernards, et al., 1987), probably also are good candidates for translation by the internal ribosome initiation mechanism. Internal initiation mechanism may play an important role in enabling the translation of a small class of mRNAs when the cap-dependent scanning mechanism is repressed.

A novel mechanism of ribosome initiation has been proposed based on the studies for the polycistronic cauliflower mosaic virus 35S RNA (Futterer, et al., 1993). The translation of the far downstream cistron appears not to be initiated by an internal ribosome entry as described above. Rather, it appears that 40S ribosome subunits initiate scanning normally from the 5'-cap of the RNA, but the scanning ribosome is shunted to the 5' region of the far downstream cistron to initiate translation instead of arriving there by linear scanning. Moreover, experimental evidences indicated that this shunting process can also occur between separate RNA molecules.

(iv) Formation of 80S initiation complex by joining of the 60S subunit to the 40S initiation complex. The 60S ribosomal subunit binds to the 40S subunit carrying mRNA and Met-tRNA\text{\textsubscript{\text{f}}} positioned at the initiator codon. The reaction requires the function of eIF-5 and the hydrolysis of the GTP molecule bound to eIF-2. The GTPase reaction is promoted by eIF-5 in the absence of 60S subunits and results in the ejection of
eIF-2-GDP and other bound factors such as eIF-1A and eIF-3 (Raychaudhuri, et al., 1985). eIF-2 can enter another round of initiation by exchanging its bound GDP to GTP; this reaction is catalyzed by eIF-2B (guanine nucleotide exchange factor) (Dholakia and Wahba, 1989; Rowlands, et al., 1988). Subsequent binding of the 60S subunit may be rapid, since the stability of bound Met-tRNA\textsubscript{i} to 40S ribosomes in the absence of eIF-2 is low.

Following 80S initiation complex formation, the elongation phase of protein synthesis commences. However, formation of the first peptide bond has unique features that distinguish it from subsequent peptide bond formation reactions: the Met-tRNA\textsubscript{i} in the ribosomal P site (donor site) has a charged $\alpha$-amino group, whereas in subsequent rounds of the elongation cycle, the aminoacyl-tRNA derivative in the P site is acylated as peptidyl-tRNA.

1.1.2 Elongation

The elongation phase of protein synthesis (Figure 1.2) is a cyclic process that adds one amino acid residue to the C-terminal end of the nascent polypeptide chain per turn of the cycle. The pathway of elongation involves three major steps: (i) binding of aminoacyl-tRNA to the A site of the ribosome; (ii) formation of the peptide bond; (iii) translocation. Elongation is promoted by elongation factors (abbreviated eEF) and considerable energy is expended during each cycle; at least one high-energy pyrophosphate bond in GTP is cleaved at both the binding and translocation steps; and two additional high-energy bonds are required to synthesize the aminoacyl-tRNA that is consumed. The process is rapid, with a ribosome incorporating up to six amino acids per second (Goustin and Wilt, 1982; Haschemeyer, 1976). Key features of elongation phase are high fidelity, i.e., the ability to match properly the aminoacyl-tRNA and the codon in the mRNA template, and processivity, i.e., the ability to synthesize long polypeptides without premature dissociation of the peptidyl-tRNA.

eEF-1\textalpha (Bermek, 1978; Kaziro, 1978; Moldave, 1985) catalyzes the binding of aminoacyl tRNA to the A site of ribosomes. The binding of GTP to eEF-1\textalpha favors energetically the subsequent attachment of aminoacyl tRNA (ternary complex formation), followed by binding of the ternary complex to the ribosome. Subsequent to ribosome binding, GTP is hydrolyzed to GDP and the factor is released as an eEF-1\textalpha-GDP.
complex. eEF-1α possess an intrinsic GTPase activity (Crechet and Parmeggiani, 1986; Slobin, 1983) which is stimulated upon interaction of ternary complexes with ribosomes.

Although eEF-1α binds GTP and GDP with about equal affinity, the spontaneous rate of dissociation of GDP from the factor is slow. To recycle, the eEF-1α bound to GDP depend on eEF-1β, which is usually found in association with eEF-1γ (Janssen and Moller, 1988). eEF-1β by itself has been found to catalyze a guanine nucleotide exchange reaction, while eEF-1γ stimulates the exchange reaction slightly (Janssen and Moller, 1988).

It has been known for some time that yeast requires, in addition to the above mentioned elongation factors, another protein factor designated as eEF-3 (Qin, et al., 1987; Skogerson and Wakatama, 1976; Uritani and Miyazaki, 1988). eEF-3 was uniquely required by yeast ribosomes for the translation of poly U (Skogerson and Wakatama, 1976) and natural mRNA (Herrera, et al., 1984; Hutchison, et al., 1984). An isolated temperature-sensitive mutant of yeast harboring an altered eEF-3 was unable to grow at a nonpermissive temperature, indicating that the factor is essential for translation in vivo (Herrera, et al., 1984; Kamath and Chakraburtty, 1986a; Kamath and Chakraburtty, 1986b). There is evidence that eEF-3 plays an important role in correctly positioning aminoacyl tRNA on ribosomes (Kamath and Chakraburtty, 1989; Uritani and Miyazaki, 1988) and that a functionally similar factor may be an essential constituent of all eukaryotic ribosomes.

When aminoacyl tRNA is correctly positioned at the ribosomal A site and the P site is occupied by peptidyl tRNA, the ribosome has the capacity to catalyze peptide bond formation. The catalytic agent is generally referred to as peptidyl transferase. Studies in prokaryotic system show that ribosomes retain considerable peptidyl transferase activity even when most ribosomal proteins are removed, indicating that rRNA may be the essential component of peptidyl transferase (Noller, 1993).

Upon completion of the peptidyl transferase reaction, the ribosomal P site is occupied with deacylated tRNA and the A site contains the newly formed peptidyl tRNA. The translocation reaction results in the movement of the ribosome and/or mRNA in relation to each other, by precisely one triplet, from the 5'- to the 3'-end of the mRNA. Translocation is dependent upon eEF-2 (Bermek, 1978; Moldave, 1985) and GTP. The translocation process commences when an eEF-2-GTP complex binds to a
pretranslocation complex containing peptidyl tRNA in the A site. At some point in the translocation reaction GTP is hydrolyzed to GDP and Pi and eEF-2 is released from the posttranslocated ribosome. The catalytic center for GTP hydrolysis appears to be located within eEF-2 (De Vendittis, et al., 1986). However, the GTPase activity of eEF-2 is very low in the absence of ribosomes (Nygard and Nilsson, 1989).

Complexes of eEF-2 and ribosomes can be formed in the presence of nonhydrolyzable analogs of GTP, indicating that GTP hydrolysis is not required to bind the factor to ribosomes. Furthermore, eEF-2 dependent translocation is supported by nonhydrolyzable analogs of GTP (Tanaka, et al., 1977). Translocation in the presence of GMP-P(NH)P was reversible and stoichiometric with eEF-2 whereas in the presence of GTP translocation was unidirectional and eEF-2 acted catalytically. It is likely that the eEF-2 catalyzed hydrolysis of GTP is required for the release and recycling of eEF-2 rather than for the translocation reaction per se. In this regard, the GTP hydrolysis reaction plays a functionally identical role in aminoacyl tRNA binding and translocation (Kaziro, 1978). Additional evidence shows that GTP is hydrolyzed by eEF-2 on posttranslocated ribosomes (Nilsson and Nygard, 1986; Nilsson and Nygard, 1988; Nygard and Nilsson, 1989), possibly because eEF-2 catalyzes the release of uncharged tRNA from the P site.

1.1.3 Termination

Peptide chain termination is considered as the last step in protein biosynthesis (Figure 1.2). It is a complex process during which the various elements of the translation machinery that were coordinately engaged in protein synthesis fall apart. This includes the release of the terminated peptide chain from the peptidyl-tRNA, and release of the tRNA from the ribosome. Release of the mRNA from the ribosome, and dissociation of the ribosomal subunits are generally also considered as part of the termination process. This series of events is brought about on the ribosome by the concerted action of a specific termination factor, GTP and a termination codon on the mRNA.

Three codons (UAA, UAG, or UGA) specify termination (Beaudet and Caskey, 1971). Whereas three release factors have been isolated from Escherichia coli to participate in the termination process, a single release factor (RF) has been purified from
rabbit reticulocytes (Caskey, 1980). This single factor recognizes all three termination codons (Konecki, et al., 1977).

After translation of the codon corresponding to the C-terminal amino acid of the polypeptide chain, the peptidyl-tRNA is translocated from the A site to the P site on the ribosome such that the termination codon now occupies the A site. The termination process is mediated by RF and GTP, the latter facilitating binding of the factor to the ribosome (Tate, et al., 1973). Binding of RF with GTP leads to the unmasking of the ribosome-dependent GTPase activity of RF that is enhanced by the presence of a termination codon. As a consequence of GTP hydrolysis to GDP, the RF is dissociated from the ribosome. Ultimately, the ribosome is released from the mRNA and is dissociated into its subunits.

The release of the peptidyl moiety from the peptidyl-tRNA is catalyzed by the peptidyl transferase contained in the 60S ribosomal subunit (Caskey, 1980). However, this event does not appear to be directly linked to GTP hydrolysis. Indeed, antibiotics such as sparsomycin that specifically act on the peptidyl transferase center inhibit release of the peptide chain without interfering with GTP hydrolysis (Goldstein, et al., 1970; Tate and Caskey, 1973; Wei, et al., 1974).

1.2 Regulation at the level of translation

Regulation of translation is an important step in controlling the level of gene expression in eukaryotic organisms (Hershey, 1991; Kozak, 1992). Translation can be affected in a variety of ways due to the complexity of translational machinery. The particular structural features of specific mRNAs can be involved in translational regulation. In this regard, the presence or absence of m$^7$G cap, secondary structures, or additional AUGs in the 5' leader can drastically affect the translation of specific mRNAs. The sequence context of a particular AUG can influence its efficiency to be used as a translation initiation codon. Binding of specific regulatory proteins to certain structural feature of mRNA can modulate the translatability. The presence and length of poly(A) tails at the 3'-terminus can also affect translation efficiency. Besides influence of the structure of mRNA, posttranslational covalent modification such as phosphorylation or methylation can regulate the activities of protein components in translational machinery.
In this category, modification of protein factors by phosphorylation/dephosphorylation appears to be the most prevalent general mechanism of translational control.

Regulation of translation requires altering the rates of one or more of the phases of protein synthesis. It is generally believed that the rate-limiting step of protein synthesis under most physiological conditions is the initiation phase (Jagus, et al., 1981). The evidence supporting this view is based in part on analyses of polysome size. Typically, ribosomes are spaced along the mRNA at intervals of 80-100 nucleotide residues. If initiation rates were fast relative to elongation, ribosomes could bind as close as one every 30 nucleotides, as is observed when ribosomes stack up behind pause sites in the mRNA (Wolin and Walter, 1988). Whether translation initiation is relatively efficient among different mRNAs can be assayed by treating cells with low concentrations of the elongation inhibitor cycloheximide to slow down the elongation rate, as shown in studies with reovirus infected SC-1 cells (Walden, et al., 1981). The major reovirus mRNAs are poor in translation initiation relative to host mRNAs, thus its translation is relatively insensitive to low concentrations of cycloheximide compared with host mRNAs.

1.2.1 The structure of mRNA

The intrinsic efficiency whereby an mRNA is translated depends on the structure of that specific RNA (Kozak, 1989c). As described above, several structural features appear to be important for affecting the efficiency of translation of a certain mRNA, including \( m^7G \) cap, sequence context of the initiation site (mostly AUG), secondary structures, upstream AUGs, and poly(A) tail.

1.2.1.1 \( m^7G \) Cap

The ubiquitous 5'-\( m^7G \) cap structure increases the efficiency of translation \textit{in vitro} in a variety of cell-free protein synthesizing systems (Banerjee, 1980). Almost all eukaryotic mRNAs are thought to be capped (Seidel and Somberg, 1978; Shatkin, 1976; Shatkin, 1985), though uncapped mRNAs are found in some virus-infected cells (Jang, et al., 1989; Pelletier and Sonenberg, 1988). Secondary structures that bury the cap can reduce translational efficiency, indicating that the accessibility of the 5'-cap structure correlates with the efficiency of mRNA translation (Lawson, et al., 1988; Lawson, et al., 1986).
Efficient translation of most mRNAs *in vivo* is stringently cap-dependent, as shown in studies with vesicular stomatitis virus mutants that are defective in methylation (Horikami, et al., 1984) and with various other test systems (Fuerst and Moss, 1989; Malone, et al., 1989). Studies with vesicular stomatitis virus distinguish nicely between the ability of the cap to stabilize transcripts and its ability to stimulate translation; a guanylylated, unmethylated cap is sufficient to protect transcripts from 5'-exonucleases, while N-7 methylation is essential for efficient translation. A methylated cap and the associated cap-binding protein may be less important for the translation of mRNAs that have a rather long, unstructured leader sequence (Gallie, et al., 1989; Kozak, 1991b).

**1.2.1.2 The sequence context of AUGs**

Most of the eukaryotic cellular mRNAs are monocistronic, i.e., only a single translational start and stop signal are recognized and used. This is in contrast to protein synthesis in bacteria, where polycistronic mRNAs are common and are translated either independently or by coupling translation of the downstream cistron to that of the upstream cistron.

Mutagenesis of nucleotides in the vicinity of the AUG codon revealed that GCC(A/G)CCAUGG is the optimal context for initiation of translation in cultured monkey cells (Kozak, 1986c; Kozak, 1987b). A purine, preferably A, in position -3 and a G in position +4 have the strongest effects, modulating translation at least 10-fold; the smaller effects of other nucleotides near the AUG codon are seen most easily in the absence of A and G. To be effective, the GCCACC motif must abut the AUG codon. Shifting the motif by just one nucleotide to the left or right abolishes its facilitating effect (Kozak, 1986c; Kozak, 1987b). The strong contributions of A or G in position -3 and G in position +4, deduced initially in transfection assays with COS cells, have been confirmed in experiments with transformed plants (Jones, et al., 1988; Taylor, et al., 1987) and with standard *in vitro* translation systems from plants (Kozak, 1989b) and animals (Himmler, et al., 1989; Kozak, 1989b; Query, et al., 1989; Stirzaker, et al., 1987; Sundan, et al., 1989). One set of constructs used for the *in vitro* translation experiments was designed with two in-frame AUG codons, positioned to produce "long" and "short" versions of chloramphenicol acetyl transferase (Kozak, 1989b). The experiments carried out with those constructs revealed that a suboptimal context around the first AUG codon
15

causes some 40S ribosomal subunits to bypass the first AUG and initiate instead at the second AUG codon. When the first AUG codon lies in a weak context, it is recognized inefficiently irrespective of the mRNA concentration and irrespective of the presence or absence of competing mRNAs (Kozak, 1989b), consistent with the scanning model. The leaky scanning that results from a suboptimal context around the first AUG codon enables some viral mRNAs to produce two proteins by initiating at the first and second AUG codons (Kozak, 1986a; Kozak, 1991a).

The experimentally determined optimal context for initiation (Kozak, 1986c; Kozak, 1987b) matches the consensus sequence derived from inspection of 699 vertebrate mRNA sequences (Kozak, 1987a). Except for yeasts where modest effects (2 fold or less) were found by mutating nucleotides in the vicinity of AUG initiation codon (Bairn and Sherman, 1988; Cigan, et al., 1988; Werner, et al., 1987; Zitomer, et al., 1984), several other eukaryotic organisms that have been examined show context effects similar to those described for vertebrates. ACC in positions -3 to -1 promotes translation in *Drosophila* (Feng, et al., 1991), although the actual consensus sequence in flies differs slightly from mammals. 95% of 252 plant mRNA sequences that were examined has the expected purine in position -3 and 74% has the expected G in position +4; and those conserved nucleotides augment translation in plants (Jones, et al., 1988; Kozak, 1989b; Taylor, et al., 1987). A recent compilation of translational start sites in *protozoa* also shows a strong preference for A in position -3 (Yamauchi, 1991).

Analysis of gene sequences from *N. crassa* deposited in GeneBank or EMBL indicate that the distribution of nucleotides surrounding translation initiation sites are clearly not random (Edelmann and Staben, 1994). A consensus sequence (CA(A/C)(A/C)ATGGGCT) was derived for sequences around the AUG translation initiation codon. The presence of A at position -3 and G at position +4 are consistent with the survey results from other eukaryotic systems. Whether the sequence context around the *N. crassa* initiation codons affects translation has not been reported.

### 1.2.1.3 upstream AUGs

Analysis of the structures of the 5'-leaders of vertebrate mRNAs (Kozak, 1987a) indicates the low abundance of upstream AUG codons, particularly within strong contexts for translational initiation. Less than 10% contained AUG codons in front of
the major ORF AUG codon. Interestingly, the population of mRNAs containing upstream initiation codons was strongly biased towards products of proto-oncogenes and genes encoding growth factors and cell-surface receptors. Two-thirds of the proto-oncogene transcripts had AUG codons preceding the major ORF. Surveys of yeast mRNA sequences (Cigan and Donahue, 1987) and *N. crassa* gene sequences (Edelmann and Staben, 1994) indicated that most of them lack upstream AUG codons in front of the major coding sequences.

Translation of a downstream open reading frame (ORF) can be achieved by either bypassing the upstream AUGs (leaky scanning) or reinitiating after the ORF(s) specified by one or more of these upstream AUGs are translated (reinitiation). Leaky scanning and reinitiation are common mechanisms in eukaryotes to avoid or reduce the effect of upstream AUGs on downstream translation (Chenik, et al., 1995; Kozak, 1992; Oliveira and McCarthy, 1995). Leaky scanning normally occurs when the sequence context of upstream AUGs is unfavorable for initiation (Cao and Geballe, 1995; Kozak, 1986c; Kozak, 1991a). Introduction of a novel strong, out-of-frame AUG codon upstream of the original one dramatically inhibits translation, while a strong, upstream, and in-frame AUG codon supplants the original site of initiation (Kozak, 1983; Kozak, 1989c).

Reinitiation normally occurs when an upstream AUG codon is followed shortly by a terminator codon, creating a small upstream open reading frame (uORF) at the 5'-end of the mRNA (Kozak, 1984; Liu, et al., 1984; Peabody, et al., 1986). Expanding the distance between the 5'- and 3'-cistrons increases the efficiency of reinitiation (Kozak, 1987c). An important feature is that reinitiation has been shown to occur fairly efficiently in eukaryotes only when the 5'-ORF is short (Abastado, et al., 1991; Kozak, 1987c; Sedman and Mertz, 1988; Werner, et al., 1987). Few naturally occurring di-cistronic mRNAs have been found that express two full-length proteins from non-overlapping cistrons through reinitiation, although low-level reinitiation after a major ORF has been reported for the xanthine-guanine phosphoribosyl transferase (Peabody and Berg, 1986) and dihydrofolate reductase (Kaufman, et al., 1987) coding regions. Artificially constructed di-cistronic transcripts of that sort allow only very inefficient translation of the second cistron (Angeonon, et al., 1989; Hasemann and Capra, 1990; Kaufman, et al., 1987). There are many plant and animal virus mRNAs that are structurally di-cistronic, encoding two full-length proteins in non-overlapping ORFs, but
they are functionally monocistronic, i.e. only the 5'-proximal ORF is translated (Kozak, 1986d).

1.2.1.4 Secondary structure

Secondary structures of mRNAs play an important role in regulating the level of translation, depending on their stability and position. The GC richness of 5' leader sequences might be taken as a general indication of the amount of secondary structure that they can form (Kozak, 1992). Only a small fraction of vertebrate mRNAs have leader sequences with a GC content of <50%. These leaders occur on mRNAs that encode globins, caseins, albumin, α-fetoprotein, and histones, all of which are abundant proteins that appear to be translated efficiently. Leader sequences on the majority of vertebrate mRNAs have a higher GC content (50%-70%). 5' leader sequences with extremely high GC content (70%-90%) are typical of mRNAs that encode oncoproteins, growth factors, signal transduction components, and transcription factors, as well as many housekeeping proteins (Kozak, 1991a). The general low abundance of such proteins is consistent with the expected difficulty of translating ribosomes passing through highly structured leader sequences.

Secondary structure in the 5'-UTR may affect cap accessibility and may impede the scanning 40S ribosomal subunit if sufficiently stable (>50 kcal/mol) (Kozak, 1986b; Kozak, 1989c; Pelletier and Sonenberg, 1985). However, when placed just downstream from the initiator codon, such structures cause the scanning 40S ribosomal subunit to pause at the AUG and thereby enhance initiation (Kozak, 1989b). If the cap is accessible, the translational machinery can melt out moderately stable structures during scanning. Secondary structure in the coding region of mammalian mRNAs, even when very stable, does not block or greatly impede the elongating ribosome (Wolin and Walter, 1988), nor do long cDNAs annealed to this region of the mRNA (Minshull and Hunt, 1986).

Introduction of downstream secondary structure (ΔG = -19 kcal/mol) can completely suppress the leaky scanning that otherwise occurs when the first AUG codon lies in an unfavorable context (Kozak, 1990), probably by slowing scanning, thus providing more time for recognition of the preceding AUG codon. Although 97% of vertebrate mRNAs have the required purine in position -3, few possess the full consensus sequence. The fact that the usual absence of leakiness exists indicate that some feature in
addition to primary sequence would seem to be required to avoid leakiness. It is possible that secondary structure near the start of the coding sequence compensates for the less-than-perfect context around the AUG codon. Consistent with this hypothesis, the experimental placement of appropriately positioned secondary structure increases initiation from cryptic non-AUG codons in test cases (Kozak, 1990), and nearly all of the mRNAs that naturally support initiation from upstream non-AUG codons have extraordinarily GC-rich (hence highly structured) leader sequences.

In contrast with the positive effects of secondary structure introduced downstream from the AUG codon, stem-and-loop structures introduced between the cap and the AUG codon never facilitate initiation. A secondary structure (ΔG=-30 kcal/mol) near the cap (i.e. within the first 12 nucleotides) can drastically inhibit translation (Kozak, 1989a). Secondary structure in this position has been shown to prevent mRNA from binding to 40S ribosomes (Kozak, 1989a), as expected if the 5'-end of the mRNA is the entry site for ribosomes.

However, stem-and-loop structures with a free energy of -30 kcal/mol positioned 50 or 60 nucleotides from the cap did not impair translation in COS cells (Kozak, 1991c) or in cell-free extracts (Kozak, 1989a), probably because 40S ribosomal subunits can get past a -30 kcal/mol hairpin by migrating through it. It seems unlikely that 40S ribosome subunits jump over the stem-and-loop structure since AUG codons that are buried in some hairpin structures remain accessible to ribosomes (Kozak, 1986b). Certain initiation factor(s) having helicase activity might participate in this process.

Translation is profoundly inhibited in vivo (Kozak, 1986b) and in vitro (Kozak, 1989a) upon inserting into the 5'-noncoding domain a stem-and-loop structure with a free energy of -50 or -60 kcal/mol. Translation is inhibited even when the hairpin affects neither the cap nor the AUG codon. A very stable base-paired structure apparently inhibits translation by blocking the migration of 40S ribosomes, as evidenced by RNase protection experiments which showed a 40S ribosome trapped on the 5'-side of the hairpin (Kozak, 1989a). In contrast with the inability of 40S ribosomal subunits to unwind a stem-and-loop structure of -60 kcal/mol, 80S elongating ribosomes can, to some extent, penetrate such structures (Kozak, 1989a).

Secondary structure can act as a stimulator for translational frameshifting. Translating ribosomes normally advance along the mRNA by precisely reading three-
nucleotide codons at each turn of the elongation cycle, then terminate synthesis when a nonsense codon is encountered in the A site. However, ribosome reading frame shifts, or binding of an aminoacyl-tRNA at the nonsense codon to continue translation, occur at a frequency estimated to be less than 10^{-3} (Hershey, 1991). The structures of some specific mRNAs have evolved so as to increase the tendency to frameshift or read through termination codons. By increasing the frequency of such events, the ribosome is able to translate a more distal part of the mRNA at low but significant levels. The frequency of the frame shift event can be increased by secondary structural elements, particularly the pseudoknots (Brierley, et al., 1989; Jacks, et al., 1988; Matsufuji, et al., 1995).

Readthrough enables the ribosome to continue to translate a downstream, in-frame region of an mRNA that is distal to a stop codon. The readthrough phenomenon is seen in murine leukemia virus, where the gag and pol genes are separated by a UAG stop codon that is read at 10% frequency by tRNA-Gln to generate a fusion protein (Yoshinaka, et al., 1985). As in frameshifting, secondary structure elements may enhance the frequency of such readthrough events (Jones, et al., 1989; Ten Dam, et al., 1990).

1.2.1.5 Poly (A) tail

Most eukaryotic mRNAs are polyadenylated soon after transcription in the nucleus. Studies on the function of the poly(A) tails reveals that poly(A) affects translational efficiency and mRNA stability (Sachs and Wahle, 1993).

A role for poly(A) in translational efficiency is indicated by in vitro studies of rabbit reticulocyte lysates, where mRNAs carrying poly(A) tails are translated more efficiently than their deadenylated counterparts (Jacobson and Favreau, 1983). The requirement of a poly(A) tail is not absolute, as considerable translation occurs with poly(A)^- mRNAs. Addition of oligo(A) molecules inhibits translation of naturally polyadenylated mRNAs in L-cell lysates, while reovirus mRNA, naturally devoid of a poly(A) tail, is much less sensitive to this inhibition (Lemay and Millward, 1986). Reversal of inhibition by the poly(A) binding protein (PABP) suggests that the oligo(A) molecules compete for the PABP and implies that the PABP and poly(A) tail together are positive effectors of translation. poly(A)^- mRNAs are recruited less efficiently into polysomes compared to poly(A)^+ mRNAs, possibly through a defect in the junction step of the 60S subunit with the 40S initiation complex (Munroe and Jacobson, 1990).
Deletion of the PABP gene in yeast is lethal and second site revertants have been obtained, one of which is a 60S ribosomal protein (Sachs and Davis, 1990). The translation of tissue plasminogen activator (TPA) mRNA is regulated by polyadenylation during meiotic maturation of mouse oocytes (Vassalli, et al., 1989). Translational activation of dormant TPA mRNA is accompanied by elongation of its poly(A) tail to 400-600 adenylates. Polyadenylation and translational activation also occur upon microinjection of an in vitro chimeric transcript carrying the 3'-UTR of the TPA mRNA inserted next to a reporter gene.

Poly(A) tails are also involved in regulating mRNA stability (Beelman and Parker, 1995). A systematic study of the timing of deadenylation relative to the degradation of several yeast mRNAs has revealed that the mRNAs are deadenylated before they are degraded (Decker and Parker, 1993). These results are consistent with the effects of two conditional mutations in yeast that lead to an enhancement of both poly(A) and mRNA destabilization (Minvielle-Sebastia, et al., 1991). Other experiments have also shown that mRNA degradation is preceded by poly(A) shortening (Muhlrad and Parker, 1992; Wellington, et al., 1993). Each of these experiments has contributed to the working model that destabilizing sequences in mRNA exert their function by first stimulating deadenylation.

1.2.2 Phosphorylation of protein factors in translational machinery

Phosphorylation/dephosphorylation of protein factors appears to play an important role in controlling the overall rate of protein synthesis in eukaryotic cells (Hershey, 1989). Phosphorylation of some of them inhibits translation, whereas phosphorylation of others can stimulate translation. A variety of protein kinases participating in signal transduction are involved in phosphorylating these protein factors, indicating that the global regulation of translation and signal transduction process are tightly linked.

1.2.2.1 Initiation factors

The phosphorylation of the α-subunit of eIF-2 was first detected in rabbit reticulocyte lysates (Farrell, et al., 1978). The phosphorylation of eIF-2α in many cell types correlates with inhibition of the initiation rate, suggesting that this is a common
mechanism for controlling protein synthesis (Hershey, 1989). An interferon-induced protein called the double-stranded RNA activated inhibitor (DAI, or dsl), phosphorylates the Ser51 residue of eIF-2α (Colthurst, et al., 1987; Pathak, et al., 1988).

Phosphorylation of only 25-30% of the factor is sufficient to cause a strong inhibition of the initiation phase in reticulocyte lysates. Phosphorylation does not directly inhibit formation of the ternary complex or ribosomal initiation complexes since these reactions proceed efficiently in vitro with completely phosphorylated factor (Trachsel and Staehelin, 1978). However, it impedes the guanine nucleotide exchange reaction that enables eIF-2 to recycle and promote multiple rounds of initiation (Pain, 1986). Phosphorylated eIF-2-GDP binds more tightly to eIF-2B, but does not exchange GDP for GTP (Dholakia and Wahba, 1989; Rowlands, et al., 1988).

eIF-2α phosphorylation causes translational repression in vivo. Point mutation of Ser51 (Ser->Asp), which is the site of eIF-2α phosphorylation, results in severe inhibition of global protein synthesis following accumulation of small amounts of the protein, possibly because the Asp residue mimicks the charge of the phosphorylated Ser51 residue (Kaufman, et al., 1989). However, the mutant factor eIF-2α(Ser51->Ala) stimulates the translation of both plasmid-derived mRNAs in transiently transfected COS-1 cells (Kaufman, et al., 1989) and viral mRNAs in human 293 cells infected with a mutant form of adenovirus lacking the VA-I gene (Davies, et al., 1989). In these cells, DAI kinase is activated (Kaufman and Murtha, 1987; Schneider and Shenk, 1987) but the repressive effects of the kinase are prevented by the mutant eIF-2α protein, which is not phosphorylated. The results implicate the importance of eIF-2α phosphorylation by the DAI kinase and indicate that eIF-2α phosphorylation is the cause of translational repression in intact cells.

In contrast to the phosphorylation of eIF-2, the phosphorylation of eIF-4F stimulates protein synthesis. The low-abundance cap-binding protein, eIF-4Fα (eIF-4E), is phosphorylated in vivo primarily at Ser53 (Rychlik, et al., 1987). The same site is phosphorylated in vitro by protein kinase C (Morley and Traugh, 1989; Tuazon, et al., 1989), where the preferred substrate is the eIF-4F complex rather than the free eIF-4Fα subunit (Tuazon, et al., 1990). Phosphorylation of eIF-4Fα does not appear to alter its affinity for m7G cap structures. However, numerous experimental evidence link phosphorylation and the active state of the factor. The factor is dephosphorylated in cells
during mitosis (Bonneau and Sonenberg, 1987) or when treated at high temperatures (Duncan, et al., 1987), both of which are conditions that inhibit protein synthesis. eIF-4Fα is phosphorylated by treatment of quiescent cells with serum, insulin, tumor necrosis factor α, or the mitogen, phorbol ester (TPA) (Kaspar, et al., 1990; Marino, et al., 1989; Morley and Traugh, 1989). These results suggest that phosphorylation of eIF-4Fα activates the factor. In vitro studies with radiolabeled protein expressed from eIF-4Fα cDNAs demonstrate that the protein binds to 40S initiation complexes, where it is mainly in a phosphorylated state (Joshi-Barve, et al., 1990). However, a mutant form (Ser53->Ala) abolishing the phosphorylation at this site does not bind to the complexes. Wild-type eIF-4Fα cDNA, when overexpressed in murine 3T3 cells or in rat 2 fibroblasts, causes the cells to undergo malignant transformation and become tumorigenic (Lazaris-Karatzas, et al., 1990). Microinjection of eIF-4F or eIF-4Fα into serum-starved NIH 3T3 cells results in the stimulation of DNA synthesis and a transient morphological transformation (Smith, et al., 1990). In contrast, overexpression or microinjection of the mutant form (Ser53->Ala) of eIF-4Fα is not oncogenic. The results above implicate the factor as a key target in the phosphorylation cascades that lead to control of cell growth through signal transduction mechanisms and protein kinase C.

The γ-subunit of eIF-4F is phosphorylated in 3T-L1 cells treated by insulin and phorbol esters (Morley and Traugh, 1990). Protein kinase C and protease-activated kinase I and II phosphorylate eIF-4Fγ in vitro (Tuazon, et al., 1990). When eIF-4F is treated with protein kinase C, there is a fivefold increase in the factor's activity in a cell-free globin synthesis assay (Morley, et al., 1991). Since the α-subunit also is phosphorylated by protein kinase C, it is not yet clear if phosphorylation of either or both the α- and γ-subunits causes enhanced activity. The site(s) of phosphorylation of eIF-4Fγ have not yet been determined, nor is it certain that protein kinase C is responsible for the protein's phosphorylation in vivo.

The phosphorylation of eIF-4B happens at eight or more serine residues. The hyperphosphorylation of eIF-4B in vivo correlates with activation of protein synthesis, and partial dephosphorylation occurs upon repression of translation caused by heat shock and serum deprivation (Duncan and Hershey, 1984; Duncan and Hershey, 1987). eIF-4B is phosphorylated in vivo when cells are treated with serum or phorbol esters (Morley and Traugh, 1990) and in vitro by protein kinase C and a variety of other kinases (Tuazon, et
al., 1989), but it remains to be shown whether or not the sites are identical to those phosphorylated \textit{in vivo}.

eIF-3 is another initiation factor whose phosphorylation on the \( \eta \)-subunit is enhanced by insulin and phorbol esters (Morley and Traugh, 1990). eIF-3 comprises at least eight different polypeptide subunits (Milburn, et al., 1990a), and there is evidence for \textit{in vivo} phosphorylation on the \( \delta \), \( \epsilon \), \( \xi \), \( \eta \) and \( \theta \) subunits (Benne, et al., 1978; Milburn, et al., 1990a). However, it remains unclear if phosphorylation of these subunits is involved in translational control.

The phosphorylation of a variety of other initiation factors has been described but not yet studied extensively. The \( \beta \)-subunit of eIF-2 is phosphorylated \textit{in vivo}, but there are only minor changes in its phosphorylation status when protein synthesis is either inhibited or stimulated (Duncan and Hershey, 1984; Duncan and Hershey, 1985). eIF-2\( \beta \) is phosphorylated on Ser2 by casein kinase II and on Ser13 by protein kinase C (Clark, et al., 1989; Clark, et al., 1988). eIF-5 is phosphorylated at multiple sites \textit{in vivo} and by protein kinase II \textit{in vitro} (Benne, et al., 1978), but changes in the extent of phosphorylation have not been determined.

\subsection{1.2.2.2 Elongation factors}

Phosphorylation of elongation factor eEF-2 inhibits protein synthesis. Evidence for the phosphorylation of this factor comes from analyses of reticulocyte lysates (Ryazanov, 1987) and mammalian fibroblasts (Nairn and Palfrey, 1987). A highly specific kinase, called the \( \text{Ca}^{2+}/\text{calmodulin-dependent protein kinase III or eEF-2 kinase, has been identified and shown to phosphorylate primarily at Thr56 (Ovchinnikov, et al., 1990; Price, et al., 1991), but Thr53 and Thr58 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 \textit{in vitro} (Ryazanov and Davydova, 1989). Therefore, the phosphorylation inactivates eEF-2 and inhibits the elongation phase of protein synthesis.

eEF-1\( \beta \) and eEF-1\( \gamma \) can also be phosphorylated. eEF-1\( \beta \) can be phosphorylated at Ser89 by an endogenous kinase present in eEF-1\( \beta \gamma \) preparations (Janssen, et al., 1988). The phosphorylation site resembles the recognition sequence for casein kinase II. The
rate of guanine nucleotide exchange on eEF-1α was moderately affected by the phosphorylation state of eEF-1β: the rate of exchange catalyzed by the phosphorylated factor was reported to be half that of the unmodified eEF-1β. 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; this activation is correlated with changes in protein synthesis (Wasserman, et al., 1982).

### 1.2.2.3 Ribosomal proteins

Ribosome protein S6 is phosphorylated *in vivo* on up to five serine residues near the C-terminus of the protein (Kozma, et al., 1989). Phosphorylation correlates with activation of protein synthesis caused by mitogens and growth factors, but no compelling evidence has been generated to show that S6 phosphorylation enhances the activity of 40S ribosomal subunits in translation (Kozak, 1992). Two types of S6 kinase has been found to be responsible for S6 phosphorylation, p70s6k/p85s6k and pp90^rsk^ (Jurivich, et al., 1991; Kozma and Thomas, 1994). The signaling pathways leading to the activation of these kinases remain obscure, although MAP-2 kinase has been shown to phosphorylate pp90^rsk^ (Chung, et al., 1991; Ferrari and Thomas, 1994). Protein kinase C might also be involved in the S6 phosphorylation pathways since treatment of cells with phorbol esters increases S6 phosphorylation (Blenis and Erikson, 1986). Phosphoprotein phosphatase type-I dephosphorylates S6 and is differentially regulated by insulin and epidermal growth factor (Olivier, et al., 1988). Besides ribosomal protein S6, the large subunit acidic proteins P1 and P2 are phosphoproteins (Arpin, et al., 1978), and the small subunit proteins S2 and S13 are phosphorylated following infection of cells by vaccinia virus (Beaud, et al., 1989). Whether or not the phosphorylation status of any of these ribosomal proteins affects translation rates remains to be determined, although phosphorylation of P1-P2 is required for their *in vitro* assembly into 60S particles (Lavergne, et al., 1987).
1.3 mRNA stability and Translation

In addition to modulating the rate of translation to control the level of gene expression, eukaryotic cells have evolved mechanisms to regulate mRNA stability. mRNA stability controls the overall amount of mRNA template available for translation. The range of mRNA stability in eukaryotic cells can vary over several orders of magnitude (Peltz, et al., 1991). The existence of highly unstable mRNAs allows cells rapid and precise reductions or elevations in transcript levels in response to physiological needs. Studies on mRNA stability revealed that sequence elements regulating mRNA stability are found throughout the message (Sachs, 1993). Besides the involvement of 5' UTR and 3' UTR in regulating mRNA stability, the stability of some mRNAs is tightly correlated with the translation process itself.

Since the translational machinery and the RNA degradation apparatus interact with the same mRNA molecules, it would be no surprise that mRNA degradation and translation may mutually influence each other. Experiments in yeast using the translational inhibitor cycloheximide revealed that almost all mRNAs were stabilized by drug treatment (Herrick, et al., 1990). Consistent with this finding, a mutation resulting in partial loss of function of a tRNA nucleotidyl transferase protein, which leads to a decrease in the rates of translation due to limiting functional tRNA, resulted in mRNA stabilization (Peltz, et al., 1992). Although inhibition of translation resulted in stabilization of mRNA, these experiments did not distinguish between a requirement for translation in mRNA degradation and a requirement for a highly labile degradation activity that fails to be synthesized but continues to be degraded upon the inhibition of translation.

An example of mRNA degradation coupled with translation is β-tubulin mRNA. Degradation of tubulin mRNAs requires translation, as indicated by it only occurring with translationally active mRNA. Determination of β-tubulin mRNA stability after slowing the elongation of polypeptide chains with various inhibitors showed that destabilization of β-tubulin mRNA requires ongoing ribosome translation beyond at least ninety codons of the coding region (Pachter, et al., 1987). The N-terminal nascent tetrapeptide encoded by the β-tubulin mRNA provided a signal to target rapid degradation of that mRNA under conditions of tubulin monomer excess (Yen, et al., 1988).
Experiments utilizing the c-fos mRNA also showed the existence of a degradation signal sequence within the ORF of the mRNA and it is only utilized when the message was transiently expressed following growth factor stimulation (Shyu, et al., 1989). Closer examination of this element showed that it is the RNA sequence and not the protein product or a bias in codon usage that is required for inducing mRNA degradation (Wellington, et al., 1993).

Another mRNA degradation signal sequence is found in the ORF of the yeast MATα1 mRNA (Parker and Jacobson, 1990). The degradation of this mRNA required translation through a 65 nt region and this region contains several rare codons. Further experiments (Caponigro, et al., 1993) have confirmed that it is the presence of rare codons in conjunction with as yet undefined sequence 3' to them that lead to stimulated degradation.

Degradation of at least some yeast mRNAs is accelerated by nonsense codons introduced into the coding region (Losson and Lacroute, 1979; Peltz, et al., 1993). The degradation of mRNAs with nonsense codons is part of a process, termed mRNA surveillance, that ensures the rapid degradation of aberrant transcripts (Beelman and Parker, 1995). In yeast, the nonsense-mediated degradation pathway is dependent on trans-acting factors encoded by UPF genes (Cui, et al., 1995; Leeds, et al., 1992). Mutations in UPF1 lead to the selective stabilization of mRNAs containing nonsense mutations without affecting the decay rates of most other mRNAs (He, et al., 1993). Experiments seeking those cellular factors that specifically interact with Upf1p resulted in identification of a novel component NMD2 involved in nonsense-mediated mRNA degradation pathway (He and Jacobson, 1995). Other than nonsense codons, a sequence motif downstream of a nonsense codon has been identified as the required element for nonsense-mediated degradation (Zhang, et al., 1995). This type of mRNA degradation has also been observed in mammalian cells (Cheng and Maquat, 1993) and Xenopus oocytes (Whitfield, et al., 1994).

1.4 uORF, a feature indicating translational regulation

As described above, one of the mRNA structure features involved in regulation of translation is the presence of upstream AUGs. Any of these AUGs, if used as a
translation initiation codon, can specify an ORF either completely upstream or overlapping the major ORF, depending on whether it runs into a stop codon before or after the initiation codon for the major ORF. The ORFs specified by upstream AUGs are generally known as upstream open reading frames (uORFs) in eukaryotes.

The presence of a uORF generally has an inhibitory effect on the translation of downstream major ORF. In some cases, this inhibitory effect can be regulated (Hill and Morris, 1992; Hinnebusch, 1994; Werner, et al., 1987). Genes embracing a uORF are not restricted to a certain type of organism; they have been found in plants, animals, animal virus, and fungi. A common feature of uORF function is that uORFs only act in cis. Analyses of the role of these uORFs in affecting the expression of the downstream major ORF indicate that they can be categorized into two groups based on their amino acid coding information: one includes those uORFs in which the amino acid coding information is not required for uORF function; the other includes those that are dependent on amino acid coding information for uORF function. Described below are several well-studied examples in these two categories.

1.4.1 uORFs which do not require amino acid coding information for regulation

1.4.1.1 Yeast GCN4

The yeast GCN4 gene encodes a transcription factor that regulates the expression of at least 40 amino acid biosynthetic enzymes in response to amino acid starvation (Hinnebusch, 1988). Analysis of GCN4-lacZ fusions suggests that GCN4 expression is enhanced 10- to 50-fold in response to single amino acid limitation (Hinnebusch, 1984; Thireos, et al., 1984), and its expression is regulated primarily at the translational level (Hinnebusch, 1984; Hinnebusch, 1985; Mueller, et al., 1987; Thireos, et al., 1984).

Four short uORFs (each consisting of two or three codons) in the GCN4 leader are involved in translational regulation of GCN4. Removal of the four uORFs from the GCN4-lacZ transcript, either by deletion or point mutations in the four AUG start codons, results in constitutively derepressed fusion enzyme expression. Amino acid biosynthetic enzymes under GCN4 control are derepressed when the same mutations are introduced upstream from the authentic GCN4 protein-coding sequences. These mutations have little or no effect on the steady-state levels of GCN4 or GCN4-lacZ mRNAs, showing that
the uORFs affect GCN4 expression at the translational level (Hinnebusch, 1984; Hinnebusch, 1985; Thireos, et al., 1984).

Regulation of GCN4 through the above uORFs seems to be independent of the amino acid sequence of their putative peptides (Tzamarias and Thireos, 1988; Williams, et al., 1988). The regulatory system can be reconstituted with just uORF1 and uORF4, with each uORF contributing a different control function (Mueller, et al., 1987). uORF4 alone constitutively represses translation of constructs containing the GCN4 leader. Replacing the 16 bp upstream from uORF1 with the corresponding sequence from uORF4 had no effect on GCN4 expression, suggesting that uORFs 1 and 4 have very similar initiation efficiencies. Consistent with this, lacZ fusions to uORFs 1 and 4 each express high constitutive levels of fusion enzyme activity in the absence of other uORFs, comparable to that seen for the GCN4-lacZ fusion lacking all four uORFs (Mueller, et al., 1988; Tzamarias and Thireos, 1988). However, replacement of the 25 nucleotides immediately following uORF1 with the corresponding sequence from uORF4 significantly lowered GCN4 expression under derepressing conditions. The last codon of uORF4 plus 10 bp sequence immediately after the stop codon of uORF4 are sufficient to convert uORF1 into a strong translational barrier when it is present as a solitary uORF, suggesting the characteristics of translation termination at uORF4 are responsible largely for its strong inhibitory effect on translation initiation at the GCN4 AUG codon (Miller and Hinnebusch, 1989).

The above results indicate that wild type uORF1 blocks translation downstream less than uORF4 does, not because of different initiation efficiencies at the two start sites, but because there is a greater probability for reinitiation following translation at uORF1 versus uORF4. This helps build up the following model: In the absence of amino acid starvation, reinitiation after termination at uORF1 occurs at uORF4, which in turn suppresses translation of the major cistron. During amino acid starvation, it seems that the start codon of uORF4 is bypassed, enhancing translation of the GCN4 ORF.

Further studies have revealed that the frequency of reinitiation at uORF4 seems to be controlled by the phosphorylation state of a subunit of initiation factor-2, eIF-2α (Dever, et al., 1992). eIF-2α is highly conserved from yeast to mammals (Cigan, et al., 1989) and phosphorylation of eIF-2 is currently the best understood mechanism for global control of protein synthesis in eukaryotic systems. In mammalian systems,
phosphorylation of eIF-2α reduces the level of active eIF-2α through formation of an inactive complex with the guanine nucleotide exchange factor, eIF-2B (Pain, 1986). The yeast eIF-2α kinase GCN2 has a kinase domain homologous to mammalian protein kinases and a regulatory domain related to histidyl-tRNA synthetase (Wek, et al., 1989). Recent studies show that the histidyl-tRNA synthetase-related sequence in GCN2 interacts with tRNA and is required for activation in response to starvation for different amino acids (Wek, et al., 1995). To account for the role of phosphorylation of eIF-2α in translational control of GCN4, it is suggested that after termination at uORF1, a finite length of time is required for the scanning ribosome to accumulate factors necessary for reinitiation, in particular the ternary complex consisting of eIF-2, GTP, and Met-tRNAi. When the level of eIF-2 activity is depressed by phosphorylation of eIF-2α, the scanning ribosome is thought to have more time to get past the suppressive uORF4 before acquiring the ability to reinitiate and translate the GCN4 coding region. Consistent with this model is the fact that mutations in subunits of the yeast equivalent of eIF-2B lead to either constitutive repression or derepression, depending on the allele (Cigan, et al., 1993).

As the GCN4 homologue in N. crassa, cpc-1 has two uORFs present in its transcripts (Paluh, et al., 1988), indicating the expression of cpc-1 could be regulated in mechanisms similar to GCN4 as described above.

1.4.1.2 Cauliflower mosaic virus (CaMV) 35S RNA

CaMV produce typical pathogenic mosaic symptoms in the leaves of systematically infected host plants (Baughman, et al., 1988). The mature CaMV virions contains a double stranded DNA genome of about 8000 bp, which is replicated by reverse transcription of an 8200 nt 35S RNA containing a 180 nt terminal redundancy (Gordon, et al., 1988). This 35S RNA consists of a 600 nt long leader sequence, containing multiple small uORFs, followed by seven to eight tightly arranged long ORFs encoding all of the viral proteins (Mason, et al., 1987).

CaMV ORFs following a long upstream ORF on a polycistronic RNA are not translated efficiently either in vitro (Gordon, et al., 1988) or in plant protoplasts (Bonneville, et al., 1989; Gowda, et al., 1989). In plant protoplasts, expression of downstream ORFs requires the presence of a CaMV transactivator (TAV) protein for
transactivation (Bonneville, et al., 1989; Gowda, et al., 1989). Transactivation occurred post-transcriptionally and was specific for the downstream ORFs (Bonneville, et al., 1989).

Analysis of the CaMV uORFs in the translation of an artificially constructed di-cistronic mRNA, with upstream ORF encoding β-glucuronidase (GUS) and downstream ORF encoding chloramphenicol acetyltransferase (CAT), revealed that it is the presence of a uORF, regardless of its amino acid coding information, that is important for stimulating the far downstream CAT gene expression. The presence of a uORF always has an inhibitory effect on the direct downstream GUS gene (Futterer and Hohn, 1991).

Further experimental analysis (Futterer and Hohn, 1992) indicated that the extent of uORF inhibition on GUS expression was correlated to the length of the uORF, with longer uORFs having more inhibitory effect. However, the uORF stimulatory effect on CAT expression was not linearly related to uORF length, with the strongest stimulation observed when the uORF has a length of 28 and 34 codons. Longer and shorter uORFs had weaker effect. Unlike GCN4 described above, alteration of sequences around the uORF termination codon has minimal effect on downstream GUS or CAT expression. The uORF effect on the far downstream CAT gene expression was explained by a model involving ribosome shunting, in that a scanning ribosome can be "shunted" from one part of the leader to another (Futterer, et al., 1993).

1.4.2 uORFs which require amino acid coding information for regulation

The findings of translational regulation depending on the amino acid coding information of a uORF provide us with new insights of uORF action in translational regulation. Four genes involving this type of uORF mediated regulation have been extensively studied (Damiani and Wessler, 1993; Degnin, et al., 1993; Hill and Morris, 1993; Werner, et al., 1987). They have drastically different functions and, coincidentally, they happen to be representatives of genes from animals, plants, animal viruses, and fungi. The universal existence of this type of uORF mediated translational regulation in very different eukaryotic cells indicate that it could be a common mechanism in translational regulation in eukaryotic cells.
The requirement for coding information implicates the peptide products of these uORFs as mediators of the regulatory effects and fusion gene studies indicate that their putative AUG initiation codon can be utilized to initiate the synthesis of fusion peptide. However, none of the predicted peptide products of these four uORFs has yet been identified, although peptides of similar size are translated from uORFs in other eukaryotic transcripts (Hackett, et al., 1986; Khalili, et al., 1987). A possible model involving stalling translating ribosomes has been proposed to explain this type of uORF-mediated regulation (Geballe and Morris, 1994).

1.4.2.1 Yeast CPA

The CPA gene of Saccharomyces cerevisiae encodes the small subunit of arginine pathway carbamoyl phosphate synthetase (Pierard and Schrotter, 1978). The expression of CPA is subjected to specific repression by Arg (Pierard, et al., 1979). Little or no change in the steady-state level of CPA mRNA was observed in response to an arginine supplement, suggesting that enzyme repression occurs at the translational level. However, a transcriptional component might also be involved in Arg-specific repression of CPA since measurements of the rate of synthesis of CPA mRNA indicate that it is partially repressed by Arg (Crabeel, et al., 1990).

The CPA transcript has a leader sequence of about 250 nucleotides which contains a single uORF of 25 codons (Nynoya and Lusty, 1984; Werner, et al., 1985). Removal of the uORF AUG start codon (AUG->UUG) by site-directed mutagenesis leads to derepression of CPA enzyme expression in the presence of excess arginine, while apparently having little effect on the CPA mRNA level (Werner, et al., 1987). Arg-specific repression was 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).

Further support for the importance of this uORF in addition to the in vitro mutagenesis experiments came from analyses of yeast mutants selected for constitutive CPA expression (Werner, et al., 1987). Three classes of constitutive mutations were identified. The first one lost the uORF AUG initiator codon (AUG->AUA). The behavior of this in vivo selected mutant is identical to that of the one made by in vitro mutagenesis. The second class of mutants involves single nonsense mutation at codon 8 (CAA->UAA) or codon 20 (UGG->UAG). These mutations result in truncated uORFs
with wild-type uORF sequence. None of these mutants show Arg repression of CPA1 expression. The third class involves single missense mutation at codon 11 (UGC->UAC) or codon 13 (GAC->AAC). They also lost Arg repressibility of CPA1 expression.

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).

1.4.2.2 Mammalian AdoMetDC

S-Adenosylmethionine decarboxylase (AdoMetDC) is a key regulated enzyme of polyamine biosynthesis (Heby and Persson, 1990). The synthesis of AdoMetDC increases in mammalian cells in response to a variety of stimuli. In T-lymphocytes, this increase occurs through modulation of both the level of AdoMetDC mRNA and the efficiency of its translation (Mach, et al., 1986).

AdoMetDC mRNA has an unusual distribution in polysomes from cells of T lymphocyte origin. It associates predominantly with monosomes and small polysomes with none located in the preribosomal or ribonucleoprotein pool. In sharp contrast, it associates broadly with larger polysomes in several nonlymphoid cell lines, including fibroblasts and the adrenal carcinoma line, Y1 (Hill and Morris, 1992).

The AdoMetDC 5'-transcript leader region is highly conserved between human and bovine mRNAs. It has a length of about 330 nucleotides and contains a six-codon uORF, MAGDIS, that represses downstream translation in normal T cells and T-cell lines (Hill and Morris, 1992). The uORF restricts the intracellular distribution of AdoMetDC mRNA primarily to monosomes in normal T-lymphocytes and in T-cell lines. In contrast, non-lymphoid cells normally carry an average of seven to nine ribosomes per AdoMetDC mRNA molecule (Hill and Morris, 1992). The initiator AUG of the uORF has a strong sequence context for recognition. Mutations that remove the uORF, or prevent its initiation by changing the uORF AUG initiator codon to GUG, abolished the suppressive influence of the uORF in T cells (Hill and Morris, 1992).
The importance of uORF amino acid coding information in translational repression comes from the following evidence (Hill and Morris, 1993). Missense mutations altering each codon from positions four to six in the uORF derepress downstream translation. The second and third codons of the AdoMetDC uORF can be altered without losing the inhibitory signal. Scrambling the order of the codons in the uORF from MAGDIS to MDSIGA abolished the suppressive effect of the uORF. However, the translation regulation mediated by this uORF was not affected by the degenerative changes of uORF codons.

1.4.2.3 Human cytomegalovirus gp48 (UL4)

Human cytomegalovirus (CMV) is a medically important herpesvirus responsible for severe infections in newborns and immunocompromised patients (Alford and Britt, 1990). Although much research has concentrated on the transcriptional events responsible for the differential expression of α (or immediately-early), β (or early), and γ (or late) genes, posttranscriptional events also influence the expression of CMV genes (Mocarski, 1991).

The glycoprotein gp48 (UL4) gene encodes a protein product which is synthesized as a β protein that is present in virions (Chang, et al., 1989b). The gp48 ORF is contained in two β (E1 and E2) and one γ (L) 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 inhibits downstream translation in cell extracts (Chang, et al., 1989b). Mutational analysis indicate that the second uORF, which contains 22 codons, is an essential component of the inhibitory signal (Schleiss, et al., 1991). Removal of the initiator codon (AUG→AAG) for uORF2 abolishes the inhibition. The amino acid coding information, particularly those involving the carboxy-terminal codons of uORF2, is important in 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. Interestingly, the uORF2 termination codon is required for the inhibitory effect (Degnin, et al., 1993).

Analysis of the gp48 leader from clinical CMV isolates showed that AUG2 was conserved in all five strains, while AUG1 and AUG3 were not (Degnin, et al., 1993).
The deduced amino acid sequence of uORF2 was at least 82% identical in uORF2 coding content, with the coding information of the carboxy-terminal six codons in uORF2 identical in all strains (Degnin, et al., 1993). Thus the preservation of uORF2 in all strains suggests that uORF2 plays a significant role during the CMV infectious cycle.

By using retroviral vectors, gp48 uORF2 has been shown to be a potent translational inhibitor that functions in multiple cell lines (Cao and Geballe, 1994). Changes in the inhibitory effect resulting from altering the context of the uORF2 AUG codons suggest that the inhibition effect involves a leaky scanning mechanism (Cao and Geballe, 1995).

1.4.2.4 Maize Lc
The pigmentation pattern of maize is determined by the genetic constitution of the R/B gene family, comprised of R locus on chromosome 10 and B locus on chromosome 2 (Styles, et al., 1973). The ease of visually distinguishing subtle differences in plant pigmentation patterns has led to the identification of nearly 100 naturally occurring variants of R and B. Members of the R and B family share more than 80% amino acid identity and contain the basic helix-loop-helix DNA-binding and dimerization motif (Goff, et al., 1992; Ludwig, et al., 1989; Perrot and Cone, 1989; Tonelli, et al., 1991).

Comparison of the 5' untranslated regions of R and B gene transcripts indicates extensive sequence divergence. upstream AUGs are present in some, but not all, members of the R/B family. In addition, among the R and B gene transcripts with upstream AUGs, some have uORFs that overlap the translation initiation site of the major ORF, whereas the transcripts from other alleles have uORFs that terminate before the start of the R/B coding regions.

The Lc gene was the first R/B family member to be cloned and sequenced (Ludwig, et al., 1989). Mapping of the transcription start site revealed that the Lc 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).

Elimination of all upstream AUGs by site-directed mutagenesis derepressed the expression of downstream ORF by 30-fold, and this derepression was not due to increased mRNA stability (Damiani and Wessler, 1993). Analysis of additional mutant constructs indicates that repression is mediated by initiation at the first AUG and
translation of the uORF. Elimination of the first AUG led to fully derepressed levels, even though the second and third AUGs were intact. Furthermore, single missense mutations, frameshift mutations, or introduction of a premature stop codon all derepress the levels of expression of downstream cistron. Interestingly, a synonymous codon change (CAU>CAC) at codon 32 partially derepress the level of expression, indicating codon usage within the uORF may be important in inhibiting the downstream translation.

1.5 *N. crassa* as a model system

*N. crassa* is a eukaryotic organism, a member of the fungal class Ascomycetes. As an ascomycete, it is related to yeasts, and as a fungus, it is more distantly related to mushrooms. The eukaryotic nature of *N. crassa* and its ease to be handled as yeast and bacteria makes it a valuable model organism to study fundamental mechanisms of gene regulation in eukaryotes.

Studies in *N. crassa* have contributed significantly to our understanding of the gene. Among these studies, analyses of *N. crassa* mutants deficient in amino acid and vitamin biosynthesis led Beadle and Tatum to propose the one gene-one enzyme theory (Beadle and Tatum, 1941). Results of studies in *N. crassa* soon led to the elucidation of the sequence of biochemical steps in metabolic pathways (Srb and Horowitz, 1944). Gene conversion (Mitchell, 1955) and intra-allelic complementation (Fincham and Pateman, 1957; Giles, et al., 1957) were discovered in *N. crassa*. The regulation of enzymes for amino acid biosynthesis by cross-pathway control, which has become a paradigm for mechanisms of gene regulation through subsequent work in *S. cerevisiae*, was first discovered in *N. crassa* (Carsiotis and Jones, 1974).

Molecular as well as classical genetic approaches can be used to study *N. crassa* gene expression. More than 600 loci have been mapped to the seven *N. crassa* chromosomes by classic genetic or RFLP studies (Perkins, et al., 1982). Chromosomes have been physically resolved and identified using contour-clamped homogeneous electric field electrophoresis (Orbach, et al., 1988b). *N. crassa* can be transformed with DNA using a variety of selectable markers; transforming DNA integrates into the genome through both nonhomologous and homologous recombination mechanisms (Fincham, 1989). Effective strategies for cloning *N. crassa* genes (Akins and Lambowitz, 1985;
Orbach, et al., 1988a), as well as the availability of cosmid and cDNA expression libraries, have facilitated the cloning of \textit{N. crassa} genes in many laboratories. No functional alleles of cloned genes have been generated by a process known as RIP (Selker, 1990) or by targeted integration of transforming DNA to create a gene disruption (Ebbole, et al., 1991; Frederick, et al., 1989; Paitetta and Marzluf, 1985). Gene function can be studied by \textit{in vitro} mutagenesis and introduction of the altered gene into the organism by transformation (Frederick and Kinsey, 1990a; Frederick and Kinsey, 1990b; Paluh, et al., 1988). On the whole, studies on gene regulation using \textit{N. crassa} as a model system can help us understand the fundamental regulatory mechanisms governing the eukaryotic world.

1.6 \textit{arg-2} and its regulation

Arginine (Arg) metabolism has been studied intensively in many organisms, beginning in earnest with the discovery of the urea cycle in mammals (Davis, 1986). Although Arg metabolism varies greatly among eukaryotes, most organisms have in common the conversion of ornithine to citrulline and this requires carbamoyl phosphate (Davis and Weiss, 1988). The \textit{N. crassa} Arg biosynthetic pathway was the first multi-enzyme pathway to be defined by genetic mutation (Srb and Horowitz, 1944). Two carbamoyl phosphate synthetases are found in \textit{N. crassa}: one specific for the Arg pathway (CPS-A) and the other specific for the pyrimidine pathway (CPS-P). These two enzymes are regulated independently and are specified by different genes (Davis, 1986). The mitochondrially located CPS-A has two subunits: the small subunit, encoded by \textit{arg-2}, provides CPS-A with the ability to use glutamine as the NH$_3$ donor; the large subunit, encoded by an unlinked gene \textit{arg-3}, carries out all other catalytic functions of CPS-A and can itself use ammonium as the NH$_3$ donor. The activities of Arg$_2$p and Arg$_3$p can be distinguished by measuring the glutamine-dependent activity and ammonium-dependent activity respectively.

The Arg$_2$p activity (glutamine-dependent) can be repressed 4- to 10-fold by Arg and rises 10-fold upon Arg starvation over values characteristic of cells grown in minimal medium (Cybis and Davis, 1975), while the Arg$_3$p activity is not repressible by Arg though its activity rises 3- to 5-fold upon Arg starvation (Cybis and Davis, 1975; Davis,
et al., 1981). It is the control of *arg-2* expression that regulates the flux of metabolites through the Arg biosynthetic pathway under most growth conditions (Davis, 1986; Davis and Weiss, 1988).

Regulation of *arg-2* expression involves at least three separate control mechanisms:

(i) *arg-2* is subject to crosstalk control. Crosstalk control is a regulatory phenomenon in which starvation for a single amino acid or amino acid imbalance leads to derepression of enzymes involved in many amino acid biosynthetic pathways. Derepression of many of the enzymes of the Arg, Lys, Trp, His, Ile, Val, and Leu biosynthetic pathways occurs upon starvation of cells for any of these amino acids (Davis, 1986). The crosstalk response is dependent on the *cpc-1* gene, where mutation of this gene prevent the increased synthesis of amino acid biosynthetic enzymes that normally accompanies amino acid starvation (Barthelmess, 1982).

The level of *arg-2* mRNA increases in response to amino acid starvation and *cpc-1* is important for this response (Orbach, et al., 1990; Sachs and Yanofsky, 1991). There are four copies of TGACTC sequence elements, which was shown to specifically bind Cpc1p *in vitro* (Ebbole, et al., 1991), in the *arg-2* 5' region that has been sequenced. Two TGACTC elements are located upstream of the mRNA 5’ ends, and two are located downstream of the mRNA 5'-ends, one in each of the introns in the uORF. The role of these Cpc1p response elements in the regulation of Arg2p synthesis remains to be determined.

(ii) *arg-2* is developmentally regulated. *N. crassa* is a haploid, filamentous fungus that undergoes complex developmental and morphological changes during its asexual and sexual cycles. At different stages of *N. crassa* development, the level of *arg-2* mRNA varies drastically (Sachs and Yanofsky, 1991). Low levels of *arg-2* mRNA were detected in dormant spores, while the highest level were found during spore germination and early exponential growth.

(iii) *arg-2* is the only component of the *N. crassa* Arg biosynthetic pathway known to be negatively regulated by Arg (Davis, 1986). Decreased CPS biosynthetic activity is associated with a reduced level of Arg2p polypeptide in mitochondria (Davis, et al., 1981). Growth with Arg also reduces the level of *arg-2* mRNA (Orbach, et al., 1990; Sachs and Yanofsky, 1991). Arg-specific control of *arg-2* expression seems to be
independent of cross-pathway control since the level of \textit{arg-2} mRNA is still subject to Arg regulation in a \textit{cpc-1} mutant strain (Sachs and Yanofsky, 1991).

Comparison of the genomic and cDNA sequences of \textit{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 sequence of the uORF peptide strongly resembles the 25-residue uORF peptide encoded from the yeast \textit{CPA1} transcript (Orbach, et al., 1990). These two uORFs specify identical residues at 9 positions, and there are conservative amino acid substitutions at several other positions. Comparison of the codons used by these uORFs indicates that most nucleotide differences in homologous regions are in codon third positions, consistent with the hypothesis that these coding regions have functional significance.

The \textit{CPA1} uORF has been demonstrated to be important in translational regulation of \textit{CPA1} expression by Arg. The highly conserved \textit{arg-2} uORF might also be involved in a similar regulatory mechanism for Arg-specific regulation of \textit{arg-2} expression. Indeed, it is the purpose of this thesis to try to address the role of \textit{arg-2} uORF in Arg-specific regulation of \textit{arg-2} and compare its function to that of the \textit{CPA1} uORF.
CHAPTER 2
MATERIALS AND METHODS

2.1 Growth of *N. crassa*

Wild type strain 74A-OR23-1VA and *arg-125* strain were obtained from D. Perkins, Stanford University. The *his-3* (1-234-723) strain FGSC #6103 was obtained from Fungal Genetics Stock Center, University of Kansas Medical Center.

Procedures described previously (Sachs and Yanofsky, 1991) were used to grow *N. crassa*, except that macroconidia were harvested with water to inoculate cultures for experimental analyses. Minimal growth medium was composed of 1X Vogel’s N (Vogel, 1956) and 2% sucrose. Arg and His media were minimal medium supplemented with 0.5 mg/ml of Arg or His respectively. His starvation medium was made by supplementing minimal medium with 10 mM 3-amino-1,2,4-triazole (AT). AT was added to media from a fresh, filter-sterilized 0.1 M stock prepared in water after the media were autoclaved and cooled. AT plus His medium was made by adding filter-sterilized AT to a final concentration of 10 mM into autoclaved His medium. In typical experiments, 30 ml of medium in 125 ml Erlenmeyer flasks were inoculated with conidia at a final concentration of 2 × 10^7 conidia per ml. Inoculated cultures were grown at 34°C with orbital shaking (125 rpm) for 6.5 hours before harvesting on Whatman #541 filter paper for further analysis. For experiments in which cells were to be switched to different media, cells grown for 6.5 h were harvested by vacuum filtration onto Whatman 541 filters. The mycelial mat was peeled off the filter and resuspended by vigorous shaking in fresh medium.

2.2 Preparation of anti-Arg2p serum

The 1828 bp *KpnI* fragment of the *arg-2* gene includes the coding region for residues 68-453 of the predicted Arg2p polypeptide (Orbach, et al., 1990). A plasmid
was constructed to fuse this region of Arg2p to glutathione-S-transferase for production of recombinant protein in *Escherichia coli*. The *arg-2* *KpnI* fragment was first placed into the *KpnI* site of pSP72 (Promega) to create pIG8; then the *BamHI-BglII* fragment from pIG8 containing *arg-2* DNA was placed in the *BamHI* site of pGEX-KG (Guan and Dixon, 1991) in the appropriate orientation to generate pDM-1. *E. coli DH5αF* was transformed with pDM1 and production of the fusion protein induced with IPTG. The glutathione S-transferase-Arg2p fusion protein was localized to inclusion bodies. Inclusion bodies were isolated, solubilized, and the fusion protein purified by SDS-PAGE (Harlow and Lane, 1988). Polyclonal antiserum directed against the fusion protein was prepared by Pocono Rabbit Farms (Canadensis, PA) using a rabbit pretested for low initial reactivity against *N. crassa* polypeptides.

Polyclonal antisera directed against cytochrome oxidase subunit V and cytoplasmic leucyl-tRNA synthetase have been previously described (Benarous, et al., 1988; Sachs, et al., 1986).

### 2.3 Immunoblot analyses

Cell-free extracts were prepared from freshly harvested mycelia. Mycelia (ca. 1 g wet weight) were added to 0.8 g of ice-cold acid-washed glass beads (0.5 mm) in 2 ml screwcap Eppendorf tubes (Sarstedt) that also contained 1 ml of breaking buffer (20 mM Hepes-NaOH, pH 7.9; 100 mM KCl; 2 mM EDTA; 10 mM DTT; 20% glycerol (Sachs and Ebbole, 1990)). Tubes were filled completely with breaking buffer and cells were broken in the cold room by two 1 min disruption cycles in a Mini-Beadbeater (Biospec, Bartlesville OK) separated by a 2 min chill on ice. Extracts were clarified by centrifugation for 10 min at 16,000xg at 4°C. Clarified whole cell extracts were transferred to a fresh tube, quick frozen in liquid nitrogen and stored at -80°C. Protein concentrations were determined by Bradford assay using BSA as the standard (Bradford, 1976). For comparisons, equal amounts of total protein (20-40 μg) for each sample were adjusted to equal volumes with SDS sample buffer (50 mM Tris-HCl, pH 6.8; 0.05% Bromophenol Blue; 1% SDS; 10% glycerol; 2% β-mercaptoethanol), boiled, and examined by SDS-PAGE in 10% polyacrylamide gels. Polypeptides were transferred to PolyScreen™ membranes (DuPont) by electroblotting according to the manufacturer’s
protocol. Membranes were incubated for 1 h in PBST (5% non-fat dry milk, 0.83 mM NaH$_2$PO$_4$, 8.1 mM Na$_2$HPO$_4$, 145 mM NaCl and 0.05% Tween 20). Antiserum was added (1:5000 vol:vol) and membranes were incubated with serum for 1 h. Membranes were washed three times (5 min each) in PBST, and incubated for 1 h in PBST containing anti-rabbit IgG alkaline phosphatase conjugate (Promega). Membranes were again washed in PBST and air-dried. Antigenically-reactive polypeptides were visualized using alkaline phosphatase-coupled antibody, BCIP and NBT (Sambrook, et al., 1989).

2.4 Pulse-labeling with $^{35}$S-methionine and immunoprecipitation

Labeling of *N. crassa* cells with $^{35}$S-methionine and immunoprecipitation of newly-labeled peptides was adapted from a procedure devised for *S. cerevisiae* (Klionsky, et al., 1988). For a typical labeling reaction, 1 ml of culture was placed in a 50 ml Falcon tube; 100 μCi $^{35}$S-methionine (800 Ci/mm, New England Nuclear) was added and the culture incubated for 5 min at 34°C with gentle agitation. The amount of $^{35}$S-Met was not limiting under these conditions, because incorporation of radioactivity into polypeptide doubled, as assayed by hot-TCA precipitation, when twice the standard amount of cells were used.

Labeling reactions were stopped by adding 150 μl of 100% TCA and chilling on ice. The TCA-treated cell suspensions were transferred to 2-ml screw cap tubes (Sarstedt); pellets were collected by centrifugation in a microfuge at 13,000xg for 10 min. After removing supernatants, the pellets were washed twice with 1 ml of acetone and dried using a Speedvac. Pellets were resuspended in 1 ml of solubilization buffer (50 mM Tris-HCl, pH 7.5; 0.1 mM EDTA; 1% SDS) by adding 0.3 g of acid-washed glass beads, vortexing for 1 min and heating to 95°C for 4 min. Samples were centrifuged for 10 min in a microfuge; the supernatants containing solubilized protein were transferred to fresh tubes, frozen on dry ice, and stored at -80°C until further analysis.

To compare the relative rates of synthesis of different polypeptides under different growth conditions, equal amounts of pulse-labeled polypeptides ($2 \times 10^6$ hot-TCA precipitable cpm) were used for immunoprecipitation. That equal amounts of TCA-precipitable cpm represented equal amounts of labeled protein was confirmed by SDS-PAGE and autoradiography: densitometric analysis revealed less than 10% differences
among samples. For immunoprecipitation, samples were diluted to 1 ml with IP buffer (50 mM Tris-HCl, pH 7.5; 150 mM NaCl; 0.1 mM EDTA; 0.5% Tween-20; 1 mM PMSF) containing antiserum (2 μl) and incubated for 1 hour at 4°C with gentle rotation. Protein A-Sepharose 6MB beads (30 μl of beads/60 μl IP buffer) were added; incubation with antiserum and beads was continued overnight at 4°C with gentle rotation. Beads were collected by centrifugation and the supernatant aspirated. Beads were washed three times with 1 ml IP buffer and resuspended in 20 μl of SDS sample buffer. Beads were heated to 90°C for 8 min and the supernatant collected for SDS-PAGE (Sambrook, et al., 1989). Gels were stained in Coomassie Brilliant Blue R-250, destained, treated with sodium salicylate (Chamberlain, 1979) dried, and exposed to X-ray film. Control experiments in which immunoprecipitations were repeated using supernatants from the initial immunoprecipitations as starting material indicated that more than 90% of immunoprecipitatable antigen was recovered in the first immunoprecipitation.

2.5 Preparation of RNA

Small-scale total RNA samples were prepared by breaking 50 to 100 mg of frozen mycelia in 2 ml screw cap tubes with 0.7 g acid-washed 0.5 mm glass beads, 840 μl phenol:chloroform, 580 μl extraction buffer (50 mM Tris-HCl, pH 7.5; 100 mM LiCl; 20 mM dithiothreitol) and 84 μl of 10% SDS for 40 sec in a Mini-Beadbeater (Biospec. Bartlesville, OK). Tubes were centrifuged in a microfuge at 13,000xg for 5 min. The aqueous phase was removed and extracted once with 840 μl phenol:chloroform, and once with 800 μl chloroform. RNA was precipitated twice with ethanol and NaOAc, dissolved in sterile DEPC-treated water and stored at -80°C.

Large-scale total RNA samples were prepared by modification of a previously described method (Sachs and Yanofsky, 1991). Cells frozen in liquid nitrogen (0.5-1 g wet weight) were broken for 40 sec in phenol (7.5 ml), chloroform (7.5 ml), 10% SDS (1.5 ml) and 15 ml of extraction buffer using a 250 ml stainless steel Waring blender. The homogenate was transferred to 50 ml centrifuge tubes and phases separated by centrifugation. The aqueous phase was removed and extracted once with 15 ml phenol:chloroform and once with 15 ml chloroform. RNA was precipitated twice with ethanol and NaOAc, dissolved in sterile DEPC-treated water and stored at -80°C.
Poly(A) RNA was prepared from large-scale total RNA samples by oligo-dT cellulose chromatography as described previously (Ebbole and Sachs, 1990; Sachs and Yanofsky, 1991).

2.6 Northern blot analyses

Radioactive probes for northern blot analyses were prepared using gel-purified *N. crassa* DNA fragments obtained by digestion of recombinant plasmids with appropriate restriction enzymes. The *arg-2* probe was the 1.3 kb *PvuII-NcoI* cDNA fragment of *arg-2* (Orbach, et al., 1990); the *cox-5* probe was the 0.77 kb *EcoRI* fragment from pSRCoX5 (Sachs, et al., 1989); the *cpc-1* probe was the 1.2 kb *BglII-BamHI* fragment from pCPC-1-2 (Paluh, et al., 1988); the *leu-6* probe was the 1.1 kb *BamHI-PstI* genomic fragment (Benarous, et al., 1988; Chow and RajBhandary, 1989) from pBXla; the *E. coli lacZ* probe was the 0.83 kb *SacI-EcoRV* fragment from pDE2 (Ebbole, 1990). Probes were labeled with [α-32P]-dCTP by random priming (Feinberg and Vogelstein, 1983). Unincorporated label was removed by spin chromatography through Sephadex G-50 columns (Boehringer Mannheim). The methods used for gel electrophoreses of RNA, membrane blotting and probe hybridization were as described (Sachs and Yanofsky, 1991), except that dextran sulfate was omitted from the hybridization solution.

2.7 Polysome analyses

The procedures for polysome preparation and analyses were adapted from previously described procedures (Cigan, et al., 1991; Sachs and Davis, 1989). Cycloheximide (100 μg/ml) was added to cultures 5 min prior to harvesting. Mycelia (0.5 g wet weight) were harvested by vacuum filtration onto Whatman #541 filters, transferred to 2 ml screw cap tubes containing ice-cold 0.5 g acid-washed glass beads (0.5 mm diameter) and 1.5 ml polysome extraction buffer (100 mM KCl; 20 mM HEPES-KOH, pH 7.5; 2 mM Mg(OAc)2; 15 mM 2-mercaptoethanol; 100 μg/ml cycloheximide) and disrupted for 50 sec in a Mini-Beadbeater at 4°C. Homogenates were centrifuged at 4°C for 5 min at full speed in a microfuge. Supernatants (0.9 ml) were transferred to
fresh 2 ml screw cap tubes containing 100 µl of 50 mg/ml heparin and 250 µl of 50% glycerol, quick-frozen in liquid nitrogen and stored at -80°C until further analysis.

For polysome analyses, equal number of A260 units of homogenate, in a maximum volume of 400 µl, was layered on 12 ml linear sucrose gradients (15-50% w/w sucrose in 10 mM HEPES-KOH, pH 7.5; 70 mM NH4OAc; 4 mM Mg(OAc)2). Gradients were centrifuged in a Beckman SW41 rotor at 40,000 rpm for 2 h at 4°C. Twelve 1 ml fractions were collected from the bottom with a Hoefer gradient tube fractionator into screw cap tubes containing 50 µl of 10% SDS; polysome profiles were generated by following the absorbance at 254 nm with an ISCO UA5 absorbance monitor. Fractions were quick-frozen in liquid nitrogen and stored at -80°C until further processing.

Polysomal RNA was extracted by mixing each fraction with 1 ml of phenol:chloroform (1:1) for 1 min in the Mini-Beadbeater. Phases were separated by centrifugation for 10 min at full speed in a microfuge. The aqueous phase was transferred to a fresh tube containing 1 ml of chloroform; mixing and centrifugation were repeated. The aqueous phase was transferred to a fresh tube and RNA precipitated with NaOAc (pH 5.5) and isopropanol. RNA was centrifuged and the supernatant removed by aspiration. RNA was resuspended in 100 µl of sterile DEPC-treated water, reprecipitated with ethanol and NaOAc, washed with 70% ethanol, resuspended in 42 µl of sterile DEPC-treated water, quick-frozen in liquid nitrogen and stored at -80°C until further analyses by northern blotting.

2.8 Plasmid constructions for lacZ expression studies

Standard procedures were used unless otherwise indicated (Sambrook, et al., 1989). *Escherichia coli* strain XL-1 Blue (Short, et al., 1988) was used as the bacterial host. Plasmid pF02 was generated by subcloning the 1.1 kb *KpnI-EcoRI* fragment of pAE1 (Berger, et al., 1992; Orbach, et al., 1990) into the corresponding sites of pUC119. This fragment of pAE1 contains the 5' region of *arg-2* including the uORF. pF02ΔAUG was generated by site-directed mutagenesis (Nakamaye and Eckstein, 1986) of pF02 with the oligonucleotide 5'-TTGTCGCAATCTGCCCCTCggAACGGGCGCCG-3'. This
oligonucleotide replaces the ATG start codon of the *arg-2* uORF with GAG and generates a novel *XhoI* site in its place.

Plasmids that contained nested deletions of the *arg-2* 5' sequence were constructed by exonuclease III and S1 nuclease treatment (Henikoff, 1984) of plasmid pFo2 following linearization of the plasmid with *BamHI* and *PstI*. DNA sequences at deletion junctions were confirmed by the chain termination DNA sequeencing method (Sanger, et al., 1977) using Sequenase (USB). Deletion plasmids included pUCZL1 (nt 634-928 of *arg-2* (Orbach, et al., 1990)), pUCZL2 (nt 634-1270), pUCZL5 (nt 634-1359), pUCZL6 (nt 634-1373), pUCZL7 (nt 634-1405) and pUCZL8 (nt 634-1587).

Plasmids containing *arg-2-lacZ* genes were constructed by subcloning appropriate *arg-2* fragments into phosphatase-treated, *SmaI*-digested vectors pDE1, pDE2, or pDE3 (Ebbole, 1990). All *arg-2* fragments were made flush-ended using the Klenow fragment of DNA polymerase I and nucleotide triphosphates to prepare them for subcloning. The structures of the gene fusions were confirmed by DNA sequencing (Sanger, et al., 1977).

pZL601 was generated by first treating the 0.85 kb *EcoRI*-*StyI* fragment from pFo2 with the Klenow fragment of DNA polymerase I and dNTPs to generate flush ends, and then ligating this fragment into the *SmaI* site of pDE3, a vector designed for placing *lacZ* reporter genes at the *his-3* locus (Ebbole, 1990). This construct creates a translational fusion of the LacZ coding region with the Arg2p coding region at codon 10 of the predicted Arg2p polypeptide. pZL610 was generated by the corresponding ligation of the filled 0.85 kb *EcoRI*-*StyI* fragment from pFo2ΔATG into the *SmaI* site of pDE3. The structures of these constructs were verified by DNA sequencing (Sanger, et al., 1977).

Plasmid pZL613, containing a 21 codon uORF with an altered primary sequence (MNGPVSLHLHSLPLQPSVEESP), was constructed in the following way: first, pZL304 was generated by subcloning the 0.97 kb *EcoRI*-*HindIII* fragment from pUCZL8 into the corresponding sites of pBS-SK+ (Short, et al., 1988). pZL304 then was cut with *NarI* (a unique site in this plasmid, positioned at uORF codon 4), the overhanging ends trimmed with mung bean nuclease, and the plasmid recircularized using T4 DNA ligase to generate pZL305. pZL613 was obtained by subcloning the 0.85 kb *EcoRI*-*StyI* fragment from pZL305 into pDE3.
pZL1 was generated by subcloning the 0.31 kb EcoRI-SphI fragment from pUCZL1 into pDE1; pZL6a, by subcloning the 0.75 kb EcoRI-SphI fragment from pUCZL6 into pDE1; pZL6b, by subcloning the 0.75 kb EcoRI-HindIII fragment from pUCZL6 into pDE3; pZL6fs, by subcloning the 0.75 kb EcoRI-SphI fragment from pUCZL6 into pDE3; pZL7, by subcloning the 0.79 kb EcoRI-HindIII fragment from pUCZL7 into pDE3; pZL2, by subcloning the 0.63 kb EcoRI-SphI fragment from pUCZL2 into pDE3; pZL5, by subcloning the 0.73 kb EcoRI-SphI fragment from pUCZL5 into pDE3.

2.9 Construction of *N. crassa* strains containing *arg-2-lacZ* integrated at the *his-3* locus

The *N. crassa his-3(1-234-723)* strain FGSC #6103 (Fungal Genetics Stock Center, University of Kansas Medical Center) was transformed with plasmids containing *arg-2-lacZ* fusion genes as described (Selitrennikoff and Sachs, 1991) and transformants obtained by selection for His prototrophy (Sachs and Ebbole, 1990). The recipient strain was chosen because the *his-3* mutation it contained mapped to the distal region of the gene (Overton, et al., 1989). Prototrophic *N. crassa* homokaryons were obtained by microconidiation (Ebbole and Sachs, 1990). Southern analyses were used to identify transformants containing a single copy of *arg-2-lacZ* integrated at *his-3*. Approximately one-half of the transformants examined fit these criteria.

2.10 Southern blot analyses

Southern blot analysis (Sambrook, et al., 1989; Sehgal, et al., 1988) was accomplished using *N. crassa* genomic DNA prepared by following a modified previously described procedure (Oakley, et al., 1987); sodium trichloracetate used in DNA isolation was obtained from Aldrich, St. Louis, MO. The amount of DNA recovered was quantified using Hoechst 33258 (Labarca and Paigen, 1980) and a Hoefer TKO-100 fluorometer using procedures supplied by Hoefer. One µg of DNA digested with an appropriate restriction enzyme was loaded per lane. The *E. coli lacZ* probe was the 0.83 kb *SacI-EcoRV* fragment from pDE2 (Ebbole, 1990); the *arg-2* probe was the
0.85 kb EcoRI-StyI fragment from pFo2ΔATG; the his-3 probe was the 1.7 kb XhoI fragment from pDE2. Hybridization and washing conditions were the same as were used for northern analyses.

2.11 β-galactosidase assays

Cell-free extracts were prepared as described above for immunoblot analyses. β-galactosidase activity was assayed using 50 µg of protein as described (Miller, 1972; Sachs and Ebbole, 1990). Briefly, cell extract was diluted to 1 ml with Z buffer (60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM KCl, 1 mM MgSO₄, 50 mM β-mercaptoethanol, pH 7.0) and incubated at 28°C for 2 min. 200 µl of 4 mg/ml ONPG was added and incubation continued at 28°C until light-medium yellow color is seen. Reaction was stopped by adding 500 µl of 1 M Na₂CO₃. Optical absorbance at 420 nm was measured and the specific activity of β-galactosidase was calculated by the following equation:

\[
\text{β-galactosidase activity} = \frac{(A_{420} \times 380)}{\text{Incubation Time in minutes} \times \text{amount of protein in milligrams used in reaction)}
\]

At least two independent transformants that contained a single copy of each construct integrated at his-3 were examined initially. In every case, the levels of β-galactosidase activity, and the magnitude of regulation by Arg, were similar in independent transformants representing a given construct. The data reported here represent one of two growth experiments in which a representative transformant containing each construct was grown and analyzed in parallel with a representative of each of the other constructs. The results of the second experiment were similar, as were other studies using smaller subsets of strains.

2.12 Immunoprecipitation of LacZ proteins from N. crassa crude cell extracts

LacZ proteins expressed from the arg-2-lacZ fusion genes integrated at his-3 were precipitated from N. crassa crude extracts as the followings: 100 µg of N. crassa crude cell extract were diluted to 1 ml with IP buffer (50 mM Tris-HCl, pH 8.0; 150 mM NaCl; 0.05% Tween 20) containing 300 ng of anti-β-galactosidase monoclonal antibody
(Promega) and incubated for 2 hours at 4°C with gentle rotation. 40 µl (1:3 dilution with IP buffer) of Protein A-Sepharose 6MB beads (Pharmacia) were added and incubation was continued overnight at 4°C with gentle rotation. Beads were collected by centrifugation and the supernatant was transferred to another tube. Beads were washed three times with 1 ml IP buffer and resuspended with 100 µl of IP buffer. β-galactosidase activities were assayed for beads and supernatants as described in section 2.11.

2.13 Quantification

The values reported in tables represent averages for at least two independent growth experiments, each examined using duplicate samples. The relative levels of polypeptides visualized by immunoblotting were analyzed by scanning the blots with a Microtek MSF 300ZS scanner, and analyzing the data using NIH Image v.1.53. The relative levels of mRNA detected by RNA blotting, and the levels of pulse-labeled polypeptide detected following immunoprecipitation and SDS-PAGE were determined by densitometric analysis of film images using a Bio-Rad Model 620 Video Densitometer, or by direct analysis using a Molecular Dynamics Phosphorimager. Quantitative methods were validated through reconstruction experiments using a dilution-series of comparable samples.
CHAPTER 3
RESULTS

The regulation of \textit{N. crassa arg-2} and \textit{cpc-1} in response to amino acid availability was examined. The role of \textit{arg-2} uORF in Arg-specific regulation was dissected by using \textit{Escherichia coli lacZ} as the reporter gene. Analyses of mRNA levels, polypeptide pulse labeling results and the distribution of mRNA in polysomes indicated that Arg-specific negative regulation of \textit{arg-2} affected both the levels of \textit{arg-2} mRNA and \textit{arg-2} mRNA translation. Negative translational effects on \textit{arg-2} and positive translational effects on \textit{cpc-1} were apparent soon after cells were provided with exogenous Arg. In cells limited for His, increased expression of \textit{arg-2} and \textit{cpc-1}, and decreased expression of \textit{cox-5}, also had translational and transcriptional components. Examination of the role of \textit{arg-2} uORF in Arg-specific regulation of \textit{arg-2} showed that the uORF appeared necessary for regulation because elimination of the uORF start codon resulted in increased, constitutive gene expression and loss of translational control. Analyses of the expression of \textit{arg-2-lacZ} genes in cells grown in the absence or presence of Arg at polypeptide and transcript levels, and examination of the distribution of ribosomes on transcripts, indicated that the primary sequence of the polypeptide encoded by the uORF was also important for translational control.

3.1 Prolonged exposure to Arg reduces the level of Arg2p, the rate of Arg2p synthesis and the level of \textit{arg-2} transcript

The expression of \textit{arg-2} was compared to the expression of \textit{cox-5} in wild type cells grown for 6.5 h in Min or Arg media. The \textit{cox-5} gene, which encodes cytochrome oxidase subunit V, was used as a control because it is not directly involved in amino acid metabolism. Exogenous Arg reduced the level of Arg2p and the rate of Arg2p synthesis, but not the level of Cox5p or the rate of Cox5p synthesis (Figure 3.1A and 3.1B). The
Figure 3.1 The effect of prolonged exposure to Arg on the expression of *arg-2* and *cox-5*. Wild type *N. crassa* were germinated for 6.5 hours in minimal medium (M) or Arg supplemented medium (R). (A) levels of Arg2p and Cox5p examined by western blot analysis; (B) protein synthesis rates of Arg2p and Cox5p examined by immunoprecipitation following a 5 min pulse-label with $^{35}$S-methionine; (C) *arg-2* and *cox-5* transcript levels examined by northern blot analysis.
level of *arg-2* transcript, but not *cox-5*, was also reduced by growth with Arg (Figure 3.1C), as previously observed (Orbach, et al., 1990; Sachs and Yanofsky, 1991). Based on quantification of experimental data, including the results shown in Figure 3.1, the observed reductions in the level of *arg-2*-dependent enzyme activity (3-fold), the cellular level of Arg2p (2.9-fold), the rate of Arg2p synthesis (3.3-fold) and the level of *arg-2* transcript (2.9-fold) were similar. Thus, within the precision of these measurements, the difference in the level of RNA appeared sufficient to account for most of the difference in the relative rate of Arg2p translation and the level of Arg2p polypeptide in wild type cells, but effects at the level of translation could not be excluded.

We also examined the distribution of *arg-2*, *cpc-1* and *cox-5* transcripts on polysomes prepared from cells grown in Min or Arg medium. Prolonged exposure to Arg did not affect the overall distribution of ribosomes between polysomes and monosomes (Figure 3.2). In both media, the *cox-5* transcript was found on larger polysomes, on average, than *cpc-1* transcript, and there was little difference in the amounts of each transcript in polysomes between Min- and Arg-grown cells (Figure 3.2). There was less *arg-2* transcript in the fractions collected from cells grown in Arg compared to cells grown in Min, consistent with the reduced level of *arg-2* RNA in total RNA pools (data not shown). In addition, the average size of the polysomes associated with *arg-2* transcripts was reduced in cells grown in Arg (Figure 3.2). This indicates that, in addition to a reduction in the level of *arg-2* transcript, translation of *arg-2* during prolonged growth with Arg was reduced when compared to *cox-5* and *cpc-1*.

### 3.2 Short-term exposure to Arg reduces the rate of Arg2p synthesis but not the level of *arg-2* transcript

The effect of short-term exposure to Arg was examined by switching cells grown for 6.5 h in Min to fresh Min or to fresh Arg-containing medium for 2 min, 10 min, or 30 min, and comparing the expression of *arg-2* to *cox-5* (Figure 3.3, Table 3.1). The levels of Arg2p and Cox5p were not affected by transfer of cells from Min to fresh Min for 2, 10 or 30 min (Figure 3.3A). In this experiment, we observed a 35% reduction in the level of Arg2p but not Cox5p in cells transferred from Min to Arg medium. This reduction in
Figure 3.2 Analysis of mRNA distribution in polysomes prepared from cells grown in Min and Arg media. Wild type *N. crassa* were germinated for 6.5 hours in minimal medium (M) or Arg supplemented medium (R). Extracts were prepared and separated through sucrose gradients. (A, C): A254 absorbance profiles of gradients from cells grown in Min and Arg, respectively, with the top of the gradient to the right. The scale bar indicates 0.2 absorbance units; the position of the 80S monosome is indicated with an arrow; 16.7 A260 were loaded. (B, D) Distribution of *arg-2* (squares), *cox-5* (diamonds) and *cpc-1* (circles) mRNA in gradient fractions from cells grown in Min and Arg, respectively. Equal volumes of each fraction were examined by northern blotting and Phosphorimager analysis; the relative amount of mRNA in each fraction was calculated with respect to the summed amount of each mRNA in all fractions. Fraction 1 is the bottom of the gradient; fraction 12, the top. The position of the monosome peak is indicated with an arrow.
Table 3.1 Relative expression of *arg-2* and *cox-5* after short-term exposure to Arg

<table>
<thead>
<tr>
<th>arg-2/cox-5</th>
<th>M to M</th>
<th>M to R</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 10 30</td>
<td>2 10 30</td>
</tr>
<tr>
<td>Protein</td>
<td>1.0 1.0 1.1 1.1</td>
<td>0.7 0.6</td>
</tr>
<tr>
<td>Prot Synth</td>
<td>1.0 1.9 1.6 0.8</td>
<td>1.1 0.5</td>
</tr>
<tr>
<td>RNA</td>
<td>1.0 2.1 1.3 1.1</td>
<td>2.1 1.1</td>
</tr>
</tbody>
</table>

*Cells were grown in minimal medium (M) for 6.5 hr and then transferred to minimal (M) or arginine-containing (R) media for 2, 10 or 30 min. Calculations were based on data as described in Materials and Methods. The relative amounts of *arg-2* compared to *cox-5* expression after transfer from minimal medium to minimal medium for 2 min were given the unit value of 1.0.*
the level of Arg2p after short-term exposure to Arg was not always reproducibly observed (data not shown).

Pulse-labeling data showed that transfer to Arg instead of Min significantly reduced the rate of Arg2p synthesis but not Cox5p synthesis (Figure 3.3B, Table 3.1). The level of arg-2 transcript was not reduced during short-term exposure to Arg (Figure 3.3C, Table 3.1); it was similar in cells switched to either Min or Arg, with a transient increase in arg-2 transcript level observed 10 min after transfer to either medium (Figure 3.3B, Table 3.1). Comparison of the level of Arg2p synthesis to the level of arg-2 transcript following shifts to Min or Arg medium indicated that net synthesis of Arg2p per unit of arg-2 transcript was reduced two-fold within 10 minutes of transfer to Arg medium compared to transfer to Min medium (Table 3.1). At 30-minute time point, the reduction was similar to what was observed for prolong cell growth in Arg. The rate of Cox5p synthesis and the level of cox-5 transcript were unaffected by switching to either fresh Min or Arg media (Figure 3.3B and 3.3C, Table 3.1). These data indicate that, following transfer to fresh Arg medium, the efficiency of Arg2p translation and/or the stability of newly synthesized Arg2p polypeptide were reduced.

In eukaryotes, polyadenylation of mRNA can play a role in its translatability (Sachs and Wahle, 1993). To determine whether the reduction of the rate of Arg2p synthesis was due to deadenylation of arg-2 mRNA, we examined the level of arg-2 and cox-5 transcripts in poly(A) mRNA prepared from cells that were switched from Min medium to either fresh Min medium or Arg medium for 30 min. Comparison of the relative amounts of arg-2 and cox-5 transcript in poly(A) preparations from cells grown in Min or Arg media indicated that the level of arg-2 mRNA containing poly(A) tails sufficient to bind to oligo-dT was not changed by short term exposure to Arg (Figure 3.4).

The reduction in Arg2p synthesis rate observed after short-term exposure to Arg, without concomitant reduction in the level of arg-2 transcript, suggested the possibility of regulation at the level of translation. Examination of polysomes from cells switched from Min to Min or from Min to Arg medium for 30 min revealed a reduction in the average size of polysomes translating arg-2 mRNA in cells switched to Arg (Figure 3.5), while short term exposure to Arg did not affect the distribution of cox-5 on polysomes (Figure 3.5). Interestingly, short-term exposure to Arg resulted in the appearance of cpc-1
Figure 3.3 The effect of short-term cell exposure to Arg on *arg-2* and *cox-5*. Wild type cells were grown in minimal medium (M) for 6.5 hours, then switched to either fresh minimal medium or arginine supplemented medium (R) for 2 min, 5 min, and 30 min respectively. (A) levels of Arg2p and Cox5p examined by western blot analysis; (B) protein synthesis rates of Arg2p and Cox5p examined by immunoprecipitation following a 5 min pulse-label with 35S-methionine; (C) transcript levels of *arg-2* and *cox-5* examined by northern blot analysis.
Figure 3.4 The effect of short-term cell exposure to Arg on the $arg\text{-}2$ and $cox\text{-}5$ poly(A) mRNA. Wild type cells were grown for 6.5 hours in minimal medium (M), then switched to either fresh minimal medium or Arg supplemented medium (R) for 30 min. Poly(A) mRNA was prepared by oligo-dT cellulose chromatography and the levels of $arg\text{-}2$ and $cox\text{-}5$ poly(A) mRNA were examined by northern blot analysis.
Figure 3.5 Polysome profile analysis of cells switched from minimal medium to minimal, Arg, AT, or AT + His media for 30 min. Wild type *N. crassa* were germinated for 6.5 hours in minimal medium and switched to Min (A, B), Arg (C, D), AT (E, F) or AT + His (G, H) media for 30 min. Extracts were prepared and separated through sucrose gradients. (A, C, E, G): A254 absorbance profiles of gradients with the top of the gradient on the right. The scale bar indicates 0.2 absorbance units; the position of the 80S monosome is indicated with an arrow; 10 A260 were loaded. (B, D, F, H): distribution of *arg-2* (squares), *cox-5* (diamonds) and *cpc-l* (circles) mRNA in gradient fractions. Equal volumes of each fraction were examined by northern blotting and Phosphorimager analysis; the relative amount of mRNA in each fraction was calculated with respect to the summed amount of each mRNA in all fractions. Fraction 1 is the bottom of the gradient; fraction 12, the top. The position of the monosome peak is indicated with an arrow.
transcript on larger polysomes (Figure 3.5), suggesting translational activation that was not observed after prolonged growth in Arg (Figure 3.2).

3.3 Comparison of short-term changes in response to Arg or His availability

We examined short-term responses to limitation for His induced by AT in parallel with analyses of short-term exposure to Arg (Figure 3.6). Exposure to Arg for 30 min reduced the level of Arg2p synthesis but not the level of arg-2 RNA (Figure 3.6, Table 3.2). A reproducible decrease in leu-6 mRNA and Leu6p synthesis in response to fresh Arg medium compared to fresh Min medium was also observed (Figure 3.6 and data not shown); the explanation for this is not known at present. The slight decrease in cpc-1 transcript following transfer to Arg seen here did not always accompany the shift to Arg medium (data not shown).

His starvation is known to increase the levels of arg-2, cpc-1 and leu-6 transcripts, and reduce the level of cox-5 transcript (Chow and RajBhandary, 1989; Orbach, et al., 1990; Paluh, et al., 1988; Sachs and Yanofsky, 1991). In addition to these effects on transcript levels (Figure 3.6), in AT-treated cells there were increased rates of Arg2p and Leu6p synthesis, and a decreased rate of Cox5p synthesis (Figure 3.6). There was slightly more synthesis of Arg2p than would be expected from the increase in transcript level alone (Table 3.2). The effects of AT were at least partially abrogated when His was added at the same time. However, His alone appeared to reduce both the level of Arg2p synthesis and the level of arg-2 mRNA, as well as affecting Cox5p synthesis (Figure 3.6).

The effect of transfer to AT medium on translation could also be seen in analyses of polysomes (Figure 3.5). Addition of AT to cells led to increased recovery of material in the monosome fraction in polysome preparations (compare Figure 3.5A and 3.5E). In AT medium, relatively large amounts of arg-2 and cpc-1 transcripts were associated with polysomes, while the distribution of cox-5 transcript shifted to monosomes and small polysomes. The effects of AT on translation were reduced when His was also added (Figure 3.5).
Figure 3.6 The effect of amino acid availability on *arg-2*, *cox-5*, *leu-6*, and *cpc-1*. Wild type cells were grown for 6.5 hours in minimal medium (M), then transferred to fresh minimal medium (M), Arg supplemented medium (R), AT-supplemented medium (AT), AT+His supplemented medium (AT+H), and His supplemented medium (H). (A) Arg2p, Cox5p, Leu6p synthesis rates examined by immunoprecipitation following a 5 min pulse-label with $^{35}$S-methionine; (B) *arg-2*, *cox-5*, *leu-6*, and *cpc-1* transcript levels examined by northern blot analysis.
Table 3.2  Relative expression of *arg-2* and *cox-5* after short-term exposure to Arg, AT, AT + His, or His\textsuperscript{a}

<table>
<thead>
<tr>
<th>arg-2/cox-5</th>
<th>M to M</th>
<th>M to R</th>
<th>M to AT</th>
<th>M to AT+H</th>
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\textsuperscript{a}Cells were grown in minimal medium (M) for 6.5 hr and then transferred to minimal (M), arginine-containing (R), AT-containing (AT), AT+His containing (AT+H) and His containing (H) media for 30 min. Calculations were based on data as described in Materials and Methods. The relative amounts of *arg-2* compared to *cox-5* expression after transfer from minimal medium to minimal medium for 30 min were given the unit value of 1.0.
3.4 Obtaining *N. crassa* strains containing *arg-2-lacZ* integrated at the *his-3* locus

In order to assess the role of the *arg-2* uORF in gene expression, we constructed a variety of *arg-2-lacZ* fusion genes containing either the wild type uORF or altered uORF sequences. These fusion genes were placed in a vector that contained a truncated wild type *his-3* gene which confers His prototrophy when it recombines with a mutant allele of *his-3* (Sachs and Ebbole, 1990). The recombination will produce a wild type, functional *his-3* gene and a truncated one flanking the *arg-2-lacZ* fusion gene (Figure 3.7A). Verification that these constructs were integrated at *his-3* in recipient strains was accomplished by analyses of Southern blots.

Representative examples of Southern blot verification were shown in Figure 3.7. In these examples, genomic DNA prepared from wild type (74A), the untransformed (His-) recipient (6103), and transformed (His+) recipient strains were subjected to a certain restriction enzyme digest. *HindIII*-digested genomic DNA (Figure 3.7B) showed a single, approximately 9.5 kb *HindIII* fragment that hybridized to both *lacZ* and *arg-2* probes in strains transformed with plasmids pZL60l or pZL610. The *lacZ* probe did not hybridize to wild type or untransformed recipient *N. crassa* strains, as expected (Figure 3.7B). A transformant containing the plasmid pDE1 contained a smaller fragment that hybridized to *lacZ* but not *arg-2*, as expected, because pDE1 has *lacZ* but not *arg-2* sequences (Figure 3.7B). The *arg-2* probe also hybridized to a 2.3 kb *HindIII* fragment representing the endogenous *arg-2* in all strains. These results are what would be predicted from homologous integration of these plasmids at the *his-3* locus (Figure 3.7A).

Further evidence confirming integration at *his-3* was obtained using a *his-3* probe (Figure 3.7C). As expected (Figure 3.7A), an approximately 7.5 kb *BglII* fragment hybridized to the *his-3* probe in untransformed strains, and two fragments (a 9.5 kb fragment and a second fragment of 7.5 kb or 6.8 kb, depending on the integrating plasmid) hybridized to the probe in strains containing plasmids integrated at *his-3* (Figure 3.7A). The sizes of these two fragments were consistent with integration of each of these plasmids at *his-3*. An additional, weakly hybridizing larger fragment was also detected in transformants; the size of this fragment appeared to be the sum of the two smaller fragments. Moreover, its intensity varied among repeated experiments with *BglII*
Figure 3.7 Homologous recombination of transforming plasmids at the his-3 locus. (A) schematic diagram of pZL601 recombining at his-3. The origins of DNA sequences are indicated by different segment patterns and markings. lacZ: E. coli lacZ coding region; arg-2: arg-2 5' region; trpC: A. nidulans trpC terminator region; pSP72: sequence from pSP72; [his-3]: his-3 sequence with a truncated open reading frame; his-3: restored his-3 sequence with wild type function. The direction of arg-2-lacZ transcription is indicated with an arrow in the plasmid. The recipient's his-3 coding sequence is represented by open rectangle with the putative region of the inactivating mutation indicated by an "x". Probes and restriction enzyme sites used in the Southern blots shown in (B) and (C) are indicated. (B) Southern blot analysis of HindIII digested genomic DNA using lacZ and arg-2 probes. 74A: wild type N. crassa; 6103: the recipient strain used for transformation; 601, 610, and DE1: representative transformants containing plasmids pZL601, pZL610, and pDE1, respectively, integrated at his-3. (C) Southern blot analysis of BglII digested genomic DNA from these strains using his-3 probe.
digested genomic DNA (data not shown). The appearance of this band is likely due to
inefficient cutting at a BgIII site, possibly because this enzyme is inhibited by substrate
methylation, and methylation of *N. crassa* during vegetative growth near repeated
sequences is known to occur (e.g., (Levin, et al., 1973; Selker, et al., 1993)).
Homologous recombination of pZL601, pZL610, and pDE1 in transformants were also
confirmed using other restriction enzymes, including *DraI* and *EcoRI* (data not shown).

### 3.5 Effects of altering *arg-2* 5' sequences on the expression of
β-galactosidase

In wild-type *N. crassa*, the level of Arg2p polypeptide is reduced approximately
2.5-fold when cells are provided with Arg. The effect of Arg on the expression of
different *arg-2-lacZ* fusion genes was examined by first measuring β-galactosidase
activity in cells grown in minimal medium (Min) or minimal medium supplemented with
arginine (Arg). Strains containing pZL601, an *arg-2-lacZ* construct containing the wild
type uORF, produced substantial β-galactosidase compared to the background levels
detected in wild type *N. crassa* (strain 74A-OR23-1VA), which lacks the *lacZ* gene
(Figure 3.8). The level of β-galactosidase activity was 2.4-fold higher in Min than in Arg
medium (Figure 3.8). Negative regulation by Arg of the fusion gene containing the wild
type uORF was thus similar to the level of regulation of the endogenous *arg-2* gene.

While the construct containing the wild type uORF retained regulation, constructs
in which the predicted translation initiation site for the uORF was eliminated by
site-specific mutagenesis (pZL610) or in which the predicted primary amino acid
sequence of the uORF was altered (pZL613) lost Arg-specific negative regulation (Figure
3.8). The construct in which the uORF start codon was removed (pZL610) produced a
higher level of β-galactosidase activity than either of the constructs containing a uORF
(pZL601 and pZL613). Thus, compared to a gene without a uORF, either of two
different uORFs reduced the level of gene expression, but Arg-specific negative
regulation was observed only with the wild type uORF.

Negative regulation by Arg was observed for construct pZL7 in which the context
of the *lacZ* initiator AUG was changed and the distance between the uORF stop codon
and this AUG shortened from 63 nt to 20 nt (Figure 3.8). In contrast, negative regulation
Figure 3.8 Effect of growth in Arg on the level of β-galactosidase activity expressed from arg-2-lacZ reporter genes. Strains were grown at 34°C for 6.5 h in Min or Arg. Crude cell extracts were prepared and β-gal activity assayed (see Experimental Procedures). The structures of the uORF and LacZ coding regions of the arg-2-lacZ reporter construct present in each strain are diagrammed schematically. β-gal activities (Miller units/mg protein) measured for strains containing each construct grown in Min or Arg medium are as indicated. Wild type (74A) lacks any arg-2-lacZ reporter construct. Black triangles indicate the wild type uORF or Arg2p AUG initiation codons; open triangles indicate novel AUG initiation codons; the dash indicates elimination of the uORF initiator AUG by site-specific mutagenesis. Black boxes indicate the wild type uORF peptide sequence; gray boxes indicate the wild type Arg2p peptide sequence; white boxes indicate the LacZ peptide sequence. Hatched boxes in pZL613 and pZL1, and the stippled box in pZL6b, indicate novel uORF peptide sequences. The white box delineated with a dashed line in pZL6fs indicates lacZ fused out-of-frame to the uORF.

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was lost in construct pZL1 (Figure 3.8), which contained a 13-codon uORF whose first 11 codons were identical to those of the 24-codon wild type uORF. Thus these results revealed that altering the relative positions of the uORF termination codon and the initiation codon for LacZ did not affect Arg-specific negative regulation, but truncating the uORF abrogated regulation.

The effects of creating direct fusions of LacZ to the uORF coding region and of creating overlapping reading frames were also examined. Transformants containing pZL6a, in which lacZ was translationally fused to the 20th codon of the arg-2 uORF, produced substantial β-galactosidase activity that was not reduced by growth in Arg compared to growth in Min (Figure 3.8). Instead, β-galactosidase activity increased slightly in Arg. Construct pZL6b contained an arg-2-lacZ gene specifying "nested" open reading frames (Figure 3.8), in which the uORF peptide should initiate normally and a second downstream AUG should initiate translation of the lacZ gene in a second reading frame that overlaps codon 20 of the uORF. Thus two overlapping polypeptides are predicted to be specified by this construct: LacZ and a 72 residue uORF in which the first 21 amino acid residues match the wild type uORF peptide sequence. Expression of this gene was also negatively regulated by Arg (Figure 3.8). In contrast, a construct that was similar but which was out-of-frame with respect to the uORF initiator and which had no in-frame AUG available to initiate translation of LacZ until codon 106 of the LacZ coding region (pZL6fs), produced little β-galactosidase activity (Figure 3.8).

Since N. crassa has its endogenous β-galactosidase activity (albeit low), it is necessary to distinguish the proportion of β-galactosidase activity contributed by the LacZ proteins expressed by the arg-2-lacZ fusion genes from the endogenous activity when low levels of β-galactosidase activity (< 10 units) were detected in the crude cell extracts. Immunoprecipitation of LacZ with anti E. coli β-galactosidase antibody showed that the β-galactosidase activities measured in the crude extracts were contributed primarily from the LacZ proteins for the strains containing pZL1, pZL7, and pZL6b (Table 3.3). The slight increase in β-galactosidase activity (approximately 0.2 units/mg protein) above background observed in pZL6fs appeared to be due to synthesis of LacZ since it could be immunoprecipitated with the antibody (Table 3.3). The antibody did not immunoprecipitate detectable β-galactosidase from the wild type strain lacking lacZ sequences (<0.02 units/mg).
The observations that a relatively high level of β-galactosidase was produced when fused in-frame with the uORF initiator (pZL6a), but not when out-of-frame with the uORF initiator (pZL6fs) indicated that the uORF reading frame was translated in vivo. Additional data were also consistent with translation of the uORF: constitutive β-galactosidase expression was also observed in transformants containing constructs pZL2a or pZL5a, in which lacZ was translationally fused to the 12th or 18th codons of the arg-2 uORF, respectively (Figure 3.8 and data not shown).

While it is possible to make legitimate comparisons of the absolute levels of β-galactosidase produced from constructs pZL601, pZL610 and pZL613, because in each case the context of the initiation codon and the N-terminus of the LacZ polypeptide are the same, this is not true for the other constructs that were examined. Thus, while it is clear that the expression of constructs pZL6a and pZL2a both increase slightly in Arg medium, the higher overall level of β-galactosidase activity observed in strains containing pZL2a could be a consequence of differences in the amino-terminus of the LacZ polypeptide. And while it can be concluded that Arg-specific negative regulation is retained in constructs pZL7 and pZL6b, but not pZL1, the lower levels of β-galactosidase activity observed with these constructs could arise from reduction in the specific activity of the β-galactosidase polypeptides and/or from differences in the context of the AUG codons predicted to initiate translation of these polypeptides.

3.6 All constructs produced similar levels of arg-2-lacZ mRNA levels that were not subject to negative regulation by Arg

In order to determine whether exogenous Arg affected the expression of arg-2-lacZ fusion genes at the transcript level, we examined the level of arg-2-lacZ transcripts produced in transformants containing arg-2-lacZ genes. Since levels of arg-2-lacZ mRNA in transformants were quite low, we analyzed poly(A) RNA rather than total RNA. As controls, we examined expression of the endogenous arg-2 gene, whose transcript level decreases following prolonged growth in Arg medium, and the cox-5 gene, whose transcript is not regulated by Arg (Orbach, et al., 1990; Sachs and Yanofsky, 1991). lacZ mRNA was not detectable in the wild type strain but was detectable in all transformants analyzed (Figure 3.9). No decrease in arg-2-lacZ
Figure 3.9 Effect of growth in Arg on the levels of arg-2-lacZ, arg-2 and cox-5 mRNA. Poly(A) mRNA was prepared from N. crassa wild type (74A) and transformants containing arg-2-lacZ reporter genes grown in Min (M) or Arg (R) medium as described in Figure 3.1. Poly(A) mRNA was examined by northern analysis with arg-2-lacZ, arg-2 and cox-5 probes as described in Experimental Procedures. Blots probed with lacZ were exposed to film for 96 h with an intensifying screen; blots probed with arg-2 were exposed for 10 h with a screen; blots probed with cox-5 were exposed for 4 h without a screen.
transcript in Arg medium was observed, as compared to minimal medium, in any of the transformants analyzed. In contrast, arg-2 mRNA was reduced by growth in Arg in all transformants in comparison to cox-5, which was unaffected by growth in Arg (Figure 3.9). The levels of arg-2-lacZ and arg-2 mRNA relative to cox-5 mRNA were similar in all transformants. Thus the differences in expression observed at the level of β-galactosidase activity in transformants containing different constructs did not appear to be a consequence of differences in mRNA levels.

3.7 The arg-2 uORF is important for negative regulation of arg-2-lacZ by Arg at the translational level

The negative regulation of arg-2-lacZ expression at the enzyme level (Figure 3.8) but not the transcript level (Figure 3.9) suggested the existence of post-transcriptional control mechanisms. To assess the importance of the uORF for translational regulation, we examined the distribution of ribosomes on arg-2-lacZ, arg-2, cox-5 and cpc-1 transcripts using polysomal profiles prepared from wild type (74A) and transformant strains containing pZL601, pZL610, or pZL613 that were grown in Min or Arg medium (Figure 3.10). We showed in previous sections that translational control of arg-2 could be detected by the shift of arg-2 transcript in polysome gradients in cells exposed to Arg. In each of the strains examined here, a smaller fraction of arg-2 mRNA was found in polysomes from cells grown in Arg (Figure 3.10). Neither cox-5 nor cpc-1 transcripts shifted in polysomes as a result of growth with Arg; the cox-5 transcript was associated with larger polysomes than cpc-1 in cells grown in either Min or Arg (Figure 3.10). The distribution of ribosomes on arg-2, cox-5, cpc-1 mRNAs was thus similar in each strain examined, and was consistent with previous observations.

In transformants containing pZL601, arg-2-lacZ transcripts were on larger polysomes in cells grown in Min compared to Arg, indicating that, like the endogenous arg-2 transcript, the arg-2-lacZ gene containing the wild type uORF was translationally regulated by Arg (Figure 3.10). In contrast, arg-2-lacZ transcripts from cells containing pZL610, in which the uORF start codon was deleted, or pZL613, in which the uORF had a different primary sequence, were not shifted to smaller polysomes following growth in
Figure 3.10 Effect of Arg on the distribution of \( \text{arg-2-lacZ, arg-2, cox-5 and cpc-1} \) mRNA in polysomes. Transformants containing pZL601, pZL610 and pZL613, and wild type \( \text{N. crassa} \) were grown for 6.5 hours in Min or Arg medium. Extracts were prepared and 6 \( A_{260} \) units of each extract separated through sucrose gradients. The bar graphs represent data from one of two independent experiments that gave similar results. The X-axis indicates the sucrose gradient fractions collected for each profile, with the sucrose concentration decreasing from fraction 1 to 8 (the four most dense fractions contained insignificant amounts of these transcripts and are not shown). Fractions 1-4 represent polysomal material; fraction 5 represents the monosome peak (arrow). Equal volumes of each fraction were examined by northern blotting with \( \text{lacZ, arg-2, cox-5 and cpc-1} \) probes as indicated; Phosphorimager analysis was used to determine the relative amount of mRNA in each fraction, calculated as the percentage of the total amount of each transcript in all of the fractions (Y-axis). Bars represent the relative amounts of \( \text{lacZ, arg-2, cox-5, and cpc-1} \) transcripts in each fraction as indicated. Black bars represent samples from cells grown in Min; open bars represent samples from cells grown in Arg.
Arg (Figure 3.10), indicating that translational regulation in response to Arg was lost in these constructs.

Additional analyses of the polysome data revealed other differences in the translation of uORF-containing mRNA consistent with the reduced expression of β-galactosidase from the uORF-containing constructs. By comparing the absolute amount of each mRNA associated with polysomes relative the absolute amount of cox-5 mRNA associated with polysomes (data not shown), less lacZ mRNA appeared to be associated with polysomes relative to cox-5 in strains containing constructs pZL601 and pZL613 than construct pZL610, regardless of medium. Similar comparisons of arg-2 to cox-5 and cpc-1 to cox-5 revealed no such medium-independent but strain-dependent differences, and in addition indicated that the absolute amount of arg-2 transcript associated with polysomes decreased in Arg, consistent with reductions in translation and transcript levels, and that the absolute amount of cpc-1 associated with polysomes was not decreased by Arg.
CHAPTER 4
DISCUSSION

4.1 Multiple levels of regulation for \textit{arg-2} and \textit{cpc-1}

We examined mechanisms regulating \textit{N. crassa arg-2}, a gene that is negatively regulated by Arg and positively regulated by cross-pathway control, and mechanisms regulating \textit{cpc-1}, a gene whose function is essential for cross-pathway control, in response to amino acid availability. The data indicate that multiple translational mechanisms, as well as transcriptional components, appear to affect the expression of \textit{arg-2} and \textit{cpc-1} relative to \textit{cox-5} in response to changes in amino acid availability.

The effect of Arg on the expression of \textit{arg-2} in wild type \textit{N. crassa} is approximately three-fold, a magnitude similar to that observed for Arg-specific regulation of \textit{S. cerevisiae CPAI} (Werner, et al., 1987). This effect is small but highly reproducible in experiments containing internal controls such as those presented here. Why should a cell bother to regulate a gene 3-fold in response to Arg availability? In fact, the amplitude of the regulatory response observed in wild type \textit{N. crassa} grown in Min compared to Arg medium is lower than the full range of regulation that is possible. Experiments in which the level of \textit{arg-2}-dependent enzyme activity was measured in Arg auxotrophs grown under conditions in which Arg was available or Arg was depleted showed that \textit{arg-2}-dependent activity could be modulated over a 100-fold range (Davis, et al., 1981). Our experiments showed 35-fold regulation of \textit{arg-2}-dependent CPS-A activity in an \textit{arg-12s} strain by Arg, compared to 3-fold regulation wild type (Luo, et al., 1995), largely because the expression of \textit{arg-2} is greater in \textit{arg-12s} in minimal medium (Cybis and Davis, 1975). Intracellular Arg concentrations are 10-fold lower in \textit{arg-12s} compared to wild type in minimal medium (Cybis and Davis, 1975). Examination of the level of \textit{arg-2} transcripts showed that the level of \textit{arg-2} transcripts is much higher in \textit{arg-12s} than in wild type cells (Sachs and Yanofsky, 1991). It is likely that this increased
level of \textit{arg-2} transcripts is due to the crosspathway control response to Arg limitation inside the cell, as suggested by the inability of \textit{arg-l2\textsuperscript{s}} \textit{cpc-1} double mutant strain to grow in minimal medium (Davis, 1979) and the nondepressibility of \textit{CPA1} expression under conditions of Arg starvation in a \textit{gcn4} mutant strain (Kinney and Lusty, 1989). Whether the effects on \textit{arg-2} transcript levels alone would account for all the difference in the range of its Arg-specific regulation for \textit{arg-l2\textsuperscript{s}} and wild type strains remains to be determined.

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 \textit{arg-2} transcripts; 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 (Figure 3.1). However, polysome analysis indicated that the average size of the polysomes associated with \textit{arg-2} transcripts was reduced in cells grown in Arg, suggesting the translation of \textit{arg-2} mRNA was also affected. A clear response of \textit{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 \textit{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 \textit{arg-2} mRNA was also observed in cells exposed shortly to Arg.

The rapid reduction in the rate of Arg2p synthesis in response to Arg was sometimes accompanied by a rapid, smaller decrease in the cellular level of Arg2p. A rapid reduction in cellular Arg2p might result from reduced synthesis of new Arg2p and a relatively high rate of polypeptide degradation. Consistent with this hypothesis, the \textit{arg-2}- and \textit{CPA1}-specified small subunits of carbamoyl phosphate synthetase have been reported to be difficult to purify, possibly due to their instability (Davis, et al., 1980; Pierard and Schroeter, 1978). The possibility that growth in Arg affects the stability of Arg2p will have to be addressed by more sensitive measurements, for example by pulse-chase studies. Similar experiments have revealed that the stability of \textit{S. cerevisiae} Gcn4p is regulated by amino acid availability (Kornitzer, et al., 1994; Vega-Laso, et al., 1993) and the stability of \textit{N. crassa} ornithine decarboxylase is regulated by polyamines (Barnett, et al., 1988).
The *cpc-l* transcript becomes associated with larger polysomes when *N. crassa* is switched from Min to Arg or from Min to His-starvation medium (Figure 3.5). In *S. cerevisiae* shifted from nutrient-rich to nutrient-poor media, the *GCN4* transcript also becomes associated with larger polysomes (Tzamarias, et al., 1989). This effect on *GCN4* appears to be mediated at the level of translation initiation (Hinnebusch, 1994; Tzamarias, et al., 1989). Two uORFs in the *GCN4* transcript, uORF1 and uORF4, are sufficient to confer translational control. The contexts surrounding the UAA stop codons that terminate *GCN4* uORF1 and uORF4 translation are critical for translational control and are different from each other (Grant and Hinnebusch, 1994; Miller and Hinnebusch, 1989). Two uORFs are present in the *cpc-l* transcript (Paluh, et al., 1988; Sachs, In press). The sequence of the 10 nt following *cpc-l* uORF1 (based on the revised *cpc-l* sequence in Genbank) resembles sequences preferred for reinitiation following *GCN4* uORF1. There are also similarities in the region downstream of *GCN4* uORF4 and *cpc-l* uORF2; specifically, the blocks of high G+C content important for *GCN4* uORF4 function appear to be conserved downstream of *cpc-l* uORF2. Thus a model for translational regulation of *cpc-l*, based on current understanding of *GCN4* regulation (Hinnebusch, 1993; Hinnebusch, 1994) would posit that translational control by amino acid availability regulates initiation at the uORF2 start codon versus the Cpc1p start codon.

Analyses of pulse labeling and polysome data revealed that the efficiency of *arg-2* translation did not decrease when cells were shifted to His-starvation medium, while *cox-5* translation decreased. It is possible that the *arg-2* uORF also has a role to maintain the level of translation of the *arg-2* gene product under conditions of limitation for other amino acids, as do the *GCN4* uORFs; this remains to be determined.

### 4.2 Defining the role of the *arg-2* uORF in Arg-specific regulation

The importance of the *arg-2* uORF in Arg-specific translational control was demonstrated by changing the sequence of the uORF. Elimination of the *arg-2* uORF by removing the translation initiation codon increased the expression of the reporter gene in both Min and Arg medium and Arg-specific regulation was lost (Figure 3.8, pZL601 vs. pZL610). 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 (Figure 3.8, pZL613). However, the presence of the frameshifted uORF resulted in reduced expression relative to the gene lacking a uORF (pZL610).

The failure of the frameshifted uORF to confer Arg-specific regulation indicated that the wild type uORF sequence was important for Arg-specific regulation. Consistent with this interpretation, a truncated uORF failed to confer Arg-specific regulation on the fusion gene (Figure 3.8, pZL1). A single missense mutation at codon 12 of the arg-2 uORF, changing Asp to Asn, also abolishes Arg-specific regulation (Freitag, et al., in press).

The levels of arg-2-lacZ fusion gene mRNA were not affected by prolonged growth in Arg, whereas the level of arg-2 mRNA was reduced (Figure 3.9). It remains to be determined whether the fusion gene lacks sequences that are important for regulation of transcript levels (e.g., the 3' region of the arg-2 transcript is absent in these constructs), or if heterologous sequences present in the reporter interfere with regulation. Reporter constructs containing the E. coli hygromycin phosphotransferase or herpes simplex virus (HSV) thymidine kinase genes instead of lacZ, and retaining the 3' region of arg-2, retain negative regulation at the transcript level in N. crassa (M. Freitag and M. Sachs, unpublished data).

Several lines of evidence indicated that control of arg-2-lacZ gene expression via the uORF was post-transcriptional and was primarily exerted at the level of translation. These results are consistent with the scanning model (Kozak, 1989c), in that the presence of an upstream AUG in arg-2-lacZ reporters had the effect of reducing expression from a downstream AUG. Thus, increased, constitutive expression is observed when the uORF start codon is removed, consistent with the prediction that initiation at an upstream start codon reduces the likelihood of initiation at a downstream start codon. In this regard, it is interesting to contrast our results using N. crassa arg-2-lacZ fusion genes with the observation that removal of the AUG in the S. cerevisiae CPA1 uORF results in the loss of Arg-specific negative regulation without increased constitutive expression (Werner, et al., 1987). This could represent a difference between the N. crassa and S. cerevisiae systems or could be a consequence of additional negative regulatory factors acting to
reduce *CPAI* expression (e.g., transcriptional or post-transcriptional control of RNA levels (Crabeel, et al., 1990)) that were not acting on *N. crassa arg-2-lacZ* reporters.

While two different uORFs both had the general effect of reducing gene expression post-transcriptionally (Figures 3.8 and 3.9), only the wild type uORF sequence conferred Arg-specific negative regulation. Several distinct possibilities (Figure 4.1) for uORF functions are suggested by these data. First, different uORFs might by a common mechanism reduce the pool of mRNA translating from the downstream AUG at a given time and thus reduce gene expression (Figure 4.1A). This is consistent with our observation that the amount of *arg-2-lacZ* mRNA associated with polysomes appeared to be less in both Min and Arg media when the mRNA contained either of two uORFs compared to an mRNA lacking a uORF. Second, the wild type *arg-2* uORF could have additional sequence-specific effects on mRNA translation (Figures 4.1B and 4.1C).

Is the *arg-2* uORF translated? Although we have obtained no direct biochemical evidence for the existence of the 24-residue uORF peptide product, in-frame translational fusions of the LacZ coding region directly to the uORF coding region produced β-galactosidase, indicating that the uORF reading frame was translated. Although the sequence context of the *arg-2* uORF, CUU AUG A appears to be a poor match to the consensus sequences surrounding *N. crassa* initiation sites (Edelmann and Staben, 1994; Williams, et al., 1992), at least two other uORFs appear to affect gene expression despite poor initiation contexts (Cao and Geballe, 1995; Parola and Kobilka, 1994).

### 4.3 Common features of the *arg-2* uORF and other uORFs

The sequences for three fungal CPS-A small subunit genes, *N. crassa arg-2*, *S. cerevisiae CPAI* and *Magnaporthe grisea arg-2* (Orbach, et al., 1990; Werner, et al., 1987; Shen and Ebbole, pers. comm.) reveal that each contains a uORF in the 5' region of the transcript and that the primary sequence of the uORF peptide is highly conserved. Arg-specific regulation of *CPAI* via its uORF has been studied by examining the effects of mutations on enzyme activity (Delbecq, et al., 1994; Werner, et al., 1987). As with *arg-2*, shortening the intercistronic distance between the *CPAI* uORF and the downstream open reading frame does not affect regulation; lengthening the distance also
Figure 4.1 Some models for arg-2 uORF function. (A) Preemptive initiation. A scanning ribosomal subunit encounters the first AUG in the mRNA, initiates and then completes translation. Initiation events at the downstream AUG are reduced. (B) Ribosome stalling because of a sequence-specific transpeptidation block. A ribosomal subunit initiates translation at the first AUG; subsequent elongation of the peptide chain is blocked, for example by uORF inhibition of transpeptidation or because of the unavailability of charged tRNA or another factor. The stalled ribosome blocks scanning ribosomes (C) Ribosome stalling because of a sequence-specific block to ribosome release. The ribosome successfully translates the uORF but does not release at the termination site, thus blocking scanning ribosomes.
has no effect. Furthermore, in *S. cerevisiae*, direct fusions of the *CPAI* uORF with LacZ retained Arg-specific regulation, and in *N. crassa*, regulation was retained when the uORF and LacZ reading frames overlapped. An interpretation consistent with these data is that ribosome reinitiation is not an important component in translational regulation by Arg in these systems. This contrasts with the well-studied translational response of *GCN4* in *S. cerevisiae* to amino acid limitation, wherein regulation is exerted by controlling the capacity of the translation machinery to reinitiate translation at the *GCN4* start codon, following initiation at a uORF (Hinnebusch, 1994).

The amino acid sequence of the uORFs appear essential for Arg-specific regulation of both *arg-2* and *CPAI*. All of the cis-acting mutations selected in vivo for constitutive *CPAI* expression map affect uORF translation (Werner, et al., 1987). Single missense mutation at codons 11 or 13 of the *CPAI* uORF, and a similar mutation at one of these sites in the *N. crassa* uORF, abolish Arg-specific regulation (Werner, et al., 1987; Freitag and Sachs, unpublished data). Mutations in the *CPAI* uORF that leave the amino acid sequence of the uORF peptide unchanged have no effect on Arg-specific regulation of *CPAI* expression (Delbecq, et al., 1994).

There are no conserved Arg residues in the *N. crassa arg-2* and *S. cerevisiae CPAI* uORFs: the *N. crassa arg-2* uORF contains two Arg residues, the *S. cerevisiae CPAI* uORF, none. Although Arg residues are found to be present in the *M. grisea arg-2* uORF, they are evidently dispensable for Arg-related functions in *S. cerevisiae*. Possibly, the level of charged tRNA or level of Arg influences the activity of some translational component and results in increased uORF-mediated inhibition of translation in the presence of Arg. It is also possible that the level of tRNA charging might directly affect translation of these Arg codons in the *N. crassa* uORF: the second Arg codon in the *N. crassa* uORF is the rarest Arg codon, and in the case of the maize *Lc* uORF, there is evidence that rare codons in an uORF reduces downstream gene expression (Damiani and Wessler, 1993).
4.4 Ribosome stalling could be involved in Arg-specific regulation of arg-2 and CPAI through uORF

Translating ribosomes can pause at various locations in an eukaryotic mRNA; pausing can occur at initiation, elongation, or termination (Wolin and Walter, 1988). Ribosome stalling (e.g., Figures 4.1B and 4.1C) has been proposed as a possible mechanism of translational regulation in the cases of several eukaryotic uORFs (Geballe and Morris, 1994). uORFs requiring amino acid coding information for function would probably interact with the translational machinery within the channel or at the peptidyl transferase center of the ribosome, thus blocking the movement of a translating ribosome. Stalling could occur either during elongation or during termination. In prokaryotes, translational regulation of cat-86 and cmIA, which are genes conferring chloramphenicol resistance to the cells, occurs by a mechanism of ribosome stalling caused by leader peptide inhibition of the activity of peptidyl transferase (Gu, et al., 1994a; Gu, et al., 1994b).

Ribosome stalling within the uORF coding region could explain why the level of uORF-lacZ hybrid protein comprising lacZ directly fused to the first 23 amino acids of the CPAI uORF peptide is still repressed by Arg (Delbecq, et al., 1994). Stalling probably occurs before the translating ribosome reaches the termination codon, since the natural uORF stop codon is eliminated in that construct. The data suggest several elements within the CPAI and arg-2 uORFs are important. A shortened CPAI uORF containing only the first 19 amino acid residues does not confer Arg-specific regulation (Werner, et al., 1987), suggesting that the carboxyl-terminal region of the uORF or the uORF length itself is important. The lack of Arg-specific repression of the arg-2 uORF-lacZ fusion gene in pZL6a (Figure 3.8) might also be a result of the loss of the last three amino acids of the uORF. Finally, the abolition of Arg-specific regulation by missense mutations in the highly conserved domain near the middle of these uORFs indicates that this region also is important for regulation. Stalling model is also consistent with the fact that the Arg-specific regulation of arg-2 and CPAI is insensitive to the length of the intercistronic region.

It seems likely that sequences in other eukaryotic genes may have functions analogous to those observed for the arg-2 uORF. Thus a gene may contain a uORF that
has sequence-independent effects on gene expression and additional sequence-dependent effects in response to special elicitors. Finally, it must be considered that, since it is possible that neither ribosome reinitiation nor translation termination may be an integral part of sequence-dependent translational control through uORFs, there may be physiologically important cases in which such sequences are not in a separate reading frame.

4.5 Future work

We have demonstrated that the expression of *N. crassa* arg-2 gene are subject to multiple levels of regulation and the uORF in the 5' leader region of arg-2 is important in Arg-specific translational control of arg-2 expression. However, the precise mechanism of this uORF-mediated translational control remains to be determined.

The importance of amino acid coding information in arg-2 uORF raises the possibility that amino acid residues other than Asp12 may be required in Arg-specific regulation. To address this question, site-directed mutagenesis (Nakamaye and Eckstein, 1986) or PCR-mediated (Landt, et al., 1990) mutagenesis can be used to introduce point mutations within the uORF for modifying every single amino acid residue. The modified uORFs will be examined to determine whether they can confer Arg-specific regulation by using the approaches as described here. In addition to the *E. coli lacZ*, other reporter genes, e.g., HSV thymidine kinase, hph, or luciferase, could also be useful in monitoring the regulatory effect elicited by the arg-2 uORF.

Although our studies indicate that arg-2 uORF AUG codon can be used to initiate the synthesis of the reporter enzyme β-galactosidase, questions still remain whether the authentic uORF peptide can be detected *in vivo* or translated and detected *in vitro*. Experiments to reconstitute Arg-specific regulation of arg-2 *in vitro* are currently underway in our lab and arg-2 has been shown to be efficiently translated *in vitro*. The successful reconstitution of Arg-regulation of translation *in vitro* would help further for examining the mechanism of Arg-specific regulation. Possible experiments include testing ribosome stalling model by toe-printing (Hartz, et al., 1988) and assaying whether the uORF peptide inhibits peptidyl transferase activity (Gu, et al., 1994b).
It remains to be determined, in addition to the contribution of cis-acting sequences like the uORF, whether trans-acting factor(s) contributes to the Arg-specific regulation of arg-2. Studies on yeast mutants affecting the GCN4 response to amino acid starvation have revealed that one of the positive regulatory proteins, GCN2, serves the function of a protein kinase for eIF-2α. The phosphorylation of eIF-2α, which in turn inhibits the eIF-2B-catalyzed exchange of GDP/GTP on eIF-2, is proposed to participate in the regulation of GCN4 by controlling the selection of the site for reinitiation. The regulation of N. crassa counterpart cpc-l might involve similar mechanisms. Isolation of yeast mutants affecting Arg-specific regulation of CPA1 expression yielded a mutant (CPAR) that appears to be required for Arg-specific repression of CPA1 expression, but is trans-recessive and unlinked to CPA1 (Thuriaux, et al., 1972). The nature of this mutant is still obscure. A class of N. crassa mutants which affect trans-acting factors involved in Arg-specific regulation have been obtained in our lab by using a novel mutant-screening approach (Freitag, et al., In Press) and they remain to be characterized.

Purification of possible protein factors associated with the arg-2 uORF peptide in vitro can be approached by precipitating the possible uORF associated protein complex from N. crassa cell extracts by using an arg-2 uORF specific antibody, which can be prepared using a fusion protein with glutathione S-transferase translationally fused at codon 24 of the arg-2 uORF peptide. Components in the uORF associated protein complex can be separated and purified by SDS-PAGE (Harlow and Lane, 1988) and subject to micro-sequencing for partial peptide sequences. Obtained sequences could be compared with known sequences in a protein sequence database to search for homology. Moreover, these sequences can also be used as a basis for the synthesis of degenerate oligonucleotides for cloning the candidate gene(s).

4.6 Other aspects of translational and transcriptional regulation in N. crassa

Much remains to be determined concerning the mechanisms regulating translation and transcript metabolism in N. crassa. N. crassa mRNA is capped (Seidel and Somberg, 1978), and presumably initiation occurs after scanning from the cap structure of the mRNA. Internal initiation, as sometimes seen in mammals (Vagner, et al., 1995), may
also occur in *N. crassa* (Vittorioso, et al., 1994). As is typical in eukaryotes, most *N. crassa* mRNAs lack uORFs, although there are other examples of functional uORF-containing genes (Plamann, et al., 1994; Vittorioso, et al., 1994) in addition to *arg-2* and *cpc-l*.

Similarities between the organization of *arg-2* and *CPA1*, and *cpc-l* and *GCN4*, would suggest that the mechanisms that regulate amino acid biosynthesis were in place before the ancestors of *N. crassa* and *S. cerevisiae* diverged. It is interesting in this regard that *N. crassa arg-2*, but not *S. cerevisiae CPA1*, specifies a polypeptide targeted to mitochondria, and that *arg-2* contains sequences that may function as intronic enhancers, features it shares with genes in mammals (Davis, 1986; Hong, et al., 1994; Orbach, et al., 1990).

There is an increasing understanding of the importance of translational control mechanisms in the regulation of uORF-containing eukaryotic genes (Damiani and Wessler, 1993; Geballe and Morris, 1994), but few instances are known in which the stimuli eliciting translational control are well-defined. The evidence indicates that *N. crassa arg-2* and *cpc-l* are examples of uORF-containing genes that are translationally controlled in response to amino acid availability. By a combination of genetic and biochemical approaches, such as described here, it should be possible to develop a greater understanding of the mechanisms responsible for eukaryotic translational control.
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Some twenty-seven years ago, I, the author of this Ph.D. thesis, emerged on the Earth. My first footstep on earth seemed to declare that I was forever and ever bound to be a native in Chengdu, Sichuan, the People's Republic of China. I have a blurred memory of my primary school time. But ever since I said good-bye to the primary school in 1979, I started to carry the traditional studying-hard torch in the best local high school, No. 7 middle school. In 1985, I carried that torch into the Department of Biology, Nankai University, Tianjin, where it burned brightly on the Biochemistry major for four years until a passport and a visa quenched it for a new career start across the Pacific Ocean. The next thing I knew was that I landed in a beautiful city in the USA, the Portland of Oregon.

In September, 1990, I started the five-year Ph.D. program under the supervision of Dr. Matthew Sachs at the Department of Chemistry, Biochemistry and Molecular Biology in the Oregon Graduate Institute of Science & Technology. During the five-year lab life, I studied the translational regulation of arg-2, which is a marvelously important gene involved in the arginine biosynthesis in *Neurospora crassa*, an indescriptably beautiful golden brown fungus.