A UV-Induced Mutation in *Neurospora crassa* Shear & Dodge that Affects Translational Regulation in Response to Arginine

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Abstract

A UV-Induced Mutation in *Neurospora crassa* Shear & Dodge that Affects Translational Regulation in Response to Arginine

by Michael Freitag

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This study concerns the elucidation of the role of a 24-codon upstream open reading frame (uORF) in the regulation of the *Neurospora crassa arg-2* gene. The *arg-2* gene, which encodes the small subunit of mitochondrial arginine (Arg)-specific carbamoyl phosphate synthetase, is negatively regulated by arginine through mechanisms affecting both the level of mRNA and mRNA translation. Previously, the uORF in the 5' region of the *arg-2* transcript had been implicated in Arg-specific regulation by analogy to the homologous *CPA1* gene of *Saccharomyces cerevisiae*.

The problem was addressed by combining methods of reverse and classical genetics with biochemistry using two major approaches:

(i) the isolation and characterization of UV-induced mutants that change *N. crassa* responses to arginine,

(ii) the biochemical analyses of wild type and mutant strains that addressed the loading of *arg-2* and control mRNAs on polysomes to assess translational efficiency under noninhibitory and inhibitory conditions.

The results indicate that the 24-codon uORF in the 5' leader of the *arg-2* gene is involved in the translational regulation of *arg-2*. Moreover, uORF function is sequence-dependent: a mutation in the uORF changed the predicted peptide sequence at codon 12 from Asp to Asn and abolished translational regulation. Comparison of the *arg-2* uORF

with six additional sequence-dependent uORFs from eukaryotes and prokaryotes supports the idea of a conserved function for the Asp residue. Ribosome stalling models can explain the action of the *arg-2* uORF peptide.

Several significant new techniques have been developed that will prove useful in the further dissection of the translational control mechanism(s) of the *arg-2* gene and other phenomena in *N. crassa*: (1) Polysome analyses demonstrated differential translational efficiency of *N. crassa* mRNAs; (2) a combination of reverse and classical genetic methods yielded regulatory *arg-2* mutants; (3) a dot blot assay was used to measure the activity of hygromycin B phosphotransferase activity in whole cell extracts of *N. crassa*.

Chapter 1

Introduction

1.1 Overview

The expression of eukaryotic genes may be regulated by multiple controls from the initiation of transcription to the degradation of the protein product. Compared to the numerous well-studied examples of transcriptional regulation of eukaryotic genes, few gene-specific translational control mechanisms are understood at a similar level (Geballe and Morris 1994). In many regulatory systems which exhibit translational control elements, the elicitor for the specific regulatory response remains unknown. However, the translation of some transcripts are regulated by simple elicitors, most notably the *Saccharomyces cerevisiae GCN4* and *Neurospora crassa cpc-1* genes, which are translationally controlled through a pathway that senses amino acid imbalance or starvation conditions (Hinnebusch 1993; Sachs in press; Luo, Freitag and Sachs 1995), and the *S. cerevisiae CPA1* and *N. crassa arg-2* genes, which are negatively translationally controlled by the presence of arginine (Arg) in the growth medium (Werner *et al.* 1987; Luo, Freitag and Sachs 1995; Luo and Sachs submitted; Freitag, Dighde and Sachs in press). The work described here focuses on the Arg-specific negative translational control of the *N. crassa arg-2* gene.

Over the past ten years, knowledge of the mechanisms of eukaryotic protein synthesis and their control has been increasing rapidly. Current models for the initiation of eukaryotic protein synthesis are summarized below. Effects of transcript structure on translation are discussed, with special emphasis on systems in which the presence of upstream initiator codons (uAUGs) or upstream open reading frames (uORFs) results in translational regulation. As discussed in section 1.5, preemptive initiation by ribosomes at uAUGs or uORFs, control of reinitiation at downstream AUGs and ribosome stalling on

uORFs are the three major models which can account for the effects of specific uORFs on translation.

The utility of the *N. crassa arg-2* system for the elucidation of translational control mechanisms exerted by uORFs is discussed. The *N. crassa* Arg pathway represents one of the best examples of compartmentalization of substrates, enzymes and products in eukaryotic intermediary metabolism (Davis and Weiss 1988). A short review of the Arg pathway of *N. crassa* is included, as it pertains to the scheme used to isolate regulatory mutants of *N. crassa* that are altered in their response to Arg.

One of the major goals of this study was the isolation and characterization of mutations that affect *N. crassa*'s responses to Arg. The hygromycin B phosphotransferase gene from *Escherichia coli*, *hph*, can confer resistance to the antibiotic hygromycin B (Hyg). Single copies of an *arg-2–hph* reporter gene, which contained a translational fusion of *hph* to codon 10 of *arg-2*, were introduced into two *N. crassa* strains by transformation. Hygromycin B phosphotransferase (Hph) expression of the reporter conferred resistance to Hyg (Hyg^r) to transformed *N. crassa* strains. Transformants in which Hph activity was negatively regulated by Arg did not grow on media containing Hyg and Arg. One such transformant was used to select for UV-induced mutants that grew on medium containing Hyg and Arg; 46 mutant strains were isolated.

One mutant, which was primarily affected in the expression of Hph, was further characterized. In genetic crosses, the Hyg^r phenotype cosegregated with the *arg-2–hph* fusion gene. Whereas Hph activity in the original transformant was negatively regulated by Arg, Hph activity in the mutant was not regulated by Arg. Recovery of the 5' region of the *arg-2–hph* gene from this mutant, followed by sequence analyses, revealed that codon 12 of the *arg-2–hph* uORF contained a missense mutation (D12N), which changes aspartic acid (Asp; D) to asparagine (Asn; N). Targeted integration of the recovered mutant *arg-2-hph (D12N)* reporter gene into the *his-3* locus of *N. crassa* yielded transformants with Hph activities that were not regulated by arginine, showing that the D12N mutation was sufficient to abolish Arg-specific regulation.

A second class of mutations resulted in altered expression of both the *arg-2–hph* gene and the *arg-2* gene. Genetic analyses indicated that mutations were unlinked to the *arg-2–hph* gene.

The regulation of *N. crassa arg-2–hph*, *arg-2* and *cpc-1* by availability of Arg and histidine (His) was examined and expression was compared to that of the gene for cytochrome oxidase subunit V, *cox-5*. Analyses of the distribution of mRNA in polysome gradients indicated that Arg-specific negative regulation of *arg-2* had a translational component. Negative translational effects on *arg-2* and positive translational effects on

cpc-1 were apparent within minutes after providing cells with exogenous Arg. These data indicated that *arg-2* and *cpc-1* represent new examples of translational regulation in eukaryotes. Importantly, translational regulation in both cases was observed under physiological conditions in response to specific elicitors. The availability of Arg regulates *arg-2* and His starvation and/or amino acid imbalance regulates *cpc-1*. The *arg-2* and *cpc-1* transcripts contain uORFs, as do their *S. cerevisiae* homologs *CPA1* and *GCN4*. Common elements in the structure and expression of these genes suggest a conserved mechanism that controls uORF action.

Examination of the distribution of *arg-2–hph (D12N)* mRNA in polysome gradients revealed that the D12N mutation in the predicted uORF peptide abrogated Arg-specific control of *arg-2-hph* translation. These results showed that the uORF peptide sequence is important for Arg-specific translational control of *arg-2–hph* and, by analogy, of *arg-2*. To date, only a few eukaryotic systems have been described in which the uORF peptide sequences are known to be important (see section 1.4). Comparison of the *arg-2* uORF peptide with prokaryotic and eukaryotic sequence-dependent uORF peptides suggests a role for conserved Asp residues: when the Asp residues in five of the Asp-containing uORF peptides (*arg-2*, *CPA1*, *hmAdoMetDC*, *cmlA* and *cat-86*) are mutated, uORF peptide function is abolished or diminished (see section 4.7).

The mutational analyses described here reveal the importance of the uORF peptide sequence for the regulation of *arg-2* in response to Arg. Similarities of the *arg-2* uORF peptide sequence to the sequences of other uORF peptides suggest a mechanism of translational attenuation in eukaryotes. The isolation of regulatory mutants by means of a reporter gene followed by genetic and biochemical analyses, represents a novel combination of techniques in the study of *N. crassa*.

1.2 Initiation of eukaryotic protein synthesis: the scanning hypothesis

The mechanisms and regulation of eukaryotic protein synthesis have been investigated in great detail (see Hinnebusch and Liebman 1991; Hershey 1991; Merrick 1992 and references therein). Translation of most cellular mRNAs is thought to be initiated by a "scanning mechanism" (Fig. 1.1.). The 43S pre-initiation complex, consisting of (i) the small 40S ribosomal subunit, (ii) eukaryotic initiation factors (eIF) eIF-3 and eIF-1A and (iii) a tertiary complex of eIF-2–GTP–Met-tRNA$_i$, enters at the 5'-cap structure; this interaction is possibly guided by eIF-3 and eIF-4. The pre-initiation complex migrates, or "scans", along the mRNA, which has already been partially unwound by eIF-4, eIF4A and eIF-4B, in the 3' direction to the first initiation codon that is present in a sequence context

favorable for translation initiation (Kozak 1989b; Hershey 1991). At present, it is unclear what the biochemical mechanism of scanning along the mRNA molecule might be (Merrick 1992). Once an initiation codon in favorable context has been recognized, eIF2-GDP is released from the pre-initiation complex. Subsequently, the large 60S ribosomal subunit binds to the pre-initiation complex to form the translation-competent 80S initiation complex.

The initiation codon is apparently recognized by the anticodon of the initiator tRNA, as more than 90% of all eukaryotic mRNAs use the first AUG 3' from the 5'-cap structure to initiate translation (Kozak 1989b). A preferred sequence context around the AUG may ensure that the initiator tRNA finds the correct initiator codon. The deduced consensus initiator start sequence has been determined as GCC (A/G)CC AUG G in vertebrates (Kozak 1989b; Kozak 1991c), although this consensus sequence is not always present in vertebrate genes or eukaryotic genes in general (Merrick 1992). An alternative hypothesis for initiation codon selection suggests involvement of a kinetic component. For example, there could be fast scanning of the initiation complex up to an initiator codon in close context with secondary structure, which would in turn slow down scanning enough to allow for translation initiation, even at an initiator codon in poor context (Kozak 1989a).

The idea that all eukaryotic translation initiation occurs by scanning from the 5' cap in 3' direction has been challenged by the observation of internal entry of ribosomes on mRNAs specifying some viral, prokaryotic and cellular genes (Chen and Sarnow 1995; Kozak 1977; Macejak and Sarnow 1991; Pelletier and Sonenberg 1989; Vagner *et al.* 1995). Internal initiation may also occur in *N. crassa* (Vittorioso *et al.* 1994). Moreover, in a subclass of transcripts containing uAUGs or uORFs, reinitiation of translation without re-scanning from the 5' cap appears to be a mechanism for translational control (Kozak 1987b; Abastado *et al.* 1991; Grant and Hinnebusch 1994); these mechanisms are discussed in detail in sections 1.4 and 1.5.

1.3 Effects of eukaryotic transcript structure on translation initiation

The present study focussed on aspects of Arg-specific regulation of *arg-2* that are mediated by the presence of an uORF. As discussed in this section, a variety of structural features of mRNAs have regulatory effects on mRNA translation. The modulation of overall translation rates by phosphorylation and dephosphorylation of components of the translational machinery has been since long understood as a mechanism to alter global protein synthesis in cells (Hershey 1991). This regulation can in principle occur during all steps of translation, initiation, elongation or termination (Hershey 1991). In contrast,

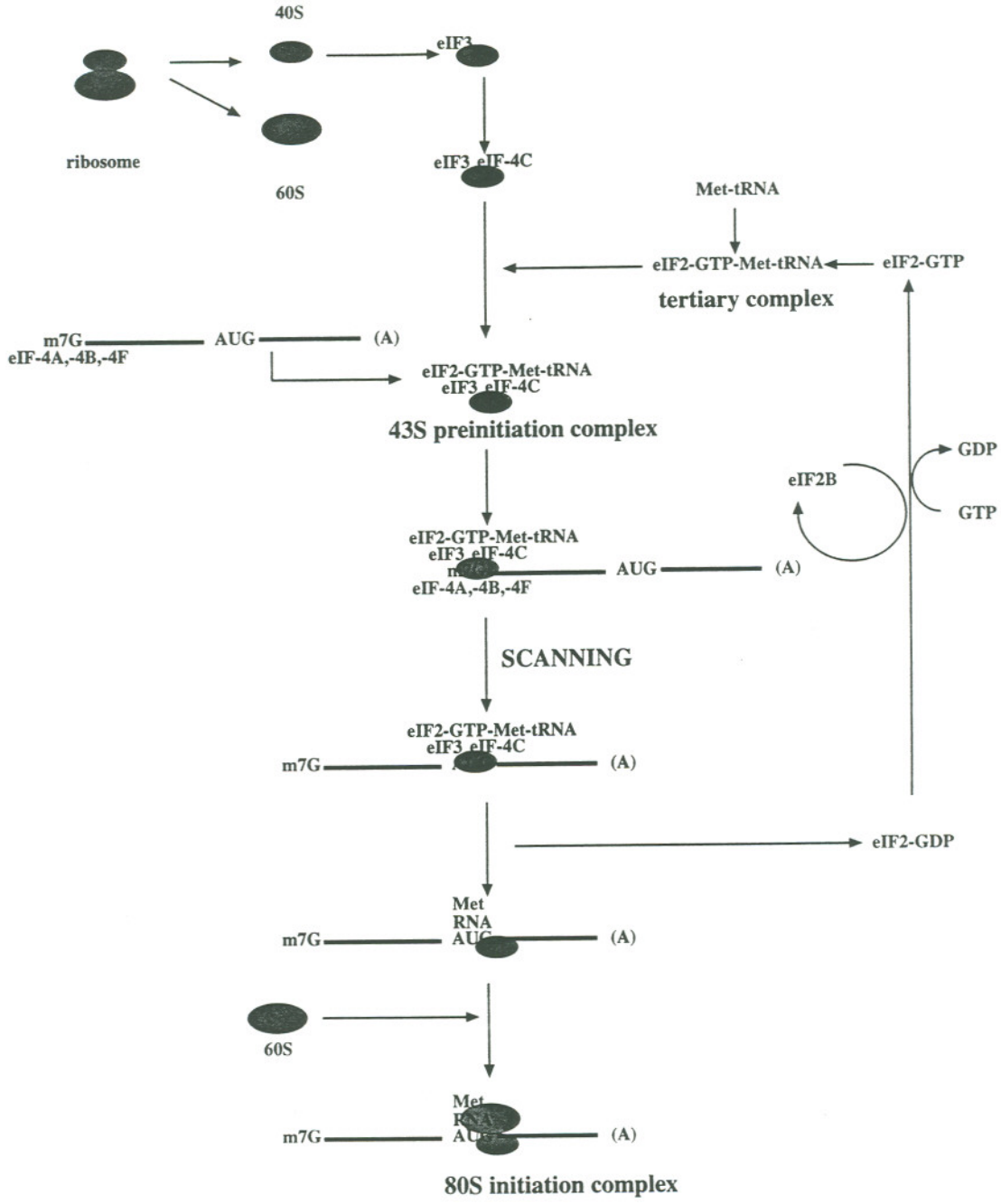

Figure 1.1: Scheme of events in the initiation of eukaryotic proteins synthesis that result in the formation of the 80S initiation complex (redrawn, from Merrick, 1992). The 60S and 40S ribosomal subunits dissociate and are stabilized by eukaryotic initiation factors (eIFs; over 20 polypeptide eIFs have so far been identified, most of them are omitted here for clarity and simplicity). A tertiary complex of eIF2-GTP-Met-tRNA binds to the 40S subunit, forming the 43S preinitiation complex, after which capped mRNA, associated with eIF-4s, binds. At this point, scanning along the mRNA to the first AUG in a favorable context occurs, eIFs dissociate and the 80S initiation complex is completely assembled by binding of the 60S subunit.

mRNA-specific regulation appears to be exerted most often at the rate-limiting step of translation, initiation, in several fundamentally different ways, determined by specific structural features of mRNAs (Kozak 1991c; Fig. 1.2.).

1.3.1 Leader length and leader secondary structures

In the presence of secondary structure within the 5' leader sequence, an N-7-methylated 5'-guanine cap (5'-cap) stimulates translation of eukaryotic mRNAs (Gerstel, Tuite and McCarthy 1992; Shatkin 1976; Sonenberg 1988). Long, unstructured leaders do not seem to require the 5'-cap or cap-binding protein (eIF-4E) for basal translation (Gallie, Lucas and Walbot 1989). The length of the untranslated 5' leader sequences by itself can influence translation initiation rates, particularly if the first AUG is close to the 5'-cap (Kozak 1991c). Thus, in some systems, shortening the leader decreased translatability (Kozak 1991a; Kozak 1991b; van den Heuvel *et al.* 1989; van den Heuvel, Planta and Raue 1990), whereas non-specific, GC-poor sequences increased translation when integrated into short 5' leaders (Kozak 1991c). The integration of multiple repeats of trinucleotides ("trinucleotide repeat expansion") in the 5' untranslated leader of the human *FMR1* gene resulted in ribosome stalling and therefore decreased translatability (Feng *et al.* 1995). This effect is most likely due to the formation of secondary structure, rather then simple "expansion" of the leader. In some studies, increasing or decreasing the 5' leader length had only modest effects *in vivo* (Baim and Sherman 1988), suggesting that scanning itself is not a rate-limiting step in translation initiation (Hinnebusch and Liebman 1991); however, expansion of the 5' leader has been suggested as a valuable method for increasing the *in vitro* expression in other cases (Kozak 1991c). Although similar studies have not been conducted with the *arg-2* gene, shortening the length of the *S. cerevisiae* *CPA1* leader or shortening the distance between the uORF termination codon and the *CPA1* start codon did not significantly alter the expression of *CPA1* (as measured by enzyme activity) or the expression of fusions of *CPA1* regulatory sequences to the *lacZ* reporter gene (Werner *et al.* 1987; Delbecq *et al.* 1994).

Inhibitory or stimulatory effects of transcript secondary structure that have been observed in numerous studies lend additional support to the scanning model of translation initiation. 5' leaders may contain significant secondary structure and thus form stable barriers for translating ribosomes. Computer modeling indicates the possibility for the presence of stable secondary structures in the 5' leader of *arg-2*. Short regions within the untranslated region theoretically would have the capacity to form short stems; however, such structures are not expected to be thermodynamically stable. Moreover, in light of biochemical and genetic evidence obtained in studies of the homologous *S. cerevisiae*

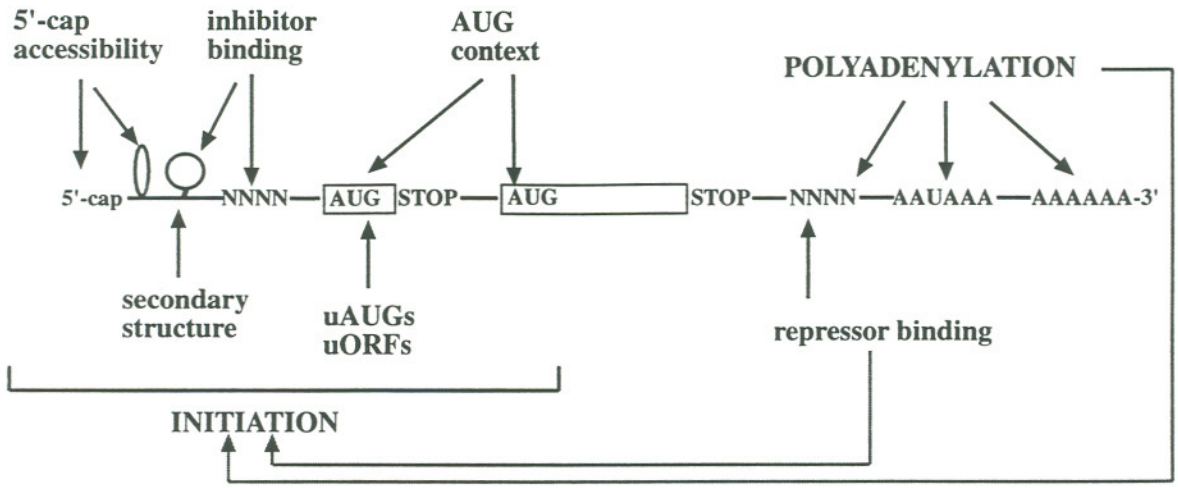

Figure 1.2: Effects of structural elements in eukaryotic mRNAs that can influence translation initiation (after Curtis *et al.*, 1995). 5'-cap accessibility, inhibitor binding, the sequence context surrounding the AUG, upstream AUGs (uAUG) or upstream reading frames (uORFs) and regulation of polyadenylation all have been shown to exert control on translation initiation.

CPA1 system (Werner *et al.* 1987; Delbecq *et al.* 1994) and evidence obtained in this study that underscored the importance of the uORF peptide sequence for Arg-specific regulation, it seems unlikely that the formation of secondary structures within the *arg-2* mRNA alone would be sufficient to explain Arg-specific translational regulation.

In other studies, introduction of stable stem-loop structure of various lengths into 5' leader of *S. cerevisiae CYC1*, *HIS4* or *PYK1* reporter genes reduced gene expression 2- to 20-fold (Baim *et al.* 1985; Baim and Sherman 1988; Bettany *et al.* 1989; Cigan, Pabich and Donahue 1988). Elegant studies in a cell-free yeast translation system with various related choramphenicol acetyltransferase (*cat*) transcripts as reporter genes showed that the degree of translation inhibition correlated well with the thermodynamic stability of the secondary structure introduced (Sagliocco *et al.* 1993; Vega-Laso *et al.* 1993). In contrast, secondary structure 3' to the initiator codon may stimulate translation initiation, by abrogating the effects of poor initiation context for certain AUGs (Kozak 1990). To obtain effects similar to those observed in *S. cerevisiae*, more stable secondary structures have to be introduced in the 5' leaders of mammalian transcripts, possibly reflecting intrinsically higher structure in mammalian transcripts (Hinnebusch and Liebman 1991).

1.3.2. Response elements for regulatory mRNA binding proteins

Proteins that bind specifically or nonspecifically to mRNA can affect translatability of particular mRNAs in response to changing environmental conditions or during differentiation and development. Protein binding sites, or response elements, are commonly found in the 5' or 3' untranslated regions of mRNAs. At present, there is no clear evidence for the involvement of specific mRNA binding proteins in the translational control of *arg-2* or *CPA1*. However, in various other systems such mRNA binding proteins proved essential. Stable stem-loop structures, for example, not only inhibit ribosome scanning along the 5' untranslated leader, but can also serve as response elements for the binding of regulatory protein or RNA factors (Kozak 1989b; McCarthy and Kollmus 1995; Standart and Jackson 1994b). Ribosome binding events are more sensitive to disruption close to the 5' cap, and such elicitor-specific binding can affect mRNA translatability or stability (Danon and Mayfield 1994; Kaspar *et al.* 1992; Kempe, Muhs and Schäfer 1993; Stripecke *et al.* 1994; Wharton and Struhl 1991). Iron-specific translation of mammalian ferritin and erythroid 5-amino-levulinate synthase, the best-studied examples of this type of translational control, is regulated by the binding of the iron regulatory protein, IRP, to an mRNA stem-loop structure, the iron response element, IRE (Hentze *et al.* 1987; Klausner, Rouault and Harford 1993; Melefors and Hentze 1993).

IRP binding inhibits the scanning of the pre-initiation complex (Gray and Hentze 1994), even in a heterologous yeast system (Oliveira *et al.* 1993).

Translational masking by sequestration of mRNA into translationally silent mRNP particles has been observed during gametogenesis (Standart and Jackson 1994a). In this fashion, mRNAs are stored for specific phases in development. Phosphoproteins bind to mRNAs with little sequence specificity (Marello, LaRovere and Sommerville 1992; Wolffe 1994) and unmasking may be achieved by changing the composition or phosphorylation of the masking proteins (Curtis, Lehmann and Zamore 1995) or by the addition of antisense RNA complementary to the 3' region of the mRNA (Standart *et al.* 1990). Binding of regulatory factors to specific sequences in the 3' untranslated regions other than polyadenylation signals has so far only been reported for the human *Lox* gene (Ostareck-Lederer *et al.* 1994), the murine protamine gene (Kwon and Hecht 1991) and the *C. elegans lin-14* gene (Ambrose 1989); in the latter case the binding factors are various complementary RNA molecules. Most likely more regulatory proteins will be identified that specifically bind transcripts, for example factors that specifically bind the direct repeats within the transcript of the *C. elegans* egg-promoting gene, *tra-2* (Goodwin *et al.* 1993) or the sperm-promoting gene, *fem-3* (Ahringer and Kimble 1991) .

1.3.3 Polyadenylation and deadenylation

Translation can be repressed or activated by selective polyadenylation that is triggered by various types of 3' untranslated regions (Richter 1995). This process is important for the programmed appearance of mRNA during development (Gebauer *et al.* 1994; Sallés *et al.* 1994; Sheets, Wu and Wickens 1995). It is likely that polyadenylation and deadenylation occur continuously. Thus, the net length of the poly(A) tail depends on both activities (Curtis, Lehmann and Zamore 1995). The polyadenylation reaction itself is required for regulation, rather than a certain length of poly(A) tail (McGrew *et al.* 1989). Conditions of efficient polyadenylation increased the rate of translational initiation as long as the mRNA is capped (Gallie 1991). Purified initiation factors complex with poly(A) (Gallie and Tanguay 1994); however, if components of the translational machinery are in excess, the length of poly(A) tails does not seem to have an effect on translation initiation (Proweller and Butler 1994). The poly(A) tail acts in *cis* to increase the local concentration of initiation factors on specific or all mRNAs (Curtis, Lehmann and Zamore 1995). This effect appears to be mediated by poly(A) binding protein (PAB). PAB is required for efficient translation in *S. cerevisiae* (Sachs and Davis 1989). Thus, it appears likely that 5' and 3' ends of mRNAs can interact.

Eukaryotic gene expression during development appears heavily controlled by selective degradation of mRNAs through deadenylation-dependent or deadenylation-independent mRNA decay pathways (Beelman and Parker 1995; Curtis, Lehmann and Zamore 1995). This type of spatial or temporal regulation can lead to deadenylation-dependent or deadenylation-independent decapping and 5' to 3' decay of transcripts, as well as interactions between 5' and 3' termini (Hagan *et al.* 1995; Hsu and Stevens 1993; Laird-Offringa *et al.* 1990; Larimer and Stevens 1990; Muhlrad, Decker and Parker 1994; Muhlrad, Decker and Parker 1995; Muhlrad and Parker 1994), which in turn can have an impact on translation initiation (Munroe and Jacobson 1990; Standart and Jackson 1994b). Transcript surveillance by deadenylation-independent decapping depends on the occurrence of early nonsense codons (Leeds *et al.* 1991; Losson and Lacroute 1979; Peltz *et al.* 1991; Pulak and Anderson 1993), unspliced introns (He *et al.* 1993), or extended 3' untranslated regions (Pulak and Anderson 1993) in transcripts that are therefore recognized as aberrant and quickly degraded. How this process is triggered or the mRNA is degraded is presently not known; the simplest model would involve a signal sent after translation termination that leads to decapping by the same nuclease that leads to normal mRNA decay (Beelman and Parker 1995).

1.3.4 Start codon sequence context

As in other eukaryotes, AUG is the primary start codon in *S. cerevisiae* (Cigan and Donahue 1987) and *N. crassa.*(Edelman and Staben 1994). The sequence context of the start codon appears to influence the efficiency with which the start codon is recognized by the scanning ribosome (Kozak 1987a). The *S. cerevisiae* (5'-(A/Y)A(A/Y) A(A/Y)A AUG UCU-3'; (Cigan and Donahue 1987; Cigan, Pabich and Donahue 1988)) consensus initiation context differs more from the vertebrate consensus (5'-GCC (A/G)CC AUG G-3'; (Kozak 1987a)) than that of *N. crassa* (5'-(A/G)(C/T)C A(C/A)(C/A) AUG GCT-3'; (Edelman and Staben 1994)). Inspection of the *arg-2* sequence (Orbach, Sachs and Yanofsky 1990) reveals that the Arg2p start codon (AAG AUG U) is in a conserved context, indicating that it represents a favored initiation site. The uORF start codon (CUU AUG A), however, is in the worst possible initiation context: a C occurs at position -3 and a T at both -2 and -1 (relative to the start codon). These are extremely rare nucleotides at the respective positions in initiator sites (Edelman and Staben 1994); in fact, they do not occur in this order in any other known *N. crassa* initiation context. Thus, based on the assumption that poor matches to the canonical consensus initiation sequence reflect inefficient initiation of translation, scanning ribosomes would be expected to frequently bypass the uORF start codon to initiate translation at the Arg2p start codon.

In contrast to *arg-2*, both the *CPA1* uORF peptide initiation context (UAU AUG U; Werner, Feller and Piérard 1985) and the Cpa1p initiation site (CAA AUG U; Werner, Feller and Piérard 1985) appear to be in a rather poor context, indicating that initiation of translation may be as efficient (or inefficient) at the uORF peptide AUG as at the Cpa1p AUG. Most importantly, both initiation contexts differ from the consensus in the -3 position, where a U or C replace the consensus A. The preference for A at position -3 is strong in all three consensus sequences; pyrimidines only occur in 3%, 7% or 6% of all initiation contexts in this position in *S. cerevisiae*, *N. crassa* or vertebrates, respectively (Hinnebusch and Liebman 1991; Edelman and Staben 1994; Kozak 1991c). That this preference is important for translation initiation has been shown by mutational analyses in *S. cerevisiae CYC1*:a 2-fold reduction of gene expression was observed when the A/G nucleotides at -3 were replaced with C/T (Baim and Sherman 1988).

In contrast to *S. cerevisiae*, where the AUG is followed most often by UCU which encodes serine (Cigan and Donahue 1987), alanine (GCN) is prevalent as the second amino acid at the 5'-terminus of *N. crassa* transcripts (Edelman and Staben 1994). While the second codon of Cpa1p encodes serine, the second codon of the uORF peptide encodes phenylalanine (Werner, Feller and Piérard 1985). Neither the uORF peptide or Arg2p second codon encode alanine, nor do they contain the consensus G at position +4 (Orbach, Sachs and Yanofsky 1990). It is unclear, whether the *N. crassa* 5' methionine is removed by an amino-terminal peptidase and therefore the coding for alanine as second residue in many *N. crassa* peptides reflects protein sequence requirements, or whether the GCT reflects nucleotide sequence requirements for translation initiation (Edelman and Staben 1994). The G residue at position +4 has been previously identified as important for efficient translation initiation in vertebrates (Kozak 1991c).

1.4 Sequence-dependent uORF peptides: the *N. crassa arg-2* and *S. cerevisiae CPA1* genes as models for uORF-mediated transcript-specific translational regulation

The presence of upstream initiation codons (uAUG; an initiator codon directly followed by a terminator codon) or upstream open reading frames (uORFs) can drastically alter translation of the downstream message (Geballe and Morris 1994; Hinnebusch and Liebman 1991; Kozak 1989a). The scanning hypothesis of translation initiation predicts that the first AUG in a favorable sequence context encountered by the scanning pre-initiation complex initiates translation (Kozak 1987b). This prediction is based on studies in which uAUGs or uORFs were introduced into the 5' leader (Kozak 1983; Kozak 1984).

A number of systems have been described in which the presence of uORFs yielded transcript-specific translational regulatory effects *in vivo*, including the best-studied example of translational control by uORFs, the *S. cerevisiae GCN4* gene. On *GCN4* transcripts, translation initiation and reinitiation are regulated by the leaky scanning of ribosomes past short uORFs (Hinnebusch 1992; Hinnebusch 1994).

The presence of even a single uORF effectively creates a eukaryotic bicistronic transcript. Such transcripts are relatively rare; they have been found in less than 10% of all vertebrate mRNAs (Kozak 1987a). The negative effect on translation initiation of a uAUG is larger when there is no in frame termination codon before the downstream AUG (Hinnebusch and Liebman 1991; Kozak 1991c). Usually, the first AUG codon encountered will be used as start codon if an A occurs at position -3 and a G occurs at position +4, even if the overall initiation context appears weak (Werner, Feller and Piérard 1985; Kozak 1991c).

Whereas uAUGs or uORFs typically inhibit translation initiation at a downstream AUG, this inhibitory effect can be regulated in some systems (Geballe and Morris 1994). Regulation by uORFs requires a way to bypass or negate the general inhibitory effect of a uORF under specific conditions. A uORF may be bypassed by "leaky scanning" due to inefficient recognition of the first AUG (Kozak 1991a; Kozak 1991b; Ruan *et al.* 1994) or by the presence of internal ribosome entry sites (IRES) on the mRNA (Jang *et al.* 1988; Macejak and Sarnow 1991; Oh and Sarnow 1992; Oh, Scott and Sarnow 1992). Alternatively, reinitiation at a downstream AUG may occur; this observation is generally explained by the continued scanning of an mRNA-associated small 40S ribosomal subunit towards the downstream AUG (Abastado *et al.* 1991; Werner *et al.* 1987). uORFs may have to be of limited length for efficient reinitiation due to the kinetics of initiation factor recycling (Hinnebusch 1994; Kozak 1987b)). This conclusion is also supported by the fact that introducing longer distances between the two ORFs leads to more efficient downstream translation initiation (Abastado *et al.* 1991; Kozak 1987b).

uORFs may be separated into three groups, depending on their arrangement with respect to the downstream AUG and the importance of the peptide coding capacity of the uORF for translational regulation. Autonomous uORFs begin and end entirely within the 5' leader; this group encompasses two subgroups, sequence-dependent (Fig. 1.3.a and Fig. 1.4) and sequence-independent uORFs (Fig. 1.3.b). A third group contains uORFs that overlap and are not in frame with the downstream ORF (Fig. 1.3.c). Since *arg-2* and *CPA1* contain sequence-dependent uORFs, the focus of this discussion will be on that particular group of uORFs. Sequence-independent and overlapping uORFs will be treated at the end of this section.

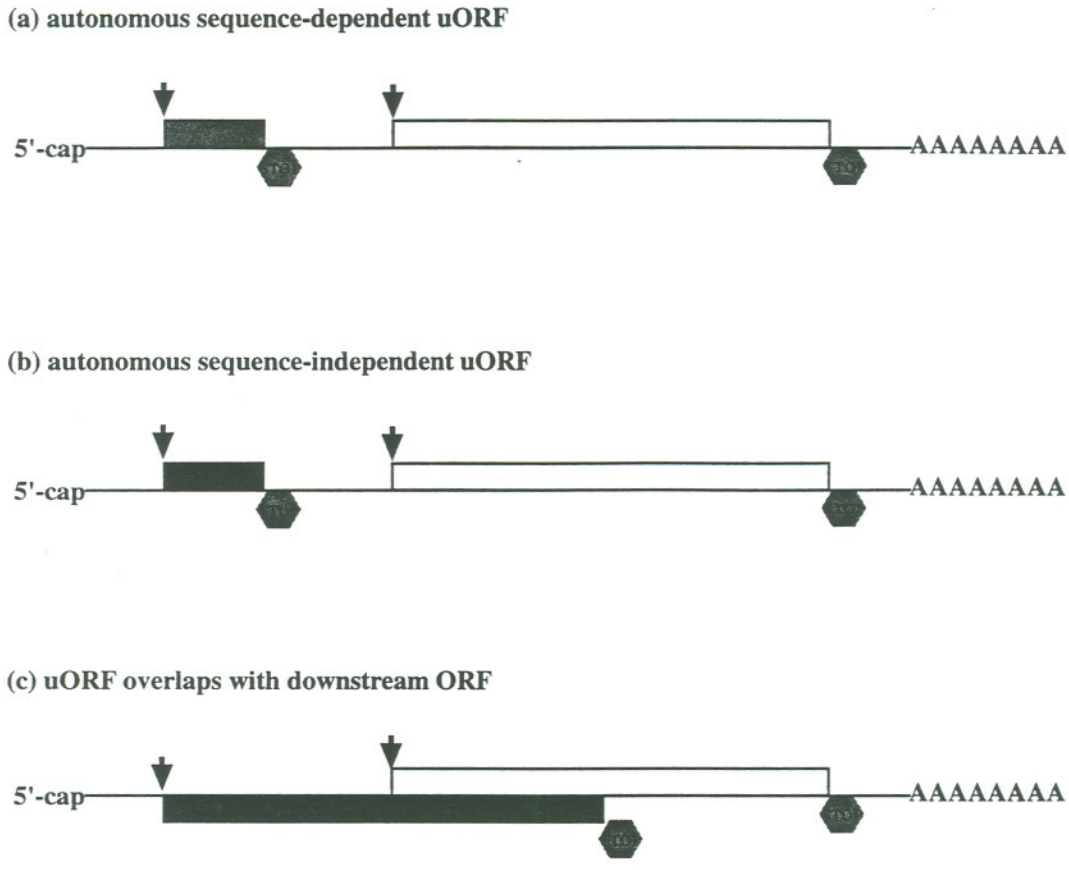

Figure 1.3: Possible arrangements of uORFs in eukaryotic mRNAs (altered; after Geballe and Morris, 1994) (a) autonomous sequence-dependent uORF (the peptide sequence is essential for regulation); (b) autonomous sequence-independent uORF (the peptide sequence is not essential for regulation); (c) the uORF overlaps with the downstream AUG. Arrows denote initiator codons, stop signs indicate terminator codons and -AAAAAA represents the poly(A) tail; open boxes represent downstream messages, filled boxes upstream messages.

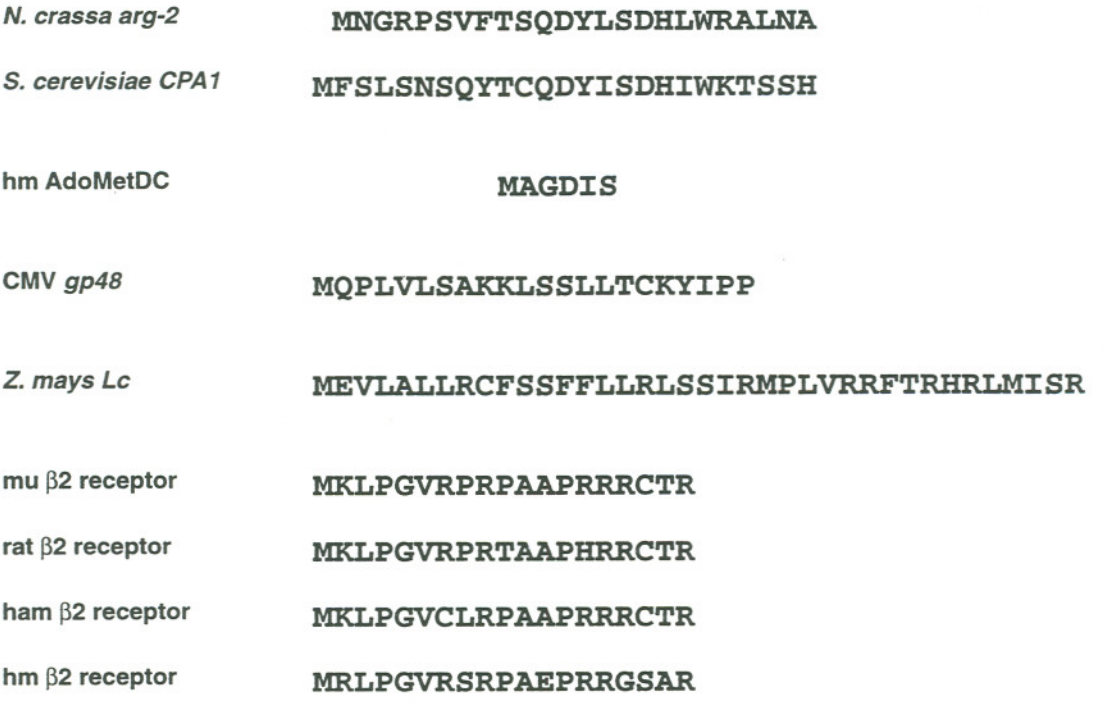

Figure 1.4: Sequence-dependent uORF peptides in eukaryotic mRNAs. The transcripts of the homologous Arg-specific carbamoyl phosphate synthetase genes of *N. crassa*, *arg-2*, and *S. cerevisiae*, *CPA1*, contain 24- and 25-codon uORFs, respectively (Orbach, Sachs and Yanofsky 1990; Werner *et al.* 1987). The human *S*-adenosylmethionine decarboxylase (hm AdoMetDC) transcript contains a 6-codon uORF, whose peptide sequence is essential for inhibition of translation (Hill and Morris 1992). Inhibition of translation by the 22-codon uORF2 of the human cyromegalovirus *gp48* (CMV *gp48*) transcript is uORF2 sequence-dependent (Degnin *et al.* 1993). The 38-codon uORF in the transcript of the maize transcriptional activator *Lc* (*Z. mays Lc*) inhibits its expression in a sequence-dependent manner (Damiani and Wessler 1993). The 5' leaders of β_2 adrenergic receptors of mouse (mu), rat, hamster (ham) and humans (hm) contain 19-codon uORFs that act in a peptide sequence-dependent manner (Parola and Kobilka 1994)

1.4.1 The uORF peptides encoded by *N. crassa arg-2* and *S. cerevisiae CPA1* genes mediate transcript-specific translational regulation

Sequence-dependent autonomous uORFs (Fig. 1.4.) have so far only been described from the carbamoyl phosphate synthetase small subunit genes *CPA1* from *S. cerevisiae* (Werner *et al.* 1987) and *arg-2* from *N. crassa* (Luo, Freitag and Sachs 1995; Freitag, Dighde and Sachs in press; Luo and Sachs submitted; Orbach, Sachs and Yanofsky 1990), both of which are negatively controlled by Arg, from the transcriptional activator *Lc* in maize (Damiani and Wessler 1993), from the mammalian *S*-adenosylmethionine decarboxylase (Hill and Morris 1992; Hill and Morris 1993), from the mammalian β_2 adrenergic receptor (Parola and Kobilka 1994) and from the human cytomegalovirus *gpUL4* (*gp48*) gene (Degnin *et al.* 1993; Schleiss, Degnin and Geballe 1991). The hallmark characteristic of this class of uORFs is that the amino acid coding capacity of the uORFs is essential for specific regulation; initiation at the uORF AUGs by itself is not sufficient to yield regulatory responses other than decreasing relative expression in the *arg-2* and *CPA1* system (this study; Delbecq *et al.* 1994; Luo and Sachs submitted; Degnin *et al.* 1993). Mutational analyses of the *S*-adenosylmethionine decarboxylase (AdoMetDC) gene (Hill and Morris 1993) and the human cytomegalovirus *gp48* gene (Cao and Geballe 1995) showed that the magnitude of the specific regulatory effect of these uORFs is dependent on the context of their initiation codons.

Among the five eukaryotic systems with sequence-dependent uORFs (Fig. 1.4), the *N. crassa arg-2* and *S. cerevisiae CPA1* genes are unique in that the specific elicitor for the translational regulatory response, Arg, is known. This fact will allow a complete dissection of the pathway that leads from a change in cellular nutritional status to the adaptation of the cellular machinery by transcriptional and translational control mechanisms.

The *N. crassa arg-2* gene specifies the small subunit of Arg-specific carbamoyl phosphate synthetase (CPS-A; Orbach, Sachs and Yanofsky 1990) and was among the first metabolic genes identified (Srb and Horowitz 1944). Control of *arg-2* expression regulates the flux of metabolites through the Arg biosynthetic pathway under most growth conditions (Davis 1986; Davis, Ristow and Ginsburgh 1981). At least three separate control mechanisms regulate *arg-2* expression. *arg-2* is positively regulated in response to amino acid starvation, as are many genes involved in *N. crassa* amino acid metabolism (Luo, Freitag and Sachs 1995; Sachs and Yanofsky 1991). The level of *arg-2* transcript increases in response to amino acid starvation; Cpc1p, the product of the cross-pathway control gene *cpc-1*, is important for this response (Ebbole *et al.* 1991; Luo, Freitag and Sachs 1995; Paluh *et al.* 1988; Sachs and Yanofsky 1991). *arg-2* is also developmentally regulated; the

highest level of *arg-2* mRNA is found during spore germination and early exponential growth (Sachs and Yanofsky 1991). Most importantly for this study, the *arg-2* gene is the only gene of in the Arg biosynthetic pathway known to be negatively regulated by Arg.

Decreased CPS-A activity in Arg-containing medium is associated with a reduced level of Arg2p polypeptide in mitochondria (Davis, Ristow and Ginsburgh 1981). In Arg-containing medium the level of *arg-2* mRNA is reduced approximately 3-fold (Luo, Freitag and Sachs 1995; Orbach, Sachs and Yanofsky 1990; Sachs and Yanofsky 1991). The *arg-2* transcript contains a 24-codon uORF (Orbach, Sachs and Yanofsky 1990), which has been implicated in Arg-specific negative regulation of *arg-2* mRNA translation in studies in which reporter genes with site-directed mutations in the 5' leader eliminated regulation (Luo, Freitag and Sachs 1995).

The *N. crassa arg-2* homolog of *S. cerevisiae*, *CPA1*, is similarly subject to positive and Arg-specific negative regulation (Davis 1986). As for *arg-2*, *CPA1* is positively regulated in response to amino acid starvation (Davis 1986). Positive regulation of *CPA1* at the transcript level in response to amino acid starvation requires the general control transcriptional activator *GCN4* (Kinney and Lusty 1989).

Sites of *cis*-acting mutations that resulted in a loss of Arg-specific negative regulation of *CPA1* (Thuriaux *et al.* 1972) were identified (Werner *et al.* 1987). These mutations were in a region of DNA specifying a 25-codon uORF and affected the formation of the predicted *CPA1* uORF peptide; this uORF peptide strongly resembles the predicted *arg-2* uORF peptide (Werner *et al.* 1987). The authors reported that growth of wild type strains in Arg-containing medium reduced enzyme activity approximately 5-fold without a concomitant reduction of the *CPA1* transcript level, indicating the possibility of translational control. In contrast, a later study found that Arg reduced *CPA1* transcription and *CPA1* transcript stability, as well as having an additional post-transcriptional effect (Crabeel, Lavalle and Glansdorff 1990).

1.4.2 Other examples of sequence-dependent uORF peptide mediated transcript-specific translational regulation

The AdoMetDC 5' leader contains a 6-codon uORF (MAGDIS) that inhibits translation of the downstream message in T cells approximately 5-fold, but fails to do so in non-lymphoid cells (Hill and Morris 1992). Missense mutations that were introduced by site-directed mutagenesis, relieved this inhibitory effect, as long as the mutations were in codons 4 to 6 (Hill and Morris 1993). The position of the termination codon was similarly of importance; carboxy-terminal extensions of the uORF peptide abolished the inhibitory uORF effect (Hill and Morris 1993). Sequences distal to the uORF coding region were not

important for regulation (Hill and Morris 1993), in contrast to findings with *S. cerevisiae GCN4*, where nucleotides downstream of uORF4 have an influence on reinitiation (Grant and Hinnebusch 1994; Miller and Hinnebusch 1989). The uORF peptide appeared to exert its regulatory influence only in *cis*, as assayed by cotransfection experiments (Hill and Morris 1993). The cell-type-specific translational regulation of AdoMetDC appears to be based on the inefficient recognition of the uORF AUG in non-lymphoid cells due to its close proximity to the 5'-cap. Because the uORF is not recognized or translated in non-lymphoid cells, there is efficient translation initiation at the downstream AUG (Ruan *et al.* 1994).

Similar results had been obtained in studies of the 22-codon uORF2 of the human cytomegalovirus *gp48* gene (Degnin *et al.* 1993; Schleiss, Degnin and Geballe 1991). Carboxy-terminal missense mutations within the uORF abolished the inhibitory effect of the uORF, as did carboxy-terminal extensions, whereas sequences between uORF2 and the downstream AUG were of no importance for regulation; the uORF2 also acted only in *cis*, as assayed by cotransfection (Degnin *et al.* 1993). In experiments with retroviral vectors to yield stably integrated reporter genes in various cell types, the same regulatory responses were observed; inhibition was enhanced 3-10-fold by placing the uORF2 AUG in an optimal initiation context (Cao and Geballe 1994; Cao and Geballe 1995). Geballe and co-workers were able to carry their mechanistic studies a significant step farther: recent studies strongly suggest that the nascent uORF2 peptide arrests ribosomes at the termination step of translation (Cao and Geballe submitted). Their report demonstrates translational termination as a regulatory control step in eukaryotic gene expression and supports a ribosome stalling mechanism as the basis for the action of the sequence-dependent uORF2 peptide.

A 38-codon uORF in the 5' leader inhibited the expression of the transcriptional activator *Lc* in maize aleurone tissues when *Lc* constructs under constitutive transcriptional regulation were delivered simultaneously with two control plasmids, encoding firefly luciferase or chloramphenicol acetyltransferase (Damiani and Wessler 1993). Removal of the entire 5' leader resulted in a 22-fold increase of relative *Lc* expression, whereas the elimination of all three AUG codons present in the uORF led to a 30-fold increase in relative expression. When the peptide sequence of the uORF was altered by substituting the uORF peptides' internal methionines with arginines, a leucine with arginine or isoleucine and a threonine with serine, or when a pre-mature termination codon was introduced, regulation of *Lc* expression was similar to that of the construct lacking the 5' leader. Interestingly, conservative substitutions of the first leucine with isoleucine or the sole threonine with serine resulted in intermediate regulation, in contrast to complete loss of

relative regulation when leucine was altered to arginine. Additionally, no significant secondary structure was detected in the 5' leader; the most stable structure was predicted to have a ΔG^0 of -78 kJ/mol (Damiani and Wessler 1993). The uORF internal AUG codons are in unfavorable initiation contexts; the first AUG is in a slightly better context and the downstream *Lc* AUG is in the best context for plant initiator codons (Damiani and Wessler 1993). A mutation altering the first AUG to CUG resulted in a 32-fold increase in relative expression, indicating that a CUG codon present in the leader did not function as a competent initiator codon. A CAU to CAC silent mutation of an histidine codon resulted in a 13-fold increase of relative *Lc* expression; the remaining regulation, compared to constructs without the leader, may be explainable by invoking codon preferences. A construct encoding the uORF peptide was also introduced by transformation to assay its effects in *trans* (Damiani and Wessler 1993). No clear effect on relative expression of *Lc* was detected when uORF-less or uORF-containing constructs were compared.

The 5' leader of the β_2 adrenergic receptor contains a 19-codon uORF which is well-conserved in the homologous mouse, rat, hamster and human genes in terms of its length, predicted alkalinity and peptide sequence, but is not homologous to any other known peptides (Parola and Kobilka 1994). The uORF AUG is in a nonfavorable initiation context; mutation to CUU prevented translation initiation (without altering the GC content of the 5' leader). Analyses of conformational polymorphisms of RNA revealed no differences in migration between these two constructs. In cotransfection experiments of mutated or wild type β_2 adrenergic receptor with α_2-10H adrenergic receptor in COS7 cells, no *trans* activity of the uORF was found (Parola and Kobilka 1994). In experiments with chimaeric β_2/α_2 receptors, sequences downstream of the uORF did not appear to be involved in modulation of expression. The uORF AUG was used as an initiator codon, as assayed by translational fusions of the uORF to the 12CA5 epitope tag. The AUG-containing fusion protein was detected by immunofluorescence microscopy *in vivo*, while a construct carrying the CUU-containing fusion did not stain with the α-12CA5 monoclonal antibody. Moreover, alteration of the uORF coding capacity, for example by substitution of the conserved arginine residues with alanine residues at codons 14 to 16, resulted in a 1.5-fold increase of expression; truncating the uORF peptide after the third residue resulted in a similar increase in expression (Parola and Kobilka 1994). The uORF peptide was synthesized and shown to inhibit the translation of the β_2 adrenergic receptor; however, the synthetic peptide was also able to non-specifically inhibit translation of mouse α_2-10H adrenergic receptor, *E. coli* β-lactamase and *S. cerevisiae* α-factor (Parola and Kobilka 1994). Several other peptides were tested for their inhibitory effects; as had been reported

before (Weeks *et al.* 1990), only the peptide rich in arginine had an inhibitory effect similar to the native uORF (Parola and Kobilka 1994).

1.4.3 Transcript-specific translational regulation mediated by sequence-independent or overlapping uORF peptides

Sequence-independent autonomous uORFs (Fig. 1.3.b) have now been reported to occur in transcripts from viruses (Donzé and Spahr 1992; Dorsky and Crumpacker 1988; Geballe and Mocarski 1988; Imataka *et al.* 1994; Yager, Marcy and Coen 1990), plants (Fütterer *et al.* 1990; Fütterer and Hohn 1992; Lohmer *et al.* 1993; Michelet *et al.* 1994), fungi (Cigan, Pabich and Donahue 1988; di Blasi *et al.* 1993; Hinnebusch 1993; Hinnebusch 1994; Hinnebusch and Liebman 1991; Paluh *et al.* 1988; Pinto *et al.* 1992; Prade and Timberlake 1993; Sherman and Stewart 1982) and animals (Aceto *et al.* 1992; Bergenhem *et al.* 1992; Dixon *et al.* 1989; Kajimoto and Rotwein 1990; Manzella and Blackshear 1990; Marth *et al.* 1988; Navone *et al.* 1992; Virbasius and Scarpulla 1988; Yiu, Gu and Hecht 1994). These citations refer to the systems that are relatively well-studied. In some of the less studied systems it remains unclear whether the uORF peptide sequence may be important for translational regulation.

Four short, sequence-independent uORFs (numbered starting from the 5' terminus of the transcript) occur naturally in the transcript of the *S. cerevisiae GCN4* gene (Hinnebusch 1984; Thireos, Penn and Greer 1984). The strong negatively regulatory effect on translation can be reconstituted with uORF1 and uORF4 alone (Abastado *et al.* 1991). The detailed mechanism of regulation by the availability of dephosphorylated or phosphorylated eIF-2α to allow reinitiation at the downstream AUG is discussed in section 1.5. Removal of all four uORFs by mutation of their ATG codons led to a 100-fold increase of translation of *GCN4-lacZ* fusion transcripts; differential effects were observed for the independent removal of one or more uORFs, with uORF4 exerting the largest effect, a 99% reduction of expression (Mueller and Hinnebusch 1986). As in the case of *S. cerevisiae CPA1*, the analyses of *GCN4* uORFs showed that sufficient distance between the upstream and downstream initiator codons allows efficient reinitiation (Mueller *et al.* 1988; Werner *et al.* 1987).

The translation of the downstream ORF within the transcript of the 35S RNA of cauliflower mosaic virus (CaMV) is enhanced by the action of uORFs and is dependent on a transctivator protein, TAV, encoded by the virus (Fütterer and Hohn 1991). The 600 nt long 5' leader is quite complex. It contains seven uORFs, several positively acting response elements and considerable possibilities to form stable secondary structures. Transactivation was weaker when translation inititated at the downstream ORF directly; it

was stronger when the downstream ORF was preceded by a short uORF (Fütterer and Hohn 1992). A mechanism for shunting scanning ribosomes has been proposed to explain the regulatory effects between far upstream sequences and the downstream ORF (Fütterer, Kiss-Laszlo and Hohn 1993).

Three uORFs have been found in the 5' leader of the maize transcriptional activator gene *opaque-2* (Lohmer *et al.* 1993). Elimination of one or combinations of the AUG codons increased relative expression of *O2*; elimination of the whole 5' leader had the same quantitative effect as the elimination of all three AUGs. The first and second uORF are primarily important for the regulatory effects. Elimination of just the first or second uORF AUG alone had an intermediate effect, whereas the elimination of the last uORF AUG or the first two uORF AUGs had negligible effects, compared to the wild type construct (Lohmer *et al.* 1993). The effects of the uORFs were determined to be *cis*-dominant by complementation analyses.

Sequence-independent uORFs, introduced by point mutations in 5' leaders of yeast or macaque erythrocyte genes, led to reduced frequency of reinitiation from the downstream AUG, as assayed by *in vitro* translation experiments (Pinto *et al.* 1992; Sherman and Stewart 1982; Bergenhem *et al.* 1992). Functional revertants of such yeast mutations fell into three classes: those that restored activity by point mutations in the upstream AUG, those that altered the intiation context around the upstream AUG, enhancing expression from 2% to 10% of normal, and those in extragenic suppressors that enhanced expression to 40% of normal (Pinto *et al.* 1992).

The second class of mutations resemble those introduced *in vitro* into the *S. cerevisiae CPA1* 5' leader (Werner *et al.* 1987). A heterologous uORF, resulting in a missense uORF peptide by a -2 frameshift, yielded a 5-fold reduction in Cpa1p activity, presumably due to the more favorable initiation context of the mutant uORF. When the mutant initiation context was altered to the corresponding wild type initiation context, expression was changed to wild type levels (Werner *et al.* 1987). Initiation context of uORF AUGs and the ability of the ribosome to reinitiate may contribute to the regulation of insulin-like growth factor (IGF-I) from *Xenopus laevis*, rat, chicken and humans (Lajara *et al.* 1989; Kajimoto and Rotwein 1990) and testis-specific cytochrome *c* of rodents (Virbasius and Scarpulla 1988). In the latter case, the uORF may lead to decreased reinitiation in two of the six potential transcripts that have been found (Yiu, Gu and Hecht 1994); the other four transcripts lack the uORF, since their 5' termini are located within its coding sequence. uORFs may have only limited importance in this system, since stable stem-loop and hairpin structures of at least -210 kJ/mol can be predicted for all six transcripts (Yiu, Gu and Hecht 1994). The same type of dual control, exerted by stable

secondary structure possibly in concert with the presence of a uORF, has been described from rat ornithine decarboxylase (Manzella and Blackshear 1990) and the human kinesin heavy chain gene (Navone *et al.* 1992).

The Rous sarcoma virus RNA leader sequence upstream of the *gag* gene carries three uORFs (Donzé and Spahr 1992). *In vitro* studies showed that ribosomes bind upstream of the first uORF and translate the 7-codon peptide (Hackett *et al.* 1986); deletion of the second or third uORF did not significantly alter the translation of the downstream message (Katz, Terry and Skalka 1986). Deletion of all three uORFs slightly increased translation of the *gag* precursor (Hensel, Petersen and Hackett 1989). Mutations in the 5' leader that eliminated the three uORF AUGs or introduced alternative uORF peptides were analyzed *in vivo* by transient transfection assays (Donzé and Spahr 1992). Elimination of the first uORF AUG resulted in a 5-fold reduction of translation of *gag*, suggesting that the first uORF acts as an enhancer of translation. The AUG of uORF3 is the only one of the three uORF AUGs in a good initiation context; when it was eliminated, a 2-fold increase of translation was observed, suggesting that ribosomes that initiate translation at uORF3 may not be able to reinitiate at the *gag* AUG (Donzé and Spahr 1992). The first two uORFs are found in a leader region that is able to form stable secondary structure and is involved in reverse transcription (Cobrinik, Sosky and Leis 1988).

uORFs that overlap and are not in frame with the downstream cistron (Fig. 1.3.c) appear to be rare in higher eukaryotes. Such uORFs are predominantly found in retro- and coronavirus mRNAs. One example from eukaryotes is the inhibition of translation of the human fibroblast growth factor-5 proto-oncogene that arises from translation of a 38-codon uORF, whose termination codon overlaps the start codon, ***ATGA*** (Bates *et al.* 1991). The modulation of rat antizyme expression, an enzyme that binds to and destabilizes ornithine decarboxylase, requires frameshifting at the codon just preceding the ORF1 terminator codon (Matsufuji *et al.* 1995). In *in vitro* experiments frameshifting was detected in 5.4% without polyamines and 19% at the optimal spermidine concentration. The termination codon of ORF1 and a pseudoknot detected in the downstream mRNA had a stimulatory effect on frameshifting. Mutational analyses of the ORF1 terminator region indicated that frameshifting was also sensitive to changes in the nucleotides encoding the last codon, serine. Another example is the regulation of reovirus S1 translation, which occurs by interference between ribosomes translating two divergent messages from one S1 transcript (Fajardo and Shatkin 1990); in this case elongation rather than initiation appears to be the rate-limiting step. Translational frameshifting has also been observed during the expression of the polymerase of barley yellow dwarf virus in carrot cells (Brault and Miller 1992). A heptanucleotide, involved in frameshifting, is followed by a region of high

secondary structure, including a potential pseudoknot. In reporter gene studies, frameshifts were detected (at ca. 1%); however, when the stop codon at the 3' terminal of the uORF was deleted, frameshifting was abolished (Brault and Miller 1992).

1.5 Models that can account for the regulatory effects of sequence-independent or sequence-dependent uORFs: leaky scanning, reinitiation and stalled ribosomes

Three classes of models have been proposed to explain how uORFs may function to affect scanning ribosomes. These models are not necessarily mutually exclusive, as a combination of mechanisms might apply to a given system (Cao and Geballe 1995; Hinnebusch 1993; Hinnebusch 1994). The first general model invokes leaky scanning of ribosomes past an upstream AUG that is in a non-favorable initiation context within a region of a 5' leader with significant secondary structure (as discussed in the preceding sections) or too close to the 5'-cap (Ruan *et al.* 1994). Alternatively, uAUGs may be bypassed by internal entry of ribosomes onto the mRNA (Macejak and Sarnow 1991). Conversely, expression and initiation at a downstream AUG is inhibited by "preemptive initiation" at a uORF initiation codon.

The second model is based on reinitiation at a downstream AUG, following termination of translation at the first AUG encountered. The likelihood of reinitiation is generally increased by the presence of short uORFs and long distances between the uORF and a downstream AUG, based on kinetic requirements of the initiation reactions. Reinitiation, combined with leaky scanning, has been observed in *S. cerevisiae GCN4* (Abastado, Miller and Hinnebusch 1991; Abastado *et al.* 1991; Hinnebusch 1993; Hinnebusch 1994). Although four short uORFs (two or three codons long) are present in the 5' leader of *GCN4*, the presence of just uORF1 and uORF4 is sufficient to exert a response similar to that observed in wild type 5' leaders (Fig. 1.5.). Under non-starvation conditions, translation of Gcn4p is inhibited by uORF1 and uORF4, whereas under conditions of amino acid starvation Gcn4p is efficiently translated (Hinnebusch 1984; Tzamarias, Alexandraki and Thireos 1986; Tzamarias, Roussou and Thireos 1989; Abastado, Miller and Hinnebusch 1991).

Reinitiation after termination at uORF1 is regulated by the availability of dephosphorylated eIF-2α (Hinnebusch 1994). eIF2 is composed of three subunits that are required for formation of the ternary complex of eIF2-GTP-Met-tRNA$_i$, which then associates with a 40S ribosomal subunit to form a 43S pre-initiation complex (Merrick 1992). As outlined above, the pre-initiation complex enters the mRNA near the 5'-cap and

scans to the first AUG in favorable context, where, after AUG recognition, GTP is hydrolyzed to GDP, resulting in the release of an eIF-2-GDP binary complex. The exchange of GDP for GTP is catalyzed by eIF-2B (Merrick 1992), a reaction which is inhibited by phosphorylation of the eIF-2α subunit at the serine 51 residue (Colthurst, Campbell and Proud 1987). In *S. cerevisiae*, the protein kinase catalyzing the phosphorylation of eIF-2α is encoded by *GCN2* (Cigan *et al.* 1989; Dever *et al.* 1992). Gcn2p possesses a protein kinase domain and a domain that resembles histidyl-tRNA synthetase, suggesting the involvement of binding of uncharged tRNA to the histidyl-tRNA synthetase domain with concomitant activation of the protein kinase domain (Hinnebusch 1994). This hypothesis is supported by the finding that phosphorylation of eIF-2α by Gcn2p in *S. cerevisiae* occurs when uncharged tRNA accumulates, such as under conditions of amino acid imbalance or starvation or presence of a defective aminoacyl-tRNA synthetase, and results in poor recycling of eIF-2-GDP into eIF-2-GTP (Dever *et al.* 1992; Hinnebusch 1994). The low level of eIF-2-GTP results in a low level of ternary complex and a lowered rate at which active pre-initiation complexes can be formed, thus increasing the timespan for potential reinitiation after termination of translation at uORF1. 40S ribosomal subunits therefore exhibit "leaky scanning" past uORFs 2, 3 and 4 and acquire a functional ternary complex in time to reinitiate at the Gcn4p start codon. Under non-starvation conditions, the recycling of eIF-2-GDP to eIF-2-GTP is efficient, due to a lack of phosphorylation on the eIF-2α subunit and active ternary complexes are available for reinitiation at uORFs 2, 3 or 4 (Dever *et al.* 1992). As described above, once uORF4 is translated, reinitiation at the downstream AUG is extremely inefficient, thus blocking translation of Gcn4p. As predicted from the scanning hypothesis, uORF4 alone is able to inhibit translation initiation from the downstream AUG; additionally, the presence of a rare codon for proline in uORF4 and the sequence immediately downstream of uORF4 result in ribosome release after termination at uORF4, making reinitiation at the downstream AUG extremely inefficient (Grant and Hinnebusch 1994).

Ribosome stalling or pausing models that attempt to explain the effects of sequence-dependent uORFs constitute the third set of models that pertain to transcript-specific translational inhibition (Fig. 1.6.). Such models have been previously proposed to explain translation inhibition due to limitation for rare tRNAs or translation attenuation in prokaryotes (Lovett 1990; Lovett 1994), or for the inhibition of translation of eukaryotic mRNAs by the signal recognition particle (Wolin and Walter 1988).

Models for this class of uORFs, however, have to incorporate the *cis*-acting nature of the uORF and the dependency on uORF peptide sequence (Geballe and Morris 1994). Presumably, the newly synthesized uORF peptide specifically interacts with the

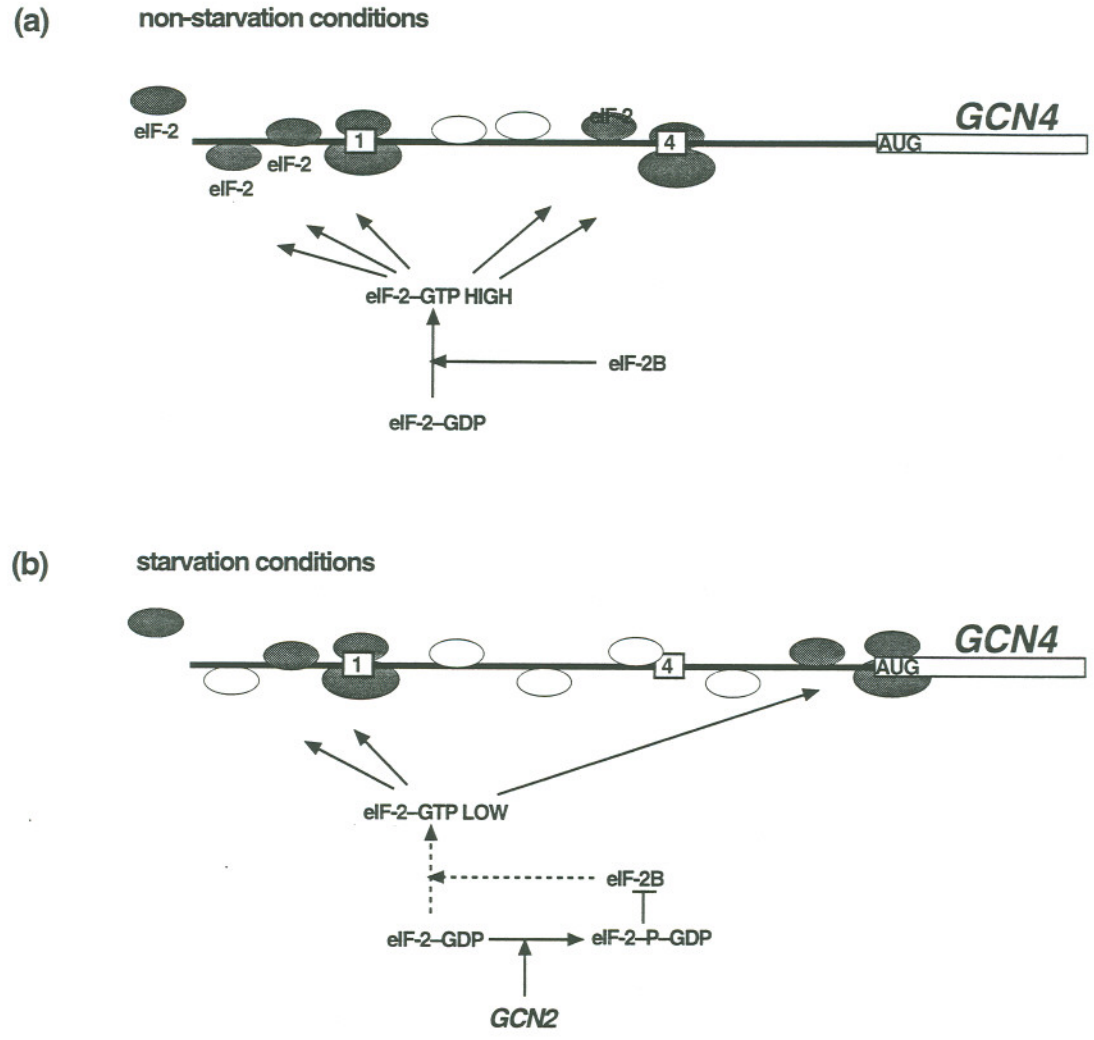

Figure 1.5: Regulation of reinitiation at the AUG of uORF4 or *GCN4* on the *GCN4* transcript under different conditions of amino acid availability (from Hinnebusch, 1993 and 1994). (a) under non-starvation conditions and high concentrations of eIF2-GTP, scanning ribosomes initiate at the AUGs of both uORF1 and uORF4, but not at the *GCN4* AUG. (b) under amino acid starvation conditions, eIF2-GTP availability is low and reinitiation cannot occur until the scanning 43S preinitiation complex reaches the *GCN4* AUG. The availability of eIF2-GTP is controlled by phosphorylation of eIF-2α by the kinase activity of *GCN2*, as explained in detail in the text.

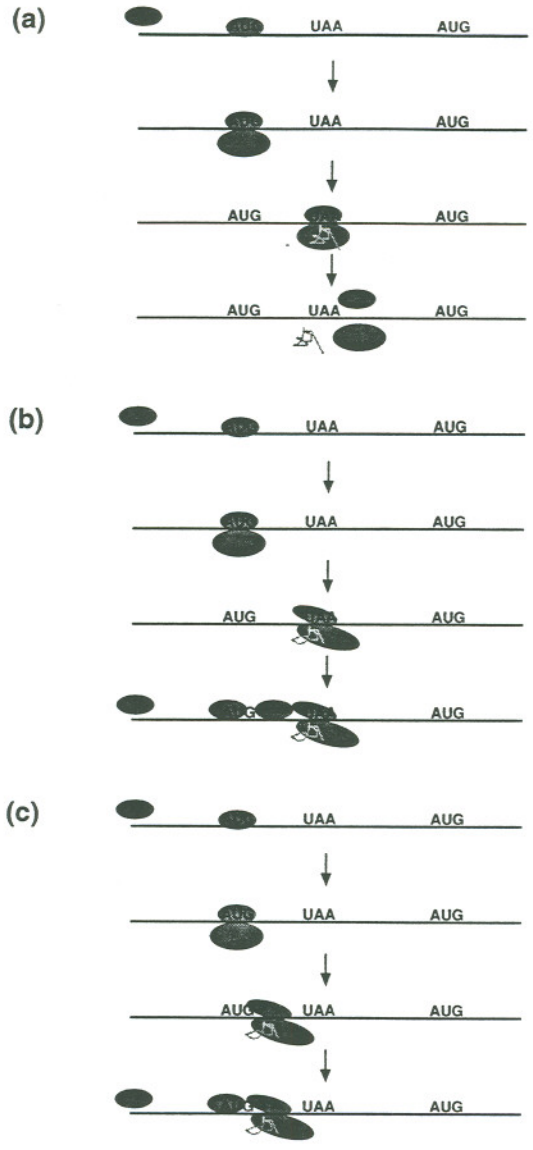

Figure 1.6: Ribosome pausing or stalling models that can explain the effects of uORFs. (a) Reinitiation of scanning ribosomes is inhibited by the uORF peptide. This model in its general form would apply to sequence-independent uORF peptides. However, the nascent peptide may also interact with the translational machinery in a sequence-dependent manner and inhibit reinitiation; (b) scanning of ribosomes past the uORF termination codon is abolished; (c) elongation or termination of the uORF peptide itself is inhibited by its own synthesis.

translational machinery and thus abolishes reinitiation (Fig. 1.6.a); this would be a specific case of preemptive initiation. The nascent peptide may also bind to the mRNA and abolish the scanning of a second ribosome past the uORF termination codon, as proposed for the β_2 adrenergic receptor uORF (Parola and Kobilka 1994); Fig. 1.6.b). Alternatively, the translation of the small peptide itself may inhibit elongation or termination of the ribosome (Fig. 1.6.c); inhibition at the point of elongation appears less likely, because carboxy-terminal missense mutations in the uORFs of both AdoMetDC (Hill and Morris 1993) and cytomegalovirus *gp48* (Degnin *et al.* 1993) abolished inhibition more efficiently than amino-terminal mutations, indicating that termination may be affected. Toeprinting experiments with uORF2 of *gp48* suggest that ribosomes stall at the translation termination step (Cao and Geballe submitted). Additionally, in both the AdoMetDC and the *gp48* systems transcripts containing the uORF are predominantly found in the monosome fractions of polysome gradients, indicating that, on average, only one ribosome per transcript is loaded (Hill and Morris 1993; Schleiss, Degnin and Geballe 1991).

1.6 Scheme to isolate regulatory mutants of *N. crassa* arginine-specific carbamoyl phosphate synthetase

Carbamoyl phosphate is a key intermediate in the biosynthesis of pyrimidine nucleotides, Arg and urea (Fig. 1.7). Most prokaryotes contain a single carbamoyl phosphate synthetase (CPS), whereas eukaryotes, like *N. crassa*, contain two CPSs (Hong *et al.* 1994). CPS-A, encoded by the nuclear *arg-2* and *arg-3* genes, is a two-subunit enzyme located in the *N. crassa* mitochondrial matrix and is specific for the Arg biosynthetic pathway, whereas CPS-P, encoded by *pyr-3*, is localized in the nucleus and functions in pyrimidine biosynthesis (Davis 1986). The level of CPS-A activity in *N. crassa* is generally the rate determining component of flux through the Arg pathway (Davis 1986). CPS-A activity in *N. crassa* is determined by the level of the holoenzyme's small glutamine amidotransferase subunit (Davis 1986), encoded by *arg-2* (Orbach, Sachs and Yanofsky 1990). *arg-2* is the only gene encoding an *N. crassa* Arg pathway enzyme that is subject to negative regulation by Arg (Davis 1986). Arg has been shown to negatively affect both the levels of *arg-2* transcript and *arg-2* translation (Luo, Freitag and Sachs 1995; Orbach, Sachs and Yanofsky 1990; Sachs and Yanofsky 1991).

Regulatory mutations of *S. cerevisiae CPA1* were first isolated by making use of the interdependence of CPS-A and CPS-P (Thuriaux *et al.* 1972). In contrast to *N. crassa*, *S. cerevisiae* CPS-A is localized in the cytosol. The *S. cerevisiae ura2C* strain lacks nuclear CPS-P activity but is prototrophic in minimal medium because of cross-feeding of

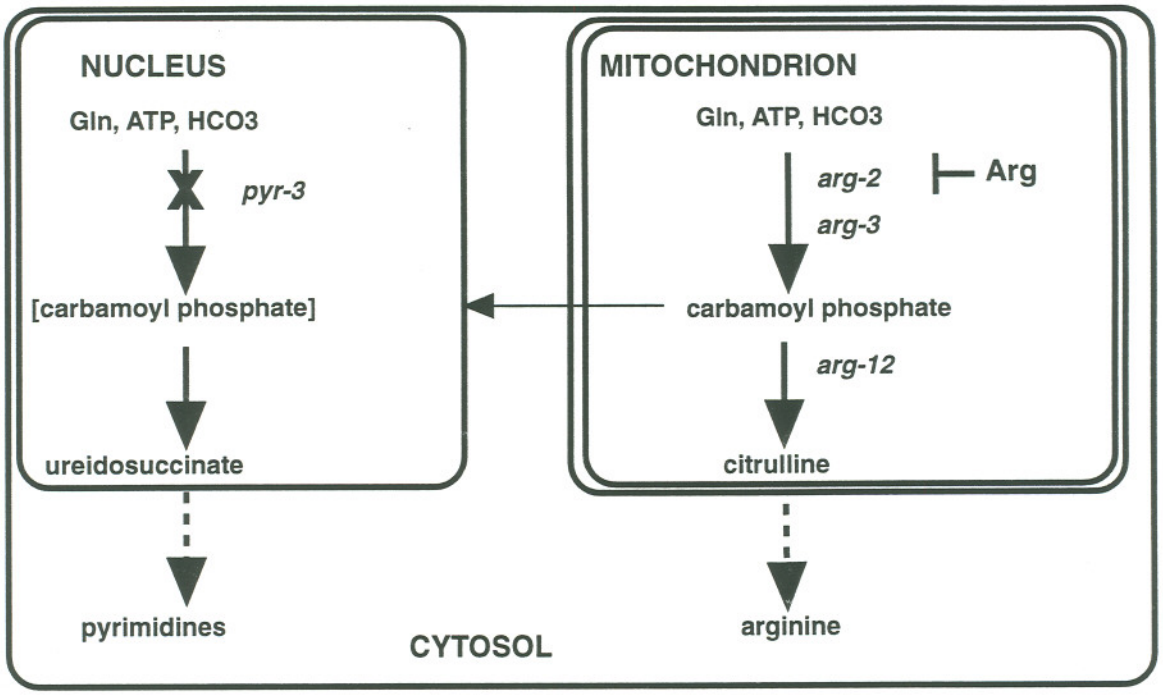

Figure 1.7: Simplified scheme of carbamoyl phosphate metabolism in *N. crassa* (after Davis, 1986). Pyrimidine-specific carbamoyl phosphate synthetase (CPS-P) is located in the nucleus and encoded by *pyr-3*. Arginine-specific carbamoyl phosphate synthetase (CPS-A) is located in the nitochondrion in *N. crassa* (in the cytosol in *S. cerevisiae*) and is encoded by *arg-2* and *arg-3*. *arg-2* is the only gene in the arginine metabolic pathway that is negatively regulated by arginine. *arg-12* encodes ornithine transcarbamylase. A leaky mutation in this gene, *arg-12^s*, permits growth on minimal medium of the double mutant *arg-12^s pur-3* due to cross-feeding of carbamoyl phosphate from the mitochondrion into the nuclear pyrimidine pathway. On arginine medium, the double mutant does not grow because of negative regulation of *arg-2* by Arg and lack of cross-feeding of carbamoyl phosphate.

carbamoyl phosphate synthesized by CPS-A from the cytosol to the nucleus. This strain acquires a pyrimidine requirement when minimal medium is supplemented with Arg, because *CPA1* is negatively regulated by Arg, leading to reduced CPS-A activity and thus to reduced cross-feeding of carbamoyl phosphate into the nucleus. *S. cerevisiae* mutants constitutive for CPS-A activity were isolated by selection for *ura2C* mutant strains that were able to grow on Arg-containing medium without adding uracil (Thuriaux *et al.* 1972)). One class (*CPA1-O*) was Arg constitutive, *cis*-dominant and closely linked to the *CPA1* locus; a second class (*cpaR*) reduced the Arg regulation of *CPA1*, was *trans*-recessive and unlinked to *CPA1* (Thuriaux *et al.* 1972). Whereas the *CPA1-O* mutations affect the sequence of the *CPA1* uORF (Werner *et al.* 1987), the involvement of the second class of mutations in Arg-specific regulation of *CPA1* is still poorly understood; this mutation may not be directly involved in Arg metabolism.

Analogous experiments in *N. crassa* that employed a double mutant, *arg-12^S pyr-3*, failed (Davis 1986). Like the *S. cerevisiae ura2C* strain, *N. crassa pyr-3* mutants lack CPS-P activity; such strains are unable to grow on minimal medium. The sequestration of carbamoyl phosphate in wild type *N. crassa* mitochondria does not allow sufficient leakage of carbamoyl phosphate into the pyrimidine pathway to enable the *pyr-3* mutant to grow without supplements. However, an additional mutation, *arg-12^S*, combined with *pyr-3*, produced a strain that is able to grow on minimal medium (Davis 1962). The bradytrophic *arg-12^S* mutation by itself results in severely reduced ornithine transcarbamoylase activity and Arg starvation, which in turn leads to overexpression of *arg-2*, increased activity of CPS-A and therefore elevated levels of carbamoyl phosphate on minimal medium (Davis 1986). The double mutant *arg-12^S pyr-3* grows on minimal medium because overexpression of CPS-A in minimal medium provides sufficient carbamoyl phosphate for both Arg and pyrimidine synthesis. Because addition of Arg to the medium reduces the expression of *arg-2*, the *arg-12^S pyr-3* strain becomes a pyrimidine auxotroph in Arg-containing medium. Therefore, the *arg-12^S* mutation acts a suppressor of *pyr-3* on minimal medium but not on Arg-containing medium. Extensive direct selections for mutants of the *N. crassa arg-12^S pyr-3* strain that lost the uridine (Uri) requirement in Arg-containing medium did not yield Arg-regulatory mutants (Davis 1986); alleles of *pmb* (*bat*), the basic amino acid transporter, were obtained by this selection scheme (see Perkins *et al.* 1982).

To select for mutants that are affected in Arg-specific negative regulation of *arg-2*, an *arg-12^S pyr-3* double mutant was transformed with a plasmid carrying a reporter gene that contains *arg-2* sequences fused to the *Escherichia coli* hygromycin B phosphotransferase (Hph) gene, *hph*. As discussed in section 1.8, Hph inactivates the antibiotic hygromycin B (Hyg) by phosphate group transfer (Rao *et al.* 1983). *N. crassa*

arg-12^{S} *pyr-3* strains were obtained that grew on Hyg+Uri, but did not grow on Hyg+Uri+Arg because the *arg-2–hph* gene was under Arg-specific negative regulation.

Mutations that affected the expression of *arg-2–hph* in Hyg+Uri+Arg and allowed mutants to grow on this medium were induced by irradiation with UV-light. At least two classes of mutants were expected. One class was expected to affect the expression of the *arg-2–hph* reporter gene and to alter the sequence of the *arg-2–hph* gene. Another class was expected to affect factors controlling the expression of the *arg-2–hph* reporter gene and the endogenous *arg-2* gene. Besides regulatory mutations in factors that are directly involved in the translational control of *arg-2*, other mutations may result in a similar phenotype. A mutation that reduces the transport of arginine into the cell, for example, may result in apparently increased expression of *arg-2* and *arg-2–hph* in Arg-containing medium.

1.7 The *Escherichia coli* hygromycin B phosphotransferase gene

Hygromycin B (Hyg; Fig. 1.8) is an aminocyclitol antibiotic with broad spectrum activity against prokaryotes and eukaryotes (Pettinger *et al.* 1953). Hyg inhibits protein synthesis by blocking ribosomal translocation and preventing polypeptide elongation by interfering with aminoacyl tRNA recognition and A-site occupation (Cabanas, Vazquez and Modolell 1978; Hausner, Geigenmüller and Nierhaus 1988). Hyg can also lead to misreading during translation in an *in vitro* system (Davies and Davis 1968; Gonzales *et al.* 1978; Singh, Ursic and Davies 1979); however, the *in vitro* effect could not be duplicated in *in vivo* studies (Bakker 1992).

Resistance to Hyg is conferred by hygromycin B phosphotransferase (Hph), an enzyme first isolated from *Streptomyces hygroscopicus* (Leboul and Davies 1982). Hph catalyzes the phosphorylation of the 4-hydroxyl group on the hyosamine moiety, thereby inactivating Hyg (Rao *et al.* 1983). Plasmid-determined resistance to Hyg had been observed in *E. coli* (Rao *et al.* 1983) and *Klebsiella pneumoniae* (Gritz and Davies 1983). Three plasmid-borne genes encoding Hph were isolated and characterized (Gritz and Davies 1983; Kaster *et al.* 1983; Malpartida *et al.* 1983).

Upon transformation, plasmids carrying fusions of a variety of eukaryotic promoters to bacterial *hph* genes can confer Hyg resistance to eukaryotic cells, for example *S. cerevisiae* (Gritz and Davies 1983; Kaster, Burgett and Ingolia 1984), *Aspergillus nidulans* and *A. niger* (Cullen *et al.* 1987; Punt *et al.* 1987) and *N. crassa* (Staben *et al.* 1989). The fact that at least twenty species of filamentous fungi have been successfully transformed to Hyg^{r} with either of two plasmids, pAN7-1 and pAN8-1, which contain a

fusion of the *A. nidulans gpd* promoter to *hph*, followed by the *A. nidulans trpC* terminator, underscores the utility of Hyg as dominant selectable resistance marker in filamentous fungi (Punt and van den Hondel 1992). Hyg resistance has also been widely used as marker in plant molecular genetics (Severin and Schöffl 1990; van den Elzen *et al.* 1985) and studies on mammalian (Blochinger and Diggelmann 1984; Giordano and McAllister 1990; Hu and Temin 1990) and virus-infected cells (Zhou *et al.* 1991).

In this study, the *hph* gene was initially used as a phenotypic reporter gene system in studies on the Arg-specific negative regulation of the *arg-2* gene. It was also desirable to quantify the expression of *hph* directly by measuring Hph activity; however the original assay procedure for aminoglycoside-modifying enzymes is both cumbersome and time-consuming (Haas and Dowding 1975). Therefore a more recently developed method to assay for Hph activity was adapted to measure Hph activity from whole cell extracts of *N. crassa*. The assay described here is an adaptation of previously published dot methods for measuring neomycin phosphotransferase and Hph activity in mammalian crude cell extracts (Duch *et al.* 1990; Platt and Yang 1987; Sørensen *et al.* 1992) and is based on the binding of the weakly positively charged radioactively labeled reaction product, hygromycin B-γ-$^{32}PO_4$, to phosphocellulose cation exchange paper. The amount of [γ-^{32}P]-labeled Hyg was determined by autoradiography followed by densitometry or, alternatively, by exposing phosphorimager plates to the [γ-^{32}P]-labeled phosphocellulose paper followed by analyses with a phosphorimager system. Results presented here indicate that *hph* can be used as a combined selectable dominant marker and reporter gene in *N. crassa* strains transformed with plasmids carrying *hph* fusion genes.

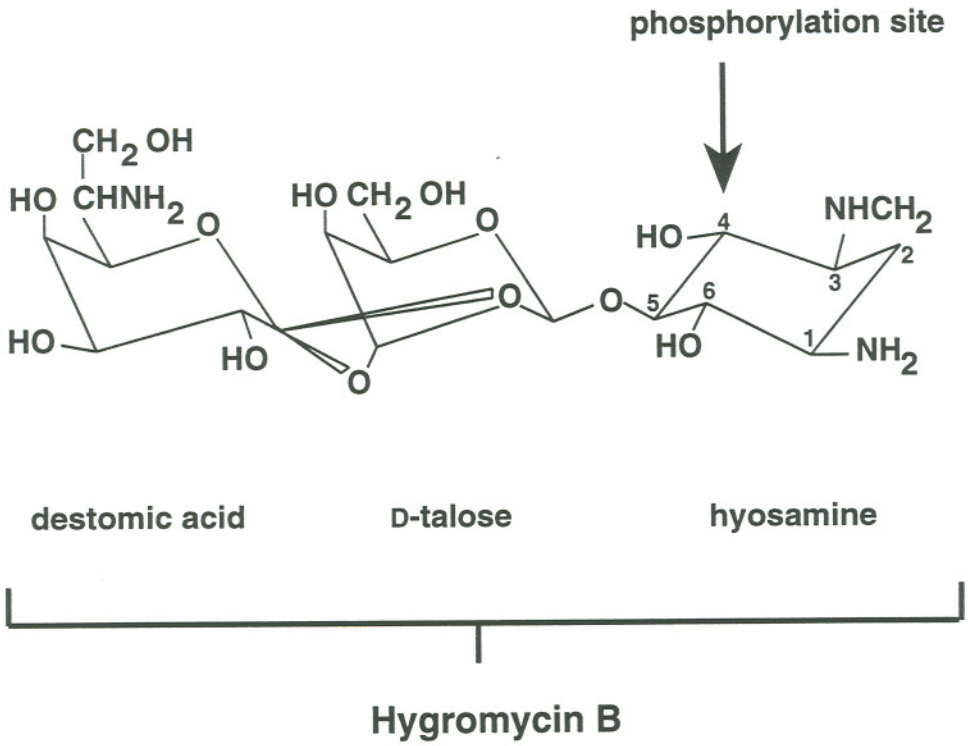

Figure 1.8: Structure of hygromycin B. The site of inactivation by phosphorylation is indicated (from Rao *et al.*, 1983).

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 *Neurospora crassa* strains

The wild type 74A-OR23-1VA (74A) and *arg-12^s a* strains were obtained from D. Perkins, Stanford University. The *arg-12^s pyr-3 a*, *fluffy A* and *fluffy a* strains were obtained from E. Selker, University of Oregon. The *his-3* strain (allele number 1-234-723; FGSC #6103) was obtained from the Fungal Genetics Stock Center, University of Kansas Medical School.

Several new *N. crassa* strains were constructed by DNA-mediated transformation of the *arg-12^s a*, *arg-12^s pyr-3 a* and *his-3* strains with *E. coli* plasmids, or by UV-induced mutation, as described below.

2.1.2 *Escherichia coli* strains

E. coli strains XL1-Blue (F'::*Tn10 proA$^+$B$^+$ lacI^q Δ(lacZ)M15/recA1 endA1 gyrA96* (Nal^r) *thi hsdR17* ($r_K^-m_K^+$) *supE44 relA1 lac*) and DH5-α (*endA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1 recA1 gyrA96* (Nal^r) *relA1 Δ(lacIZYA-argF)U169 deoR* (*ϕ80dlacΔ(lacZ)M15*) were stocks originally obtained from Stratagene (LaJolla, CA) or the laboratory of C. Yanofsky (Stanford University).

2.1.3 *E. coli* plasmids

Several plasmids that contain genomic *N. crassa arg-2* DNA, chimeras of *arg-2* genomic DNA and cDNA and fusions of *arg-2* 5' leader sequences to the *E. coli hph* gene were constructed by M. S. Sachs. Plasmid pAB1 contains the genomic 3681 nt *Bam*HI-*Pst*I *arg-2* fragment from pAR201 and plasmid pAE1 contains the genomic 3048 nt *Sac*I-

*Pst*I *arg-2* fragment from pAR201; both fragments were cloned into the corresponding sites of pDSP72 (Orbach, Sachs and Yanofsky 1990). The 1866 nt *Pvu*II-*Nco*I fragments of pAB1 or pAE1, containing *arg-2* genomic DNA, were replaced with the intronless 1367 nt *Pvu*II-*Nco*I fragment from pAH4, a pSP72-based plasmid containing an *arg-2* cDNA insert from pARCG228 (Orbach, Sachs and Yanofsky 1990), resulting in plasmids pAR1 and pAU1, respectively.

Four vectors that contain different translational fusions of *arg-2* 5' leader sequences to the *E. coli hph* gene were constructed (see Fig. 3.1.). To construct these plasmids, pDH25 (Cullen *et al.* 1987) was digested with *Cla*I and *Bam*HI to yield a 1035 nt fragment containing *hph.* The 5' *Cla*I site was replaced with a *Bam*HI/*Xba*I linker and ligated to pCon10 to give pCon10/ΔKCHyg (D. Ebbole, Texas A&M University). The 1040 nt *Bam*HI *hph* fragment from pCon10/DKCHyg was incubated with dNTPs and the Klenow fragment of *E. coli* DNA polymerase I to yield blunt ends. This fragment was ligated to the large *Sty*I fragment of pAB1, pAE1, pAR1 and pAU1, respectively, which was made blunt-ended by incubation with dNTPs and the Klenow fragment of *E. coli* polymerase I. The *Sty*I sites of *arg-2* and the *Bam*HI sites of the *hph* cassette were regenerated by this ligation. Constructs were transformed into *E. coli*, plasmid DNA isolated and analyzed by restriction enzyme mapping to confirm the orientation of the *hph* insert with respect to the plasmid. The resulting vectors, pGS3, pGT6, pGU1 and pGV4, respectively, contain four different translational fusions of the 5' *arg-2* sequences to *hph.*

Plasmid tgCMV/HyTK (Lupton *et al.* 1991), which contains a translational fusion of the *E. coli* hygromycin B phosphotransferase resistance gene, *hph*, to the herpes simplex virus type 1 thymidine kinase gene, *tk*, driven by the human cytomegalovirus immediate early promoter (IE94), was obtained from S. Lupton, Targeted Genetics Co., Seattle, WA.

Plasmid pH303 (K. Hager, Yale University) contains a 5'-truncated 2.6 kb *Hind*III-*Cla*I *N. crassa his-3* gene fragment from pNH60 (Ebbole 1990; Legerton and Yanofsky 1985), cloned into pBS+ (Stratagene, LaJolla, CA). Plasmid pDE1 contains *E. coli lacZ*, followed by the *A. nidulans trpC* terminator and the 2.6 kb *Hind*III-*Cla*I fragment from pH303 (Ebbole 1990).

Two vectors for targeted integration of PCR-amplified *arg-2–hph* genes at the *N. crassa his-3* locus were constructed. The 5' truncated *his-3* fragments of pDE1 (Ebbole 1990) or pH303 (K. Hager, Yale University) can complement certain *N. crassa his-3* mutants by homologous recombination at *his-3* following transformation and selection on minimal medium (Sachs and Ebbole 1990). To construct pMF1 (7334 nt), the 2470 nt *Bgl*II - *Hind*III fragment of pGV4, which contains the *arg-2–hph* reporter gene, was

cloned into the unique *Bgl*II and *Hind*III sites of pDE1. Analagously, pMF2 (6691 nt) was constructed by integrating the 2509 nt *Eco*RI - *Cla*I *his-3* fragment from pH303 into the unique *Eco*RI and *Cla*I sites of pGV4. pMF2 reproducibly resulted in higher *N. crassa* transformation frequencies when strains were selected for His prototrophy by plating transformants on minimal medium, whereas transformation frequencies were equal for pMF1 and pMF2 when transformants were selected for Hyg resistance in His-supplemented media. pMF2 was used in experiments leading to targeted integration at the *his-3* locus. Plasmids pMF11-wt, pMF11-D12N and pMF11-37 contain PCR-amplified, 638 nt *Eco*RI 5' *arg-2–hph* fragments from a single copy *arg-12^S pyr-3 arg-2–hph* transformant (strain MF13-3), *arg-12^S pyr-3 arg-2–hph (D12N) cis*-mutant (MF13-3-40) and *arg-12^S pyr-3 arg-2–hph* class II mutant (MF13-3-37), respectively, cloned into the unique *Eco*RI site in plasmid pMF2.

Plasmids for sequencing analyses were constructed by cloning *Eco*RI-digested amplification products obtained from PCR of pGV4 plasmid or genomic DNA templates from wild type and mutant *N. crassa arg-12^S pyr-3 arg-2–hph* strains into the unique *Eco*RI site of pBS SKII+ (Stratagene, La Jolla, CA). Plasmids pSP72, pSP65 (Promega, Madison, WI) and pBS SKII+ (Stratagene, LaJolla, CA) were stocks from this laboratory.

2.2 General Methods

2.2.1 Growth and and storage of *N. crassa* strains

Solid minimal medium (Min) contained 1X Vogel's N salts (Vogel 1956), 2% sucrose and 2% agar. Colonial growth was induced by growing strains on solid medium in which 0.05% fructose, 0.05% glucose and 2% sorbose (FGS) were substituted for sucrose (Davis and deSerres 1970). FGS was autoclaved separately at 10x concentration and added to the medium after cooling to 55°C. For analyses of phenotypes, the *arg-12^S pyr-3 arg-2–hph* transformants and mutants derived from it were grown on the following media: Min; Min supplemented with 0.5 mg/ml uridine (Uri); Min supplemented with 0.5 mg/ml arginine (Arg); Min supplemented with 2 mg/ml hygromycin B (Hyg; obtained from Calbiochem, La Jolla, CA); Uri+Arg; Hyg+Uri; Hyg+Arg; and Hyg+Uri+Arg (abbreviations for media denote the concentrations of supplements stated here, unless otherwise indicated). Supplements were added prior to autoclaving The *his-3* auxotrophic strain was grown on minimal medium supplemented with 0.5 mg/ml histidine (His). Media were also supplemented with 2 mg/ml thialysine (Sigma, St. Louis, MO) or 10 mM difluoromethyl ornithine (a gift of R. Davis, University of California at Irvine) as indicated.

Cultures were routinely grown on 1 ml solid medium in 10 x 75 mm culture tubes and stored at 4°C or -20°C after the onset of conidiation. Large scale cultures were grown on 250 ml of solid medium with supplements as needed in 2.5 L Fernbach flasks for 2 days at 34°C and for an additional 7-10 days at room temperature to obtain macroconidia as inoculate for studies in liquid media.

Procedures described previously (Sachs and Yanofsky 1991) were used to grow *N. crassa* in liquid media, except that water, not Soltrol 170, was used to obtain conidia as inoculum. Strains were routinely grown in Min, Uri, Arg, Uri+Arg or His medium. In typical experiments, 30 ml of liquid medium in 125 ml Erlenmeyer flasks were inoculated with 2 x 10^7 conidia per ml, cultures incubated at 34°C with orbital shaking (200 rpm) for 6.5 h and harvested by vacuum filtration onto Whatman #541 filter paper.

The effects of Arg or His starvation, induced by 10 mM 3-amino-1,2,4-triazole (3AT), were examined in experiments in which cells were switched from Min, Arg, Uri or Uri+Arg medium to Min, Arg, Uri, Uri+Arg, 3AT, 3AT+Arg, 3AT+Uri, 3AT+His or 3AT+Arg medium. Cells were grown for 6.5 h, harvested by vacuum filtration onto Whatman 541 filters, resuspended in fresh medium and incubated for an additional 30 min at 34°C with orbital shaking (200 rpm). 3AT was added from a fresh, filter-sterilized 0.1 M stock prepared in water to the fresh media immediately before cells were resuspended. After collection by vacuum filtration onto Whatman #541 filter paper, the mycelial mat was divided and either used immediately to make whole cell extracts for use in enzyme assays or polysome analyses, or quick frozen in liquid nitrogen for analyses of total RNA.

2.2.2 Growth and and storage of *E. coli* strains

General methods for maintaining *E. coli* strains were as described (Sambrook, Fritsch and Maniatis 1989). *E. coli* strains were grown in LB medium or Super Broth at 37°C with orbital shaking (200 rpm). To select for plasmid-containing cells, ampicillin was added to LB medium at 100 µg/ml. Strains were stored at -80°C in Nalgene Cryovials in LB medium with 15% glycerol.

2.3. Genetic Analyses

2.3.1 UV-mutagenesis

Conidia (1 x 10^8) of *N. crassa* strains transformed with *arg-2–hph* reporter gene plasmids were suspended in 10 ml of water in an uncovered 100 mm glass Petri dish and irradiated with 254 nm UV light at 0 to 1500 J/m^2 in a UV Stratalinker 1800 (Stratagene,

LaJolla, CA); strains were handled in dim light to avoid photoreactivation repair of UV damage. A kill curve was constructed for the *arg-12s pyr-3* strain. To generate UV-induced mutants conidia were irradiated with 700 or 900 J/m^2. These doses yielded 25% or 10% spore survival, respectively. Then, 2 x 10^6 conidia per 100 mm Petri plate were spread onto Hyg+Uri+Arg medium, supplemented with FGS to induce colonial growth. Colonies derived from single spores were isolated after 2 to 5 days of incubation at 34°C; no colonies from mock-irradiated plates were Hyg^r. Putative primary mutants were analyzed phenotypically on Min, Uri, Arg, Uri+Arg, Hyg+Uri, Hyg +Arg or Hyg+Uri+Arg. Homokaryotic cultures of putative mutants were obtained by serial isolation of single asexual spores (Davis and deSerres 1970) or by isolation of microconidia (Ebbole and Sachs 1990).

2.3.2 Genetic Analyses of transformants and UV-induced mutants

Genetic crosses of *N. crassa* strains were performed and analyzed as described (Davis and deSerres 1970). The *arg-12^S pyr-3 arg-2–hph a* transformant MF13-3 or UV-induced mutants derived from this transformant were backcrossed at least twice to the 74*A* wild type strain. Strains were co-inoculated onto Petri plates containing 1X synthetic crossing medium ((Davis and deSerres 1970)) and incubated for 2 to 3 weeks at 25°C under constant light. When perithecia were visible, plates were inverted and shot ascospores collected on the plate lids.

Ascospores were heat-shocked in water for 45 min at 62°C and spread onto FGS Uri or FGS Uri+Arg medium in 100 mm Petri plates. Spread ascospores were incubated for 8 to 12 h at 34°C and single germinated spores picked to small culture tubes containing either Uri or Uri+Arg medium. The percentage of germinated ascospores on plates was determined after 1-2 days. Alternatively, 50 or 100 random ungerminated ascospores per cross were picked to 10 x 75 mm culture tubes containing Uri or Uri+Arg medium.

2.4 Methods for DNA

2.4.1 Molecular cloning techniques

General methods for cloning DNA were as previously described (Sambrook, Fritsch and Maniatis 1989). Restriction endonucleases from New England Biolabs (NEB, Beverly, MA) were used for DNA digestions. Nucleotides (Life Technologies, Bethesda, MA) and Klenow enzyme (NEB) were used for 5′-overhang fill-in reactions. When necessary, calf-intestinal alkaline phosphatase (Pharmacia, Piscataway, NJ) was used to

dephosphorylate DNA fragment ends. Ligation reactions were incubated with T4 DNA ligase from NEB, either for 6 h at 17°C or overnight at 25°C.

2.4.2 Small scale plasmid DNA preparations

Plasmid DNA was prepared by the rapid boiling method (Sambrook, Fritsch and Maniatis 1989). Bacterial cultures (5 ml of LB) from single ampicillin-resistant *E. coli* colonies were grown overnight at 37°C with orbital shaking (200 rpm). Cultures were pelleted in 1.5 ml Eppendorf tubes in a microcentrifuge for 25 sec at 16,000 x*g* and supernatants were removed by aspiration. Pelleted cells were resuspended in 0.5 ml of STET buffer (8% sucrose, 50 mM EDTA, 5% Triton, 50 mM Tris-HCL [pH 8]) by vortexing; 0.02 ml of 10 mg/ml lysozyme was added and mixed by vortexing. Samples were incubated at room temperature for 5 min and boiled in a water-filled heat block for 2 min. Cell debris was pelleted by centrifugation for 10 min in a microcentrifuge at 16,000 x*g* at room temperature. Pellets were removed from tubes with sterile toothpicks and 0.4 ml isopropyl alcohol was added to the tubes to precipitate DNA. Following centrifugation for 10 min at 16,000 x*g* in a microcentrifuge at room temperature, the supernatants were removed by aspiration. Nucleic-acid containing pellets were redissolved in 40 µl of 20 mM Tris (pH8) + 10 mM EDTA buffer. Typically, 3 µl of DNA were used for restriction enzyme analyses.

2.4.3 Large scale plasmid DNA preparations

Large scale preparations of plasmid DNA were purified by equilibrium centrifugation in CsCl gradients (Sambrook, Fritsch and Maniatis 1989). Single ampicillin-resistant *E. coli* colonies were grown overnight in 5 ml of LB medium. One ml of starter culture was used to inoculate 200 ml of Terrific Broth and grown for 4 to 6 h at 37°C with orbital shaking (200 rpm). Cultures were transferred into 250 ml GSA bottles and cells pelleted at 1,500 x*g* at 4°C for 5 min in a Sorvall RC-5B centrifuge. Supernatants were decanted and the pellets were resuspended in 6 ml of TE buffer. Cell suspensions were diluted with 13 ml of fresh lysis buffer (0.2 N NaOH/1% SDS), swirled and incubated at room temperature for 10 min. Then, 10 ml of NH_4OAc was added. The samples were mixed by inversion, held on ice for 15 min and centrifuged at 10,500 x*g* for 20 min at 4°C. The resulting plasmid-containing supernatants were filtered through cheesecloth into GSA bottles containing 34 ml of isopropanol. Nucleic acids were precipitated at -20°C for at least 2 h. Precipitated nucleic acids were pelleted by centrifugation at 10,500 x*g* for 15 min at 4°C, supernatants were discarded, bottles

inverted and allowed to air dry. The pellets were resuspended in 4.0 ml of 10 mM Tris-HCl (pH 8.0)/1 mM EDTA (TE) buffer.

Plasmid-containing solutions for CsCl equilibrium centrifugation were prepared by adding the 4.0 ml of plasmid-TE solution and 0.3 ml of 5 mg/ml ethidium bromide (EtBr) to 15 ml centrifuge tubes containing exactly 4.46 g of CsCl. DNA solutions (4.2 ml) were then transferred to Beckman QuickSeal ultracentrifuge tubes (#342412). Tubes were balanced with CsCl solution in TE buffer and centrifuged at 45,000 rpm for 15–18 hr at 20°C using a VTi65.2 rotor in a Beckman L8-70M Superspeed Centrifuge.

The plasmid DNA bands were removed from tubes by inserting #18 gauge needles attached to 3 ml syringes through the tube walls. The EtBr was removed by serial extraction using isopropyl alcohol equilibrated with a saturated solution of NaCl in sterile water. Nucleic acids were precipitated at room temperature by adding 2 volumes of sterile water followed by 6 volumes of ethanol. Samples were centrifuged at 10,500 x*g* for 15 min at 4°C in a Sorvall RC-5B centrifuge (SS34 rotor). Pellets were washed twice with 70% ethanol, vacuum dried, and redissolved in 400–800 µl of TE buffer.

2.4.4 Preparation of genomic *N. crassa* DNA

Macroconidia were collected from stock cultures. Conidial suspensions (10 µl containing approximately 10^3-10^4 conidia) were inoculated into 25 ml Min in 125 ml Erlenmeyer flasks, and cultures were grown at room temperature for 2 to 4 days (until aerial hyphae and conidia begun to form). Cells were harvested onto #1 Whatman filter paper in a Büchner funnel. Samples were frozen at -80°C in 15 ml Falcon tubes (#2059).

Genomic DNA was extracted by modification of a published procedure (Oakley *et al.* 1987) . Frozen mycelia were lyophilized overnight and pulverized by vortexing with a spatula for 30 sec. Samples were resuspended by vortexing at high speed for 20 sec in 1 ml of salt-detergent stock solution (4 mg/ml sodium deoxycholate, 10 mg/ml Brij 58 [polyoxyethylene20 cetyl ether] and 2 M sodium chloride; stored at 4°C), incubated at room temperature for 20 min while mixing on an end-over-end rotator and centrifuged at 8000 x*g* for 10 min in a Sorvall SS-34 rotor. The supernatant (300 µl) from each sample was transferred to Eppendorf tubes and gently mixed with 1.2 ml of sodium trichloroacetic acid (TCA):ethanol (1:4 vol:vol). The TCA/ethanol solution was prepared by dissolving 41.7 g NaTCA salt (Aldrich #19,078-0) in sterile water, adjusting to 50 ml to make 4.5 M TCA and adding 50 ml of ethanol. TCA/ethanol was stored at 4°C (a precipitate forms upon long-term storage of TCA/ethanol; it does not interfere with nucleic acid precipitation, as long as one uses the TCA/ethanol supernatant only). Nucleic acids were precipitated at -20°C for at least 30 min. Precipitated nucleic acids were collected by centrifugation in a

microcentrifuge at 16,000 x*g* for 15 sec, washed with 300 µl of 70% ethanol, pelleted by another 15 sec spin, and dried briefly in a SpeedVac centrifuge (Savant, Farmingdale, NY). Pellets were resuspended in 100 µl of 10 mM NH_4OAc, gently mixed with 100 µl of 0.3 mg/ml RNaseA in 10 mM NH_4OAc and incubated at 50°C for 1 hr with gentle vortexing every 15 min to resuspend the pellet. Chloroform (200 µl) was added, the tubes vortexed and centrifuged in a microcentrifuge for 5 min at 16,000 x*g*. The upper, aqueous phase was transferred to a new tube to which 107 µl of 7.5 M NH_4OAc and 0.8 ml isopropanol were added. The samples were mixed well by inversion, immediately centrifuged in a microcentrifuge for 25 sec, washed with 300 µl of 70% ethanol, recentrifuged and dried briefly in the SpeedVac. Pellets were resuspended in 100 µl of TE buffer overnight at 4°C. This procedure yielded 40-90 µg of genomic DNA per culture, as determined by fluorometry (Labarca and Paigen 1980) using a TKO 100-dedicated Mini Fluorometer (Hoefer, San Francisco, CA), following the manufacturer's instructions. Aliquots were stored at -80°C.

2.4.5 Polymerase chain reactions

Polymerase chain reactions were performed on an Ericomp Single Block Thermocycler (Ericomp, San Diego, CA) to amplify DNA fragments of the *arg-2–hph* fusion gene from genomic DNA of various *N. crassa* mutants and transformants. The right-hand primer ARG2-Eco (5'-CGG AAT TCT ACC AGA TCC AAT CAA) was identical to nucleotides 654-674 of the published *arg-2* sequence (Orbach, Sachs and Yanofsky 1990); an *Eco*RI site was created by adding a CGG clamp at the 5' end. The left-hand primer HPH-Eco (5'-GAT GCA ATA GGT CAG GCT CTC) was identical to nucleotides 463-483 of the non-coding strand of the published *E. coli hph* sequence (Gritz and Davies 1983), 3 nt downstream of the unique *hph Eco*RI site. These two primers were used to amplify 662 nt DNA fragments from the *arg-2–hph* reporter gene, containing the 386 nt of the intronless 5' *arg-2* sequence and 276 nt of the *hph* sequence.

In a typical reaction (50 µl), 5 ng plasmid DNA or 100 ng genomic *N. crassa* DNA as template, 0.5 µM primers, 400 µM of each dNTP, 1X VentPolymerase buffer (10 mM KCl, 20 mM Tris-HCl [pH 8.8], 10 mM $[NH4]_2SO_4$, 0.1% Triton X-100; New England Biolabs, Beverly, MA) and VentPolymerase (1 u) were overlaid with 25 µl of light mineral oil (Aldrich, St. Louis, MO) and cycled 35 times through the following temperature profile: 90 sec at 94°C, 30 sec at 50°C, 60 sec at 72°C. The last extension step was carried out for 5 min. Reactions were cooled on ice and extracted with 60 µl chloroform, vortexed and phases separated by centrifugation for 5 min at 16,000 x*g*. One tenth of each reaction was examined by electrophoresis through an analytical 0.8% agarose gel. Reactions that

yielded discrete bands of the expected size were incubated with *Eco*RI (5 u per reaction) and the products subcloned into the unique *Eco*RI site of either pBS SKII+ or pMF2, following standard procedures (Sambrook, Fritsch and Maniatis 1989). Orientations of inserts were determined by restriction enzyme mapping. The *arg-2–hph* region from pGV4 or from wild type and mutant *N. crassa arg-12^S pyr-3 arg-2–hph* genomic DNAs were amplified in at least three independent PCR reactions for sequence characterization; three reactions were judged sufficient to minimize the possibility that errors introduced during the PCR went undetected.

2.4.6 Purification of DNA fragments from agarose gels

Double-stranded genomic *N. crassa* or plasmid DNA was separated in agarose gels (0.8-1.2%). The gel buffer was usually 40 mM Tris base, 20 mM sodium acetate and 2 mM EDTA (pH 8.0).

Purified DNA fragments were used for cloning procedures or as radiolabeled probes for Southern or northern blotting. Several different methods (phenol extraction, agarase digestion or DEAE membrane elution) were used to isolate and purify DNA fragments following agarose gel electrophoresis.

DNA fragments were isolated from gel fragments excised from SeaPlaque™ GTG (FMC, Rockland, ME) low-melting point agarose gels by phenol extraction. Fragments were excised with a glass cover slip, extra agarose trimmed and fragments melted at 67°C for 15 min in a 1.5 ml centrifuge tube containing 50 µl of 4 M NaCl, 10 µl of 1 M Tris-HCl (pH 8.0) and 460 µl sterile distilled water. Pre-heated (67°C) phenol (0.5 ml) was added, tubes vortexed and centrifuged at 16,000 x*g* for 5 min at room temperature. The top, aqueous layer was transferred to a fresh tube. The phenol extraction was repeated twice and followed by a single chloroform extraction. The aqueous layer was transferred to a fresh tube and DNA precipitated at -20°C with 0.3 M sodium acetate and 2.5 volumes of ethanol after the addition of 20 µg of glycogen as carrier. Tubes were centrifuged at 16,000 x*g* for 15 min at 4°C, the supernatant aspirated and the pellet washed once with 300 µl of 70% ethanol, re-centrifuged and dissolved in 12 µl of TE buffer or sterile distilled water.

Alternatively, fragments were trimmed to remove excess agarose and DNA purified by β-agarase (New England Biolabs, Beverly, MA) digestion of SeaPlaque™ GTG low-melting point agarose in 1.5 ml centrifuge tubes. Fragments were washed in β-agarase buffer (10 mM Bis Tris-HCl [pH 6.5], 1 mM EDTA) for 30 min and melted at 65°C for 15-20 min. Tubes were cooled to 40°C and incubated for 1.5 h with 2 u of β-agarase for each 200 µl of agarose. Then, the salt concentration was adjusted to 0.3 M sodium acetate and

tubes held at -20°C for at least 20 min. Undigested agarose was pelleted by centrifugation at 16,000 x*g* for 15 min at 4°C. DNA was precipitated at -20°C with 2.5 volumes of ethanol and 20 µg of glycogen as carrier, washed with 70% ethanol and dissolved in TE buffer as described above.

Most often, DNA fragments were eluted onto DEAE-membranes (NA45; Schleicher & Schuell, Keene, NH) by band interception. Membranes were equilibrated by a 10 min wash in 10 mM EDTA (pH 8.0), followed by a 5 min wash in 0.5 M NaOH and three sequential 5 min washes in sterile distilled water (membranes were stored up to two months in sterile distilled water at 4°C). DNA was electrophoresed in 0.8% SeaKem™ GTG (FMC) agarose gels. Gels were stained with ethidium bromide. Fragments were excised, equilibrated membranes inserted just ahead of the band of interest and fragments re-inserted. Gels were electrophoresed at high voltage (100-120 V) until the band of interest was run completely onto the membrane. Membranes were removed from the gel and washed briefly in low salt buffer (0.15 M NaCl, 0.1 mM EDTA, 20 mM Tris-HCl [pH 8.0]) to remove adhering agarose pieces. DNA was eluted from membranes by incubation in 300-600 µl elution buffer (1 M NaCl, 0.1 mM EDTA, 20 mM Tris-HCl [pH 8.0]) at 55°C for 30-60 min. Tubes were centrifuged briefly at 16,000 x*g* and supernatants transferred to a fresh tube. DNA was precipitated with 0.3 M sodium acetate, 20 µg glycogen and 2 volumes of ethanol as described above.

2.4.7 DNA sequencing by dideoxynucleotide chain termination

The Sequenase™ Version 2.0 DNA sequencing kit (USB, Cleveland OH) was used to sequence double-stranded DNA templates according to the manufacturer's protocols. Reactions were examined by electrophoresis through 6% polyacrylamide/urea gels on a Model S2 sequencing gel electrophoresis system (Life Technologies, Bethesda, MD), according to the manufacturer's directions. Double-stranded DNA templates were purified by CsCl density gradient centrifugation as described above. Seven sequencing primers were designed to allow complete sequencing of both strands of 638 nt *arg-2–hph Eco*RI fragments derived from *N. crassa* strains containing the *arg-2–hph* reporter gene by PCR amplification and subcloning into pBS SKII+. Primers ARG2-Eco and HPH-Eco were described in section 2.5.4. Primers CC-1 (5'-ACT TTC GGA CTC TTT) and CC-2 (5'-CAG CAG CCG TAC CAA) are identical to nucleotides 805-819 and 1403-1417 of the *arg-2* sequence, respectively. Primer CC-6 (5'-GGA GAT CTT GAC TTG AAT G) is complementary to nucleotides 1438-1456 of the *arg-2* sequence, with the exception of nucleotides 1453 and 1455 which were both altered to G to create a *Bgl*II site. Primers

CC-3 (5'-AGA GGT GAA GAC TGA) and CC-4 (5'-TGA GTT GTA GTG TCG) were complementary to nucleotides 885-899 and 731-745 of the *arg-2* sequence, respectively.

2.4.8 Preparation of uniformly ^{32}P-labeled DNA

Probes for Southern and northern analyses were labeled by the random priming method (Feinberg and Vogelstein 1983). Appropriately digested and gel-purified DNA fragments (100 ng in 6 µl) were denatured by boiling for 3 min and cooled on ice for 3 min. The denatured DNA was diluted with 13 µl of LS (25 parts 1 M HEPES [pH 6.6], 25 parts DTM [100 µM dATP, 100 µM dGTP, 100 µM dTTP], 7 parts OL [1 mM Tris-HCl [pH 7.5], 1 mM EDTA, 90 O.D. units/ml hexamers], 5 parts 10 mg/ml BSA). Then, 5 µl of α-^{32}P-dCTP (50 µCi; 3000 Ci/mmol and 1 u of Klenow enzyme were added. Reactions were allowed to proceed at room temperature for 3 hr. Probes were recovered by centrifugation through Sephadex G-25 spin columns (Boehringer, Indianapolis, IN) into 150 µl of TE; 2 µl samples were counted (Cerenkov) in a Beckman LS6500 scintillation counter.

2.4.9 Southern analyses of genomic *N. crassa* DNA

For Southern analysis, genomic DNA from *N. crassa* strains was digested to completion with appropriate restriction endonucleases (usually 5 u of enzyme/µg of DNA) by incubation at the appropriate temperature for 5 h or overnight. Samples (1-5 µg) were mixed with loading buffer (6X = 0.25% xylene cyanol, 0.25% bromophenol blue, 15% Ficoll 400,000 in sterile distilled water), loaded into the wells of 0.8% agarose gels and electrophoresed at 60–80 V in TAE buffer until the bromophenol blue indicator was 1-2 cm from the bottom of the gel. Gels were stained for 20-40 min in 200 ml of water containing 0.25 mg/ml EtBr.

Gels were rinsed in deionized water and photographed. The positions of molecular weight standards (phage λ digested with *Hind*III) were marked by making plug-holes next to bands with a Pasteur pipette. Gels were depurinated in 0.25 M HCl for 15 min, rinsed with deionized water, soaked for 1 hr in 0.5 M NaOH, 1.5 M NaCl and 0.004% thymol blue, rinsed and transferred into neutralization solution (0.5% Tris-HCl [pH 7], 1.5 M NaCl) for 1 hr or until the blue indicator turned yellow.

Nucleic acid transfer was accomplished by capillary action. Three large sponges were placed in a glass baking dish containing 10x SSPE (20x SSPE = 174 g NaCl, 27.6g NaH_2PO_4, 7.4 g Na_2EDTA adjusted to pH 7.4 per 1 L). Filter paper (#1514A; Micro Filtration Systems; Fisher Scientific, Pittsburgh, PA) was soaked in 10x SSPE and placed on top of the sponges (2 sheets/ gel). Gels were placed upside-down on the filter paper

without trapping air bubbles. Plastic wrap was placed along all four edges of the gel to prohibit transfer of buffer outside of the gel area. Nylon membranes (Magna NT [0.45 μm]; Micron Separations Inc., Westboro, MA) were appropriately marked, pre-wet in distilled water and placed on top of the gels. Two sheets of filter paper were placed on the membranes, again without trapping air bubbles, followed by stacks of paper towels. Stacks were compressed by placing a large textbook on top. Transfers were usually accomplished overnight. The assembly was dismantled carefully, keeping the gel in contact with the membrane. Standards were marked on the membrane by poking holes with a dissecting needle. The membranes were separated from the gel, rinsed in 5x SSPE, and nucleic acids were cross-linked to the membranes using a UV Stratalinker 1800 (Stratagene, LaJolla, CA).

Membranes were placed in plastic bags containing fresh prehybridization solution (50 ml prepared in a 50 ml screwcap tube: 25 ml formamide, 10 ml of 5x P [1% BSA, 1% polyvinylpyrollidone, 1% Ficoll 400,000, 250 mM Tris-HCl [pH 7.5], 0.5% sodium pyrophosphate, 5% SDS], 2.92 g NaCl, 0.5 ml of 10 mg/ml denatured and sheared salmon sperm DNA) and prehybridized with gentle shaking for a minimum of 5 hr.

The following fragments, derived from digested and gel-purified recombinant plasmid DNA, were used to probe blots: the *arg-2* probe was the 1.4 kb *Pvu*II-*Nco*I fragment from pAR1(Luo, Freitag and Sachs 1995); the *cox-5* probe was the 0.8 kb *Eco*RI fragment from pSRCOX5 (Sachs *et al.* 1989); the *cpc-1* probe was the 1.2 kb *Bgl*II-*Bam*HI fragment from pCPC-1-2 (Paluh *et al.* 1988); the *his-3* probe was the 1.7 kb internal *Xho*I fragment from pDE1 (Ebbole 1990). The *hph* probe was the 1 kb *Bam*HI fragment from pGV4.

Radioactive probe (10^7 cpm α-^{32}P/bag) was injected into plastic bags with either a needle and syringe or a micropipettor. Following 12-18 hr of hybridization, filters were washed twice for 10 min and then twice for 15-30 min with 100-250 ml of 0.1x SSPE and1% SDS (lauryl sulfate, Sigma grade; Sigma, St. Louis, MO) at 67°C. Membranes were air-dried, wrapped in plastic wrap and exposed to Kodak XAR-5 film.

2.5 Transformation procedures

2.5.1 *E. coli* transformation

Competent *E. coli* XL1-Blue or DH5-α cells were prepared and transformed by a modified $CaCl_2$ procedure (Chung and Miller 1988). Single colonies of *E. coli* grown on LB medium containing plates were inoculated into 5ml of liquid LB medium and grown

overnight at 37°C with orbital shaking (200 rpm). One ml of these starter cultures was inoculated into 500 ml of LB medium in 2.5 L Fernbach flasks and grown at 37°C with orbital shaking (200 rpm) to an OD_{600} of 0.4 to 0.6 (ca. 4 h). Cells were centrifuged for 5 min at 1,500 x*g* at 4°C in a GSA rotor in a Sorvall centrifuge, the supernatant carefully poured off and cells gently resuspended on ice in 1/20 of the original volume in LB medium with 10% PEG 3350, 5% DMSO, 10 mM $MgCl_2$ and 10 mM $MgSO_4$. Cells were quick-frozen in 1 ml aliquots in liquid nitrogen for later use or used directly. Cells retained competence (1 x 10^6 colonies per µg of plasmid DNA) for up to one year when stored at -80°C. For transformation, competent cells were thawed on ice and 100 µl of cells added to plasmid DNA in 100 µl KCM buffer (100 mM KCl, 30 mM $CaCl_2$, 50 mM $MgCl_2$), mixed by gentle inversion and incubated on ice for 30 min. Cells were diluted with 1 ml LB medium, incubated for 1 hr at 37°C with orbital shaking (200 rpm) and plated onto LBA medium (LB medium with 100 µg/ml ampicillin and 2% agar). Plates were incubated for at least 16 h at 37°C.

Competent *E. coli* DH5-α cells were also prepared and transformed by electroporation with an Electro Cell Manipulator®600 (BTX Inc., San Diego, CA). To generate competent cells, single bacterial colonies and starter cultures were grown as described above. One ml of starter cultures was inoculated into 500 ml of low salt LB medium (10 g bacto tryptone, 5 g bacto yeast extract, 5 g NaCl per 1L) in 2.5 L Fernbach flasks and grown at 37°C with orbital shaking (200 rpm) to an OD_{600} of 0.5 to 1.0. Cells were chilled on ice and pelleted as above. Cells were washed and pelleted (Sorvall GSA rotor at 4,000 x*g*, 4°C, 15 min) three times in 250 ml sterile distilled water, followed by one wash in 20 ml sterile distilled water. Cells were centrifuged as above and resuspended in 2 to 3 ml of 10% glycerol (filter-sterilized in distilled water) quick-frozen, and stored at -80°C. Cells retained competence (1 x 10^7 colonies per µg of plasmid DNA) for up to two years under these conditions.

Transformation by electroporation was performed at 2.45 kV, 129 ohm, at a field strength of 12.25 kV/cm for 5 msec in a BTX Disposable Cuvette P/N620 (2 mm gap). The sample volume was 40 µl of competent cells mixed with 1 µl of plasmid DNA (1 ng) in ligation mix diluted with sterile distilled water (1:5) and cooled to 0°C. Cells were electroporated, immediately diluted with 1 ml of LB medium and incubated at 37°C for 1 hr with orbital shaking (200 rpm). Cells were plated and selected on LBA medium as described above.

2.5.2 *N. crassa* transformation

Competent *N. crassa* cells were prepared and transformed based on a previously described procedure (Selitrennikoff and Sachs 1991). Strains were grown in 50 ml Min (to generate spheroplasts of wild type, *arg-12^s* or *arg-12^s pyr-3* strains) or His (to generate spheroplasts of the *his-3* auxotrophic strain) in 250 ml Erlenmeyer flasks at 34°C for 4-7 days. Conidia were harvested in a fume hood by adding distilled sterile water to each flask, shaking and filtering conidia through cheesecloth into 50 ml sterile centrifuge tubes. Tubes were stored at 4°C for at least 2 h to activate conidia evenly. Then, tubes were centrifuged at 3,000 x*g* for 3 min, the supernatant was decanted and conidia were washed in distilled sterile water. Conidia (1×10^7 to $2 \times 10^{9)}$ were incubated in 150 ml Min for 3-6 h at 34°C with orbital shaking (200 rpm). After 3 h conidia were examined for the appearance of germ tubes; at least 70 % of conidia were germinated with germ tubes 1- to 4-fold the length of conidia. Conidia were then harvested and centrifuged at 3,000 x*g* for 5 min, washed twice with water and once in 1 M sorbitol (15 ml for each 10^9 conidia). Conidia were resuspended in 1 M sorbitol. For every 10^9 conidia 2.5 ml of 5 mg/ml NovoZym 234 (NovoIndustri A/S, Bagsvaerd, DK) were added. Conidia were incubated at 34°C for 60-70 min with orbital shaking (100 rpm; extended exposure to Novozym 234 decreases the viability of spheroplasts). Spheroplasts were gently decanted into 50 ml centrifuge tubes and centrifuged at 1,000 x*g* for 5 min. Spheroplasts were resuspended in 1 M sorbitol, mixed gently by inversion and recentrifuged. In this manner, spheroplasts were washed twice with 1 M sorbitol and once with sterile sorbitol-$CaCl_2$-Tris (1M sorbitol, 20 mM Tris-HCl [pH 8.0], 20 mM $CaCl_2$; pH 8.0). The final pellet was resuspended in 1M sorbitol-$CaCl_2$-Tris:PEG-Tris-$CaCl_2$ (40% PEG 4000 [Sigma, St. Louis, MO], 1 M Tris-HCl pH 8.0, 1 M $CaCl_2$; pH 8.0):DMSO added, in a ratio of 8:2:0.1 for each 10^9 conidia. Spheroplasts were either used immediately for transformations or aliquots were stored at -80°C (without quick-freezing).

For transformation, 2-5 µg of plasmid DNA was suspended in ice-cooled polystyrene Falcon tubes (#2058) with 25 µl of heparin-solution (5 mg heparin/ml in 1M STC buffer [1 M sorbitol, 50 mM Tris-HCl pH 8.0, and 50 mM $CaCl_2$ (adjusted to pH 8.0)] and 100 µl of slowly-thawed competent cells (ca. 10^7 spheroplasts). This mixture was incubated on ice for 30 min and 1 µl (3.5 u) lipofectin (Life Technologies, Bethesda, MD) added. The tubes were incubated at room temperature for an additional 15 min, 875 µl of PTC (40% PEG 4000 [Sigma, St. Louis, MO], 50 mM Tris-HCl [pH 8.0] and 1 M $CaCl_2$ [adjusted to pH 8.0]) added, and mixed by pipetting gently up and down. Samples were incubated at room temperature for 20 min. Transformed cells (250 µl) were transferred into 10 ml of top (regeneration) agar (FGS-Min with 1 M sorbitol), and inverted

gently before being poured onto plates containing 15-20 ml of solidified bottom agar (FGS-Min with 1.5 % agar). Transformants were selected either for prototrophy of auxotrophic *his-3* mutants which were transformed with plasmids containing truncated *his-3* genes and grown on FGS-Min bottom agar, or for Hyg^r of wild type, *arg-12^s* or *arg-12^s pyr-3* strains which were transformed with various plasmids containing the *hph* gene and grown on Hyg (0.2 mg/ml). Hyg was added to the bottom agar before autoclaving. Transformation efficiencies of newly prepared competent cells were tested by transformation with plasmid pGV4. Plates were incubated for 2-5 days at 34°C.

Plasmids pGS3, pGT6, pGU and pGV4 were ectopically integrated into the genome of *N. crassa arg-12^s* or *arg-12^s pyr-3* strains; transformants were selected on FGS-Hyg (0.2 mg/ml). Plasmid tgCMV/HyTK was ectopically integrated into the genome of the wild type 74*A* strain; transformants were selected on FGS-Hyg (0.2 mg/ml). Plasmids pMF1, pMF2, pMF11-wt, pMF11-D12N and pMF11-37 were targeted to the *N. crassa his-3* locus of the *his-3* (FGSC #6103) strain for analyses of PCR-amplified wild type and mutated *arg-2–hph* reporter genes; transformants were selected on FGS-Min or FGS-Hyg (0.2 mg/ml).

2.6 Methods for RNA

2.6.1 Small scale preparation of *N. crassa* total RNA

Small scale RNA preparations were obtained as described previously (Sachs and Yanofsky 1991). Buffers (except for solutions containing Tris) and water used for RNA analyses were treated overnight with 0.1% diethylpyrocarbonate (DEPC) and autoclaved. *N. crassa* mycelium (50-100 mg frozen in liquid nitrogen and stored at -80°C) was added to 2 ml screw-cap centrifuge tubes containing 1 g acid-washed glass beads (0.5 mm), 580 µl of extraction buffer (100 mM Tris-HCl [pH 7.5], 100 mM LiCl and 20 mM dithiothreitol), 420 µl phenol, 420 µl chloroform and 84 µl of 10% SDS. The tubes were capped and nucleic acids were immediately extracted by homogenizing with a Mini-Beadbeater (Biospec, Bartlesville, OK) for 50 sec. The homogenate was mixed for at least 5 min in an end-over-end rotator, and tubes were centrifuged at 16,000 x*g* for 30 sec. The aqueous phase was transferred to a fresh tube and extracted once with 0.8 ml of phenol:chloroform (1:1) in the Mini-Beadbeater for 30 sec. The phases were separated by centrifuging at 16,000 x*g* for 5 min. The aqueous phase was transferred to a fresh tube and extracted once with 0.8 ml chloroform by vortexing. Phases were separated by centrifugation at 16,000 x*g* for 5 min and the aqueous phase transferred to a fresh tube.

Nucleic acids were precipitated with 0.3 M sodium acetate and 2.5 volumes of ethanol, and dissolved in 100 µl sterile DEPC-treated water. Following an additional precipitation step and an 80% ethanol wash, pellets were dried in a SpeedVac centrifuge (Savant, Farmingdale, NY) and redissolved in 100 µl sterile DEPC-treated water. The amount and quality of RNA was assessed by measuring the absorbance at 260 and 280 nm (A_{260} = 1 was assumed to equal 40 µg of total RNA; the $A_{260/280}$ ratio was typically between 1.7 and 1.9). Aliquots of RNA were quick-frozen in dry ice and stored at -80°C.

2.6.2 Preparation of *N. crassa* polysomal RNA

The procedures for polysome preparation and analyses were adapted from previously described procedures (Cigan *et al.* 1991; Sachs and Davis 1989). *N. crassa* cultures were grown in Min or Arg (for wild type cultures), or in Uri or Uri+Arg (for *arg-12s pyr-3* strains). Cycloheximide (100 µg/ml) was added to cultures 5 min prior to harvesting to keep polyribosomes associated with transcripts. Mycelia (ca. 0.5 g wet weight) were harvested by vacuum filtration onto Whatman #541 filters, transferred to 2 ml screw cap centrifuge 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 magnesium acetate, 15 mM 2-mercaptoethanol, 100 µg/ml cycloheximide) and disrupted for 50 sec in a Mini-Beadbeater at 4°C. Homogenates were centrifuged at 16,000 x*g* at 4°C for 5 min. Supernatants (0.9 ml) were transferred to fresh 2 ml screw cap centrifuge 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; in later analyses heparin was omitted.

For polysome analyses, 10 to 20 A_{260} units of homogenate, in a maximum volume of 400 µl, were layered on 12 ml linear sucrose gradients (15-50% w:w sucrose in gradient buffer [10 mM HEPES-KOH [pH 7.5], 70 mM ammonium acetate; 4 mM magnesium acetate]) in Beckman #331372 12 ml centrifuge tubes. Gradients were centrifuged in a Beckman SW41 rotor at 41,000 rpm (280,000 x*g*) for 2 h at 4°C. Gradients were prepared as step gradients by adding, consecutively, 2.3 ml of 50, 42, 33, 24 and 15% sucrose (w:w) in gradient buffer (10 mM Hepes-KOH [pH 7.5], 70 mM NH_4OAc, 4 mM MgOAc) to centrifuge tubes. Layers were quick-frozen in liquid nitrogen before the next layer was added. Gradients were stored in sealed bags at -80°C. Before use, step gradients were thawed overnight (or for at least 6 h) at 4°C to let a linear gradient form.

In early experiments, twelve 1 ml fractions were collected from the bottom with a Hoefer gradient tube fractionator into 2 ml screw cap centrifuge tubes containing 50 µl of 10% SDS. Polysome profiles were generated by following the A_{254} with an ISCO UA5

absorbance monitor. Fractions were frozen and stored at -80°C until further processing. Polysomal RNA was extracted by mixing each fraction with 1 ml of phenol:chloroform (1:1) in the 2 ml tubes and shaking for 1 min in the Mini-Beadbeater. Phases were separated by centrifugation for 10 min at 16,000 x*g* in a microcentrifuge. The aqueous phase was transferred to a fresh 2 ml tube, vortexed with 1 ml of chloroform and centrifuged as before. The aqueous phase was again transferred to a fresh tube and RNA precipitated with sodium acetate (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 sodium acetate, 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.

In later experiments, fractions were collected as before. Then, 1 ml of isopropanol was added and fractions stored at -80°C until further processing. To extract polysomal RNA, the precipitate in fractions was collected by centrifugation at 16,000 x*g* at 4°C for 15 min and the supernatant removed by aspiration. Pellets were resuspended in 300 µl lysis buffer (20 mM Tris-HCl [pH 7.4], 100 mM NaCl, 2.5 mM EDTA, 1% SDS in DEPC-treated water; (Cigan *et al.* 1991)) and extracted in 300 µl of phenol:chloroform (1:1) for 1 min in a Mini-Beadbeater. Phases were separated by centrifugation for 10 min at 16,000 x*g*. The aqueous phase was transferred to a fresh tube containing 300 µl of chloroform; mixing and centrifugation were repeated. The aqueous phase was transferred to a fresh tube and RNA precipitated with sodium acetate (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 sodium acetate, washed with 70% ethanol, resuspended in 42 µl of sterile DEPC-treated water, quick-frozen in liquid nitrogen and stored at -80°C until northern blot analyses.

2.6.3 Northern analyses

For northern analyses, 15 µl (5-6 µg) of total RNA or equal volumes (15 µl) of polysomal RNA from wild type, transformed or mutant *N. crassa* strains was denatured in 38 µl sample denaturation mix (646 µl formaldehyde, 226 µl formamide, 130 µl 10X E buffer per 1 ml), mixed with 15 µl RNA loading buffer (50% glycerol, 0.3% xylene cyanol, 0.3% bromophenol blue, 1 mM EDTA), loaded into the wells of 1.5% denaturing agarose/formaldehyde gels, and electrophoresed in 1X E buffer (20 mM MOPS, 5 mM sodium acetate, 0.5 mM EDTA [pH 8.0]) in a fume hood at 80V until the bromophenol indicator was located 1-2 cm from the bottom of the gel. RNA gels were either stained with EtBr for 5 min and subsequently destained in deionized water for several hours, or

directly blotted to nylon membranes (section 2.4.9). If gels had been stained with EtBr, they were photographed and RNA ladder standards (Life Technologies, Bethesda, MD) and the location of large and small rRNA were marked by making plug-holes next to the bands with a Pasteur pipette prior to transfer to membranes. Nucleic acid transfer was accomplished by capillary action as described for Southern analyses (section 2.4.9). The blotting assembly was dismantled carefully while keeping the gel in contact with the membrane. Standards and the location of large and small rRNA were marked on the membranes with a dissecting needle. The membranes were separated from the gel and RNA cross-linked to the membranes using a UV Stratalinker 1800 (Stratagene, LaJolla, CA). If gels had not been stained with EtBr, membranes were stained with methylene blue (Sambrook, Fritsch and Maniatis 1989). A record of the stained membranes was made by either photocopying or scanning. Membranes for northern analyses were prehybridized and hybridized as described for Southern analyses, except that prehybridization solution was changed once after 1 h if membranes had been stained with methylene blue. Radioactive probes for northern blot analyses were the same as for Southern analyses. Membranes were wrapped in plastic wrap and either exposed to Kodak XAR-5 film or phosphorimager storage plates.

2.6.4 Quantitation of mRNA levels

The relative levels of mRNA detected by RNA blotting were determined by direct analysis using a phosphorimager system (PhosphorImager SI; Molecular Dynamics, Sunnyvale, CA) running IPLab Gel software (Version 1.5; Signal Analytics Corporation, Vienna, VA); autoradiographs from polysome analyses were also analyzed with a Bio-Rad Model 620 Video Densitometer. The values reported for polysome analyses represent averages for two independent growth experiments, each examined using duplicate samples. Only fractions from similarly shaped polysome profiles were analyzed. Quantitative methods were validated through reconstruction experiments using a dilution-series of comparable samples.

2.7 Methods for proteins

2.7.1 General methods

Whole cell extracts of *N. crassa* were prepared as described (Luo, Freitag and Sachs 1995; Sachs and Ebbole 1990). Freshly harvested mycelia (0.5-1 g wet weight) were added to ice-cold acid-washed glass beads in 2 ml screw cap centrifuge tubes which

were then filled completely with breaking buffer (20 mM Hepes-NaOH [pH 7.9], 100 mM KCl, 2 mM EDTA, 10 mM DTT, 20% glycerol). Mycelia were broken at 4°C by two 1 min treatments in a Mini-Beadbeater (Biospec, Bartlesville OK); treatments were interrupted by a 2 min rest on ice. Extracts were clarified by centrifugation at 16,000 x*g* at 4°C for 10 min. Clarified whole cell extracts were transferred to a fresh tube, quick frozen in liquid nitrogen and stored at -80°C. Total protein concentrations were determined by Bradford assay (Bradford 1976), with BSA as the standard. Enzyme activity levels remained stable for at least one year under these conditions.

2.7.2 Enzyme activity assays

2.7.2.1 Hygromycin B phosphotransferase (Hph)

The Hph activity assay from *N. crassa* cell extracts (Freitag and Sachs 1995) was adapted from a previously published protocol (Sørensen *et al.* 1992). Serial dilutions of 5, 2.5, 1.25 and 0.63 µg of total protein from whole cell extracts in a 10 µl total volume were added to wells of non-sterile 96-well microtiter dishes. The reactions were started by the addition of 50 µl of reaction buffer (13.4 mM Tris-maleate [pH 7.1], 8.4 mM $MgCl_2$, 80 mM NH_4Cl, 60 µM hygromycin B, 15 µM ATP and 25 µCi/ml of [γ^{32}P]-ATP) and incubated at room temperature for 1 hr. Filter paper (#1514A; Micro Filtration Systems; Fisher Scientific, Pittsburgh, PA), phosphocellulose paper (P81 cation exchanger; Whatman, Hillsboro, OR) and nitrocellulose membrane (Nitrobind [0.45 µm]; MSI, Westboro, MA) were placed in this order onto a Bio-Dot filtration apparatus (Bio-Rad Laboratories, Hercules, CA). Completed Hph reactions were mixed with 150 µl water and directly loaded into wells. Samples were filtered by application of a light vacuum and the wells were washed three times with 250 µl water. Both nitrocellulose membrane and phosphocellulose paper were washed in three changes of water for 15 min at 65°C, air-dried and exposed to either Kodak XAR-5 film (12 h to 3 days with screen at -80°C) or phosphorimager storage plates. Amounts of radioactivity in dots on the phosphocellulose paper were determined with a phosphorimager system (PhosphorImager SI; Molecular Dynamics, Sunnyvale, CA) running IPLab Gel software (Version 1.5; Signal Analytics Corporation, Vienna, VA). Values reported are averages from at least two independent growth experiments, assayed in duplicate or triplicate.

2.7.2.2 Arginine-specific carbamoyl phosphate synthetase

Arginine-specific carbamoyl phosphate synthetase was assayed in preparations of purified *N. crassa* mitochondria (Davis, Ristow and Hanson 1980). Cultures were grown in 700 ml of minimal or arginine medium in 2 L Erlenmeyer flasks for 24 h at 34°C with

gentle agitation (125 rpm), harvested onto cheesecloth and washed with extraction buffer (10 mM TES [pH 7.5], 0.33 M sucrose, 0.3 % BSA, 1 mM EDTA). To isolate mitochondria, cells (7-12 g wet weight) were suspended in extraction buffer and 50-80 g of acid washed glass beads (0.5 mm) in a 250 ml beadbeater cup; the cup was completely filled with extraction buffer. Cells were broken with three successive 30 sec bursts followed by 30-60 sec rests on ice. The homogenate was filtered through cheesecloth and centrifuged for 6 min at 600 x*g* in a GSA rotor (Sorvall). The supernatant was transferred to a fresh bottle and centrifuged for 20 min at 15,000 x*g* in a GSA rotor. The pellet was resuspended in 10 ml extraction buffer containing 1 mM PMSF, layered onto 20 ml sucrose step gradients (1.6 M and 1.2 M sucrose in extraction buffer) and centrifuged for 20 min at 45,000 x*g* in an SS34 rotor (Sorvall). Pure mitochondria from the interphase between the gradient steps were resuspended in 1 ml extraction buffer containing 1 mM PMSF and frozen at -80°C until further analysis.

Each enzyme preparation was assayed for *arg-2*-dependent (glutamine-dependent) and *arg-3*-dependent (ammonium-dependent) activities. Purified mitochondria (350 µl) were suspended in 1.35 ml extraction buffer and 1 g of 0.5 mm acid-washed glass beads and broken in 2 ml screw cap tubes with a Mini Beadbeater for 60 sec. Samples were centrifuged at 13,000 x*g* for 10 min and the membrane-free mitochondrial extract transferred to a fresh tube. Mitochondrial extracts (100 µl) were mixed with 400 µl reaction buffer (100 mM Tris-HCl [pH 8.5], 12 mM $MgCl_2$, 12 mM ATP, 20 mM $NaH^{14}CO_3/KHCO_3$, 12 mM L-glutamine or 120 mM NH_4Cl or water) and incubated at 25°C for 20 min. The reactions were stopped by adding 200 µl of 1.5 M NH_4Cl and boiled for 10 min in a fume hood. This step converts [^{14}C]-carbamoyl phosphate to [^{14}C]-urea. Small amounts of residual $^{14}CO2$ were removed by adding 100 µl of 1 M HCl. Samples were cooled to room temperature, centrifuged and passed through Dowex-1X8 columns (in the OH^- form) into scintillation vials containing an equal volume of ScintiVerse scintillation fluor (Fisher Scientific, Pittsburgh, PA). The yields of reaction products were determined by scintillation counting (Beckmann LS6500 scintillation counter). One relative unit of activity was defined as 1000 cpm per mg of mitochondrial protein.

2.7.2.3 Fumarase

Fumarase was assayed as previously described (Tolbert 1974). Briefly, 300 µl of purified mitochondria were added to 670 µl of reaction buffer (100 mM TES [pH 9.5], 100 mM $(NH_4)_2SO_4$; 30 µl 0.5 % Triton X-100), the reaction initiated by addition of 30 µl of 10 mM potassium fumarate and the decrease in A_{240} monitored. Activity is defined as the change in absorbance per minute per mg of protein.

2.7.3 Protein electrophoresis and immunoblotting

Total protein (5-100 µg) for each sample was adjusted to equal volumes with SDS sample buffer (50 mM Tris-HCl [pH 6.8], 0.05% bromophenol blue, 1% SDS, 10% glycerol, 2% ß-mercaptoethanol), boiled, and examined by SDS-PAGE in 10% or 12.5% polyacrylamide gels (Sambrook, Fritsch and Maniatis 1989) in a Mini PROTEAN II Dual Slab Gel system (Bio-Rad, Hercules, CA).

Polypeptides were transferred to PolyScreen™ membranes (DuPont, Wilmington, DE) by electroblotting in a Mini PROTEAN Trans Blotting apparatus (Bio-Rad) according to the manufacturer's protocol. Antigenically reactive polypeptides were visualized by the alkaline phosphatase reaction (Sambrook, Fritsch and Maniatis 1989). Membranes were incubated for 15 min in PBST (0.83 mM NaH_2PO_4, 8.1 mM Na_2HPO_4, 145 mM NaCl and 0.05% Tween 20) with 5% non-fat dried milk. Anti-Arg2p serum was added (1:1,000 - 1:10,000 v:v) and membranes were incubated with serum for 1 h. The fusion polypeptide to obtain anti-Arg2p antiserum was prepared and characterized by D.T. Mooney in this laboratory; (Luo, Freitag and Sachs 1995). Membranes were washed three times (2 min each) in PBST, and incubated for 1 h in PBST with 5% non-fat dried milk containing anti-rabbit IgG alkaline phosphatase conjugate (1:5000 - 1:13,000 v:v; Promega., Madison, WI). Membranes were again washed three times in PBST and then washed for 5 min in alkaline phosphatase buffer (100 mM Tris-HCl [pH 9.5], 100 mM NaCl, 5 mM $MgCl_2$). Alkaline phosphatase reaction mix was prepared immediately before use by adding 66 µl of 50 mg/ml nitro blue tetrazolium chloride (NBT) and 33 µl of 50 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP) to 9.9 ml of alkaline phosphatase buffer. The membranes were immersed in the reaction mix and incubated without shaking. Reactions usually took less than 10 min to result in visible bands. The membranes were rinsed in sterile distilled water and air-dried. Band intensities were quantified by scanning blots and analyses with the IPLab Gel software (Version 1.5; Signal Analytics Corporation, Vienna, VA).

Chapter 3

Results

3.1 Construction and characterization of *N. crassa* strains used for UV mutagenesis

3.1.1 Construction of *arg-2–hph* reporter gene plasmids

The selection for mutants which affected Arg-specific regulation required Arg-regulated expression of the *E. coli hph* reporter gene in *N. crassa.* Four plasmids (pGS3, pGT6, pGU1 and pGV4; constructed by M. Sachs) were used to transform *arg-12^s* and *arg-12^s pyr-3* strains (Fig. 3.1). In each plasmid, the *hph* gene was translationally fused at codon 10 of the *arg-2* reading frame. Sequences comprising the *arg-2* transcript's 5' and 3' ends were retained. The four plasmids differed from each other with respect to the presence of two *arg-2* introns and additional 5' genomic DNA. The elimination of the uORF introns (pGU1 and pGV4) removed two consensus response elements (5'-TGACTC-3') for the transcriptional activator protein Cpc1p (Ebbole *et al.* 1991; Paluh *et al.* 1988); similarly, elimination of the genomic *Bam*HI-*Sac*I fragment (pGT6 and pGV4) removed another two TGACTC elements. Thus, pGS3 contains four Cpc1p response elements, pGT6 and pGU1 contain two such elements and pGV4 lacks these elements.

3.1.2 Characterization of *N. crassa* strains for mutagenesis

Plasmids were introduced into the *arg-12^s* and *arg-12^s pyr-3* strains by DNA-mediated transformation. It was anticipated that some transformants would be unable to grow at some specific concentrations of Hyg+Arg (in experiments with *arg-12^s* strains) or Hyg+Uri+Arg (in experiments with *arg-12^s pyr-3* strains) because the *hph* gene was predicted to be under the control of the 5' regulatory sequences of the *arg-2* gene and addition of Arg was expected to reduce the expression of *hph.* Such transformants would

then be subjected to UV mutagenesis to select for mutants that would grow on Hyg+Arg (in experiments with *arg-12^s* strains) or Hyg+Uri+Arg (in experiments with *arg-12^s pyr-3* strains).

Isolates were obtained from plates containing Uri+Hyg (0.2 mg/ml) after 2 to 5 days of incubation at 34°C. Transformants that grew on Uri+Hyg (0.2 mg/ml) were further characterized. The growth of primary transformants on Min, Arg, Hyg (0.2 mg/ml) and Hyg (0.2 mg/ml) + Arg medium (*arg-12^s* transformants), or Min, Uri, Arg, Uri+Arg, Hyg, Hyg+Uri, Hyg+Arg and Hyg+Uri+Arg (*arg-12^s pyr-3* transformants) was examined. Homokaryons of Hyg resistant (Hyg^r) transformants were isolated and tested for growth on media containing various concentrations of Hyg and Arg; in experiments with *arg-12^s pyr-3* strains the amount of Uri was always 0.5 mg/ml.

arg-12^s pyr-3 transformants that contained either pGS3, pGT6 or pGU1 were always resistant to Hyg on plates that also contained Arg and Uri, at concentrations as high as 4 mg/ml for Hyg and 2 mg/ml for Arg. However, strains grew and conidiated faster at low Hyg (0.2-1 mg/ml) +Arg (0.5 mg/ml) concentrations. These strains only failed to grow when Uri was omitted from the Hyg+Uri+Arg media, as was expected due to the *arg-12^s pyr-3* double mutation. Presumably the ability to grow on Hyg+Uri+Arg was attributable to multiple copies of the reporter gene that had been integrated in the genomes of transformants and/or a high level of gene expression mediated by the additional 5' sequences or introns present in the reporter genes of these transformants. These transformants were not analyzed further.

arg-12^s pyr-3 transformants that contained pGV4 grew on Hyg+Uri, but not on Hyg+Uri+Arg when the Hyg concentration was 2 mg/ml and the Arg concentration was 0.5 mg/ml or higher. However, these strains could grow slowly on Hyg(0.2)+Uri+Arg. Of 15 *arg-12^s pyr-3 a* strains transformed with pGV4, two independent homokaryotic transformants, MF13-3 and MF13-4, contained single copies of the *arg-2–hph* fusion gene at a location distinct from the *arg-2* gene, as determined by Southern blot analyses (Fig. 3.2). Southern analyses showed that ^{32}P-labeled *arg-2* probe hybridized to the expected 3.8 kb *Sac*I fragment of wild type or *arg-12^s pyr-3* genomic DNA that represented the endogenous *arg-2* gene, whereas an additional 3.2 kb fragment, corresponding to the *arg-2–hph* gene, was detected in a *arg-12^s pyr-3 arg-2–hph* strain (Fig. 3.2.b). As anticipated, the ^{32}P-labeled *hph* probe failed to detect fragments in wild type or *arg-12^s pyr-3* genomic DNA, whereas the 3.2 kb fragment was detected by this probe in transformed *arg-12^s pyr-3 arg-2–hph* strains (Fig. 3.2.b). One of the single copy transformants, strain MF13-3, was chosen for UV mutagenesis.

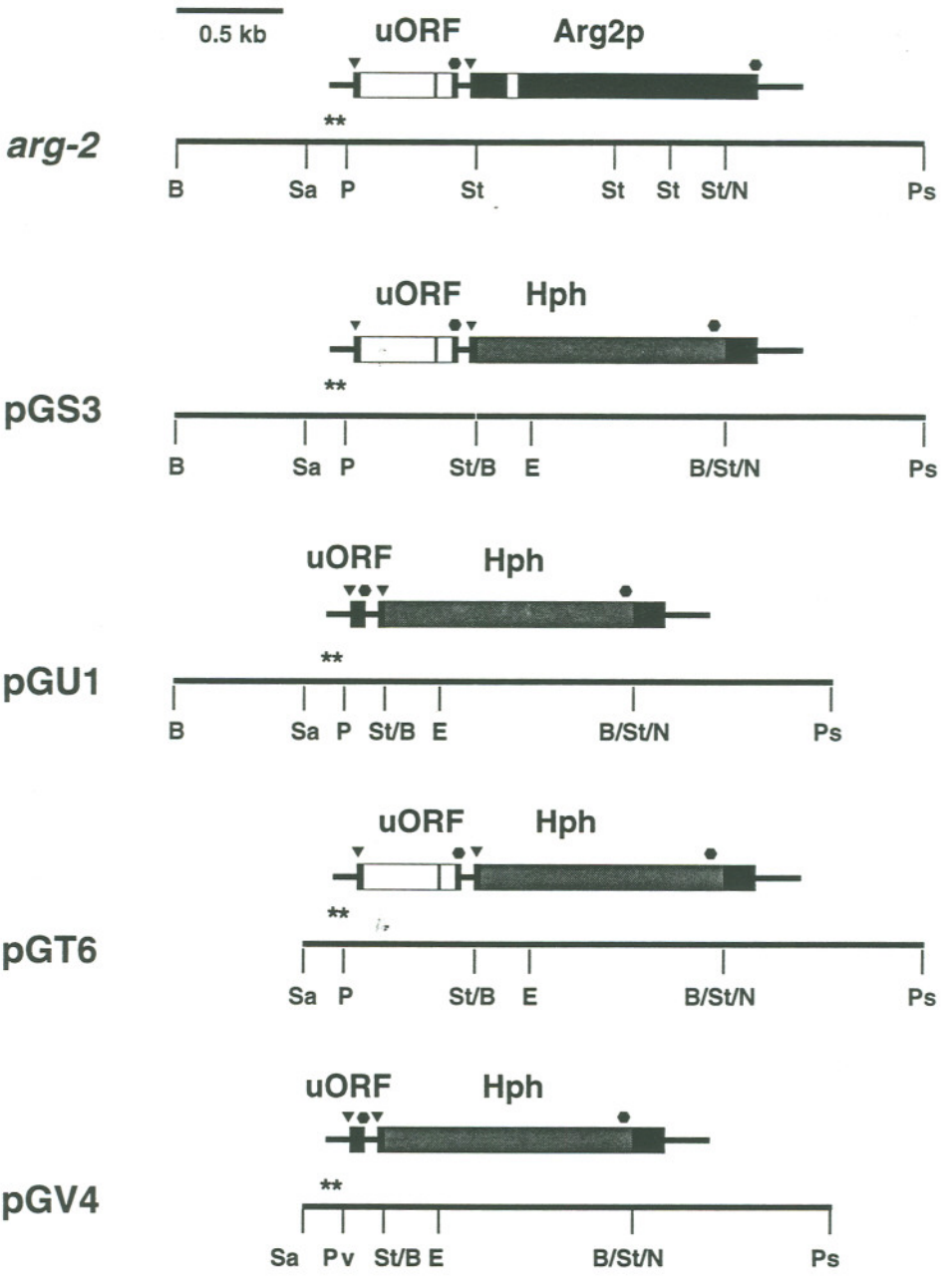

Figure 3.1: Structure of the *N. crassa arg-2* gene and structure of *arg-2–hph* reporter genes in plasmids pGS3, pGT6, pGU1 and pGV4. Plasmids contain translational fusions of 5' *arg-2* sequences to the *E. coli hph* gene at codon 10 of the *arg-2* reading frame. *arg-2* translated regions are shown as filled boxes and *arg-2* introns are indicated by open boxes. *hph* sequences are indicated by grey boxes. The transcriptional start sites are indicated by stars. Restriction enzyme recognition sites are: B = *Bam*HI; E = *Eco*RI; N = *Nco*I; P = *Pvu*II; Ps = *Pst*I; Sa = *Sac*I; St = *Sty*I. Plasmids were constructed as described in Materials and Methods.

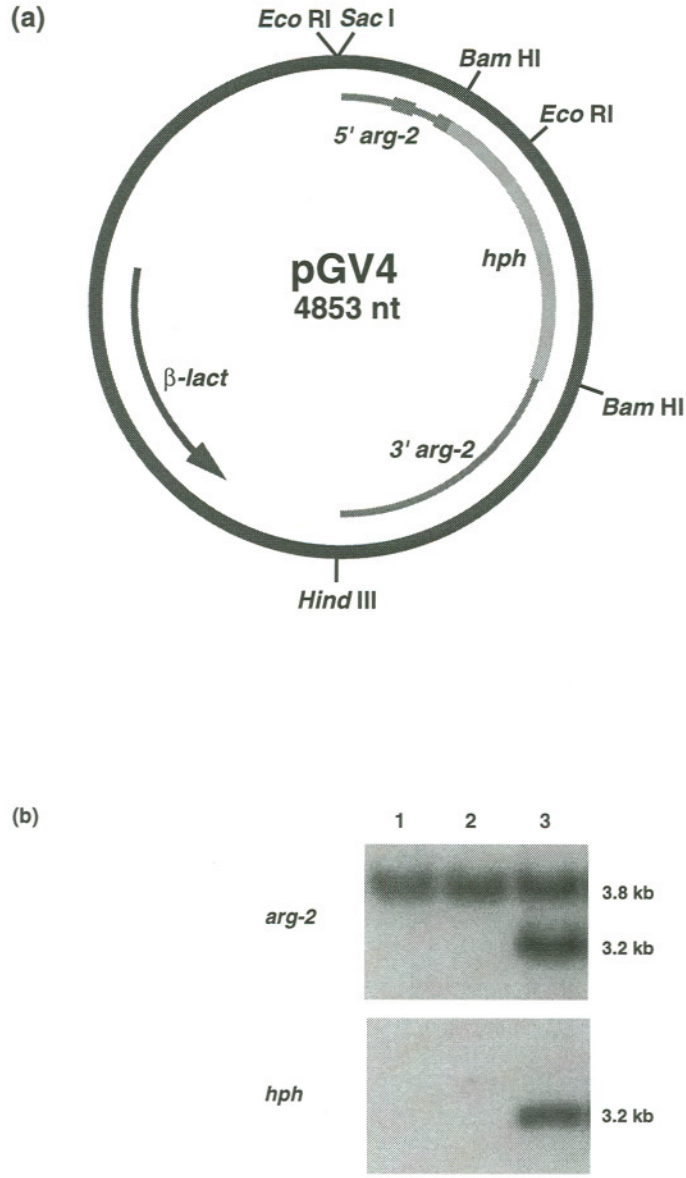

Figure 3.2: Structure of pGV4 and Southern analyses of transformant MF13-3. (a) Structure and partial restriction map of plasmid pGV4 which contains an intronless *arg-2-hph* reporter gene. *arg-2* sequences are indicated by thin dark grey lines; the 5' translated regions, including the uORF and the first 10 codons of the *arg-2* message are indicated by heavy dark grey lines. The translated region of *hph* is indicated by a heavy light grey line. (b) Southern analyses of wild type (1), *arg-12^s pyr-3* (2) and *arg-12^s pyr-3 arg-2–hph* (3) strain MF13-3 indicated that MF13-3 contains a single copy of the *arg-2-hph* gene. Genomic DNA was digested with *Sac*I and analyzed by Southern blotting as described in Materials and Methods. In all strains, ^{32}P-labeled *arg-2* probe detected a 3.8 kb fragment which corresponds to the endogenous *arg-2* gene. One additional 3.2 kb band was detected by this probe in MF13-3. The same band was detected by an *hph* probe in MF13-3, whereas no bands could be detected in the wild type or *arg-12^s pyr-3* strain.

In initial experiments, *arg-12^s* strains were transformed with pGS3, pGT6, pGU1 and pGV4. These transformants were grown on Min, Arg, Hyg and Hyg+Arg medium. Transformants containing pGS3, pGT6 or pGU1 were resistant to Hyg+Arg at all concentrations tested; these strains also contained multiple copies of the *arg-2–hph* gene, as determined by Southern blot analyses. *arg-12^s* transformants that contained pGV4 were able to grow on Hyg, but did not grow on Hyg+Arg when the Hyg concentration was higher than 1 mg/ml and the Arg concentration was at least 0.5 mg/ml. Three transformants were analyzed further by Southern blot analyses; all three strains contained multiple copies of the reporter gene. These strains were not analyzed further.

3.2 Isolation of UV-induced mutants

Conditions for the UV mutagenesis of *N. crassa* conidia in a Stratagene Stratalinker 1800 were optimized in initial experiments. A survival curve for the *arg-12^s pyr-3 arg-2-hph* strain was constructed (Fig. 3.3). The inhibitory effects of plating various amounts of killed conidia on the germination of viable conidia were examined in mixing experiments. As a result of these preliminary studies, conidia of the MF13-3 strain were subjected to two levels of UV irradiation, 700 or 900 J/m^2, which resulted in approximately 25% or 10% survival of conidia, respectively, and plates with selective Hyg+Uri+Arg were inoculated with 2 x 10^6 mutagenized conidia. After 2 to 5 days of incubation at 34°C, 47 independent colonies were picked from a total of four 100 mm Petri plates containing Hyg+Uri+Arg into 10 x 75 mm culture tubes containing the same medium. No Hyg^r colonies were obtained parallel experiments in which conidia were mock-irradiated and streaked onto Hyg+Uri+Arg plates.

Putative mutants were analyzed phenotypically on Min, Uri, Arg, Uri+Arg, Hyg+Uri, Hyg +Arg or Hyg+Uri+Arg. In these experiments, 33 strains were able to grow on Min, Uri, Uri+Arg, Hyg, Hyg+Uri and Hyg+Uri+Arg, but not on Arg or Hyg+Arg (class I); 13 strains were able to grow on all media (class II; some of these mutants grew slowly on Arg and Hyg+Arg medium); one strain failed to grow on Hyg in subsequent tests. Homokaryotic cultures of putative mutants were obtained by serial isolation of single asexual spores (Davis and deSerres 1970) or by isolation of microconidia (Ebbole and Sachs 1990). Twenty strains (7 of class I and 13 of class II) were selected for further analyses.

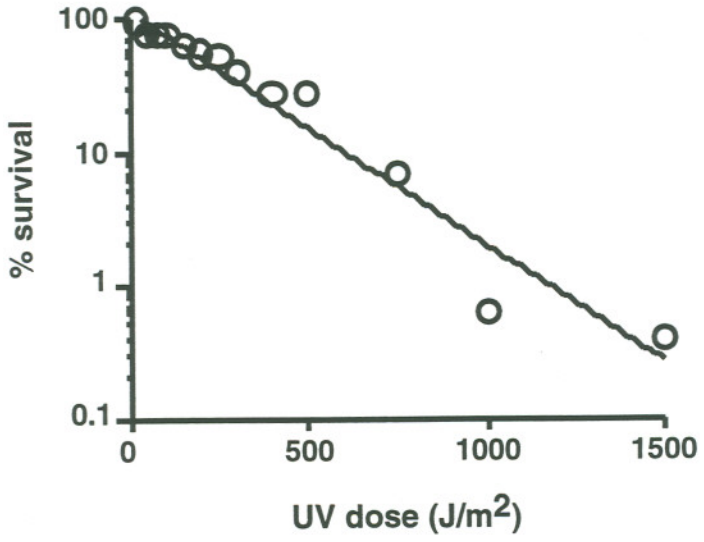

Figure 3.3: Effect of UV irradiation on the survival of conidia of the *arg-12^S pyr-3 arg-2–hph* strain MF13-3. Conidia ($1x10^8$) were irradiated with varying doses of UV light (254 nm) in a Stratalinker 1800 and dilutions of spores (500, 1000, 5000 per plate) streaked on 100 mm Petri plates containing minimal medium supplemented with 0.5 mg/ml uridine.

3.3 Phenotypic characterization of UV-induced mutants

Homokaryotic mutant and reference strains were grown on eight different media and mutants were sorted into two classes with respect to the new phenotypes they exhibited on Arg-containing media. The wild type strain was able to grow on all media lacking Hyg, but was sensitive to Hyg at concentrations as low as 0.15 mg/ml (Fig. 3.4, column 1). As expected, the *arg-12*^{S} *pyr-3* strain was able to grow on minimal medium and medium containing Uri or Uri+Arg, but unable to grow on medium containing Arg alone; as the wild type, this strain was sensitive to 0.15 mg/ml Hyg (Fig. 3.4, column 2). The *arg-12*^{S} *pyr-3 arg-2–hph* transformant MF13-3 grew on Min, Uri or Uri+Arg, but did not grow on Arg (Fig. 3.4, column 3), as expected. As observed during the initial characterization, this transformant grew on medium containing Hyg or Hyg+Uri, at Hyg concentrations as high as 4 mg/ml,but not on Hyg+Arg or Hyg+Uri+Arg, when the Hyg concentration was at least 1.5 mg/ml (Fig. 3.4, column 3).

Class I mutants, isolated after UV-mutagenesis of transformant MF13-3, grew on Min, Uri and Uri+Arg, but, like the unmutagenized parent MF13-3, not on Arg. These mutants also grew on medium containing Hyg, Hyg+Uri or Hyg+Uri+Arg (Fig. 3.4, column 4). Class II mutants grew on all media (Fig. 3.4, column 5). These data indicated that class I mutations primarily affected the expression of the *arg-2–hph* gene, whereas class II mutations affected the expression of the *arg-2–hph* gene and the *arg-2* gene.

3.4 Genetic characterization of UV-induced mutants

The genotypic basis for the phenotypes observed in 20 selected mutants was examined by comparing progeny from crosses of the 7 class I and 13 class II mutants to wild type to crosses of the unmutagenized parent MF13-3 to wild type. Ascospores from crosses of wild type to MF13-3 or wild type to mutants were germinated on Uri; in all crosses, 85-90% of the ascospores tested were viable on Uri.

Growth of progeny was scored on Min, Uri, Arg, Uri+Arg, Hyg, Hyg+Uri, Hyg+Arg and Hyg+Uri+Arg. In all crosses, *arg-2–hph* appeared to segregate as a single genetic locus in a Mendelian manner; in a total of 1059 progeny examined from the 21 Hyg^{r} x Hyg^{s} crosses, Hyg^{r}:Hyg^{s} progeny segregated 525:534. No linkage to *arg-12* (LG IIR), *pyr-3* (LG IVR) or mating type (LG IL) was observed (Table 3.1). Thus, *arg-2–hph* was also unlinked to *arg-2*, since *arg-2* and *pyr-3* are separated by only three map units on linkage group IVR (Perkins *et al.* 1982).

Segregation of mutant phenotypes of second generation backcrosses of the untransformed parent and selected mutants to wild type was scored on Hyg+Uri and on

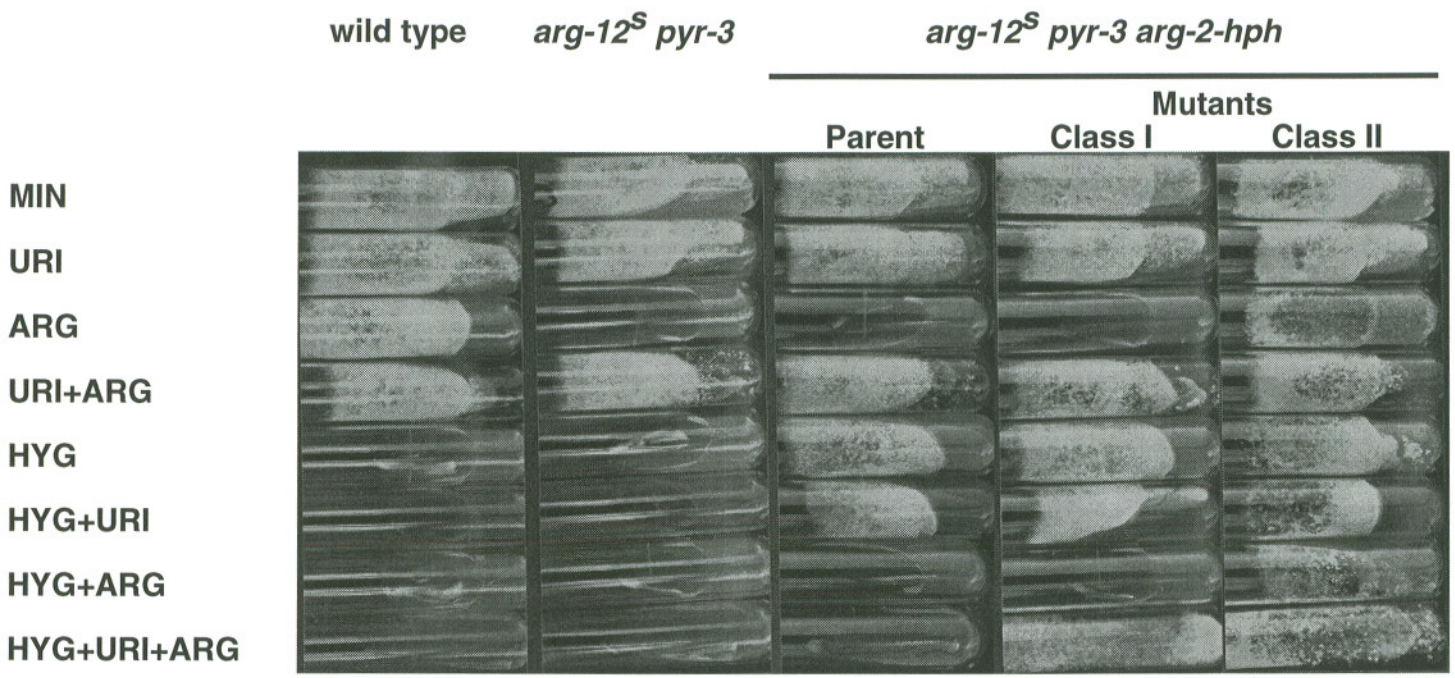

Figure 3.4: Growth responses of wild type and mutant *N. crassa* strains to Arg, Uri and Hyg. Strains were grown on: minimal medium (MIN); MIN supplemented with 0.5 mg/ml (URI); MIN supplemented with 0.5 mg/ml Arg; (ARG); URI+ARG; MIN supplemented with 2 mg/ml Hyg (HYG); HYG+URI; HYG+ARG; and HYG+URI+ARG. Tubes were inoculated with conidial suspensions of the strains indicated (parent = MF13-3; class I mutant = MF13-3-12; class II mutant = MF13-3-47) and incubated at 34°C for 4 d to score for growth.

Table 3.1: Segregation of *pyr-3*, *arg-12* and *arg-2–hph* in crosses of wild type to *arg-12^{s} pyr-3 arg-2–hph* strains[a]

wild type	X		Parent	Mutants									
pyr-3	*arg-12*	*hph*	13-3	13-3-11	13-3-12	13-3-16	13-3-20	13-3-27	13-3-28	13-3-31	13-3-32	13-3-34	13-3-36
+	+/s	+	9	15	20	29	9	8	7	9	18	12	11
+	+/s	–	10	11	26	20	11	13	19	15	9	11	9
–	+	+	13	7	13	9	8	3	5	5	6	5	9
–	+	–	6	5	10	6	5	9	7	8	5	6	5
–	s	+	5	4	11	13	3	6	4	7	5	6	5
–	s	–	3	7	20	10	6	8	8	2	7	6	10

pyr-3	*arg-12*	*hph*	13-3-37	13-3-38	13-3-39	13-3-40	13-3-42	13-3-43	13-3-44	13-3-45	13-3-46	13-3-47	SUM
+	+/s	+	10	13	13	13	13	14	10	15	9	15	272
+	+/s	–	10	6	9	11	10	12	13	10	15	12	262
–	+	+	6	6	9	6	5	11	4	6	4	3	143
–	+	–	9	2	4	5	8	4	6	5	11	5	131
–	s	+	2	1	7	6	4	4	2	7	4	4	110
–	s	–	7	5	5	3	8	6	6	5	4	5	141
													1059

[a]Parent (MF13-3) and mutants (MF13-3-11 to MF13-3-47) were crossed and progeny grown on Min, Arg, Uri, Uri+Arg, Hyg, Hyg+Uri, Hyg+Arg and Hyg+Uri+Arg to score for *arg-12*, *pyr-3* and *arg-2–hph*..

Table 3.2: Segregation of Hyg^r phenotypes on Hyg+Uri and Hyg+Uri+Arg medium of progeny of crosses of wild type with (a) the transformant MF13-3, (b) class I mutants and (c) class II mutants[a]

(a)

wild type X	Hyg+Uri	Hyg+Uri+Arg
MF13-3	27	0

(b)

wild type X	Hyg+Uri	Hyg+Uri+Arg
MF13-3-40	25	25
MF13-3-45	28	28
MF13-3-11	26	23
MF13-3-32	29	17
MF13-3-34	19	17
MF13-3-12	42	39
MF13-3-16	43	38

(c)

wild type X	Hyg+Uri	Hyg+Uri+Arg
class II		
MF13-3-37	28	14
MF13-3-39	26	13
MF13-3-20	20	7
MF13-3-28	16	7
MF13-3-36	25	11
MF13-3-46	31	8
MF13-3-44	16	4
MF13-3-43	29	11
MF13-3-47	22	6
MF13-3-38	20	5
MF13-3-31	28	12
MF13-3-27	20	6
MF13-3-42	25	11

[a]Strains were analyzed as described in the legend to Table 3.1. Values for growth on Hyg+Uri indicate all Hyg^r progeny recovered.

Hyg+Uri+Arg. As expected, none of the Hyg^r progeny from the cross of MF13-3 to wild type that grew on Hyg+Uri also grew on Hyg+Uri+Arg (Table 3.2). In striking contrast, all (or > 90 %) of the Hyg^r progeny from crosses of the class I mutants MF13-3-11, MF13-3-12, MF13-3-16, MF13-3-34, MF13-3-40 and MF13-3-45 to wild type that grew on Hyg+Uri also grew on Hyg+Uri+Arg (Table 3.2). These data showed that mutations that allowed these strains to grow on Hyg+Uri+Arg was tightly linked to *arg-2–hph*.

Crosses of class II mutants to the wild type revealed that approximately half of the progeny that grew on Hyg+Uri also grew on Hyg+Uri+Arg (Table 3.2). This indicated that the mutations that allowed growth on Hyg+Uri+Arg were unlinked to *arg-2–hph*.

3.5 Recovery of *arg-2–hph* reporter gene sequences

It was anticipated that some of the class I mutants would carry mutations in the 5' *arg-2–hph* region. Therefore, primers were designed to amplify DNA fragments from the *arg-2–hph* reporter gene contained in plasmid pGV4 and genomic DNA of transformant MF13-3 and class I strains using the polymerase chain reaction (PCR; Fig. 3.5.a). Primers ARG2-Eco and HPH-Eco amplified a single 662 nt fragment from pGV4 and genomic DNA of MF13-3, class I mutants MF13-3-12, MF13-3-16 and MF13-3-40 and class II mutants MF13-3-37, MF13-3-39, MF13-3-45 and MF13-3-47 (Fig. 3.5.b and data not shown). No discrete fragment could be amplified from pDE1 (Ebbole 1990), a plasmid which lacked *arg-2–hph* sequences, or from untransformed *N. crassa* strains (Fig. 3.5.b and data not shown).

Three independent PCRs were performed to obtain products from each strain; the strains analyzed included the original transformant MF13-3 and original mutants, as well as two progeny from the backcrosses of transformant MF13-3, class I mutants MF13-3-12, MF13-3-16 and MF13-3-40, or class II mutants MF13-3-37, MF13-3-39, MF13-3-45 and MF13-3-47 to the wild type strain.

3.6 Characterization of *arg-2–hph* reporter gene sequences

PCR products were digested with *Eco*RI and the 638 nt *Eco*RI fragment subcloned into the unique *Eco*RI site of pBS SKII+ or pMF2 for further analyses. Southern analyses and restriction enzyme mapping of subcloned fragments revealed that (i) these fragments hybridized to ^{32}P-labeled *arg-2* and *hph* probes and (ii) contained *Pvu*II and *Rsa*I sites that corresponded with the *arg-2–hph* reporter gene fragment in pGV4.

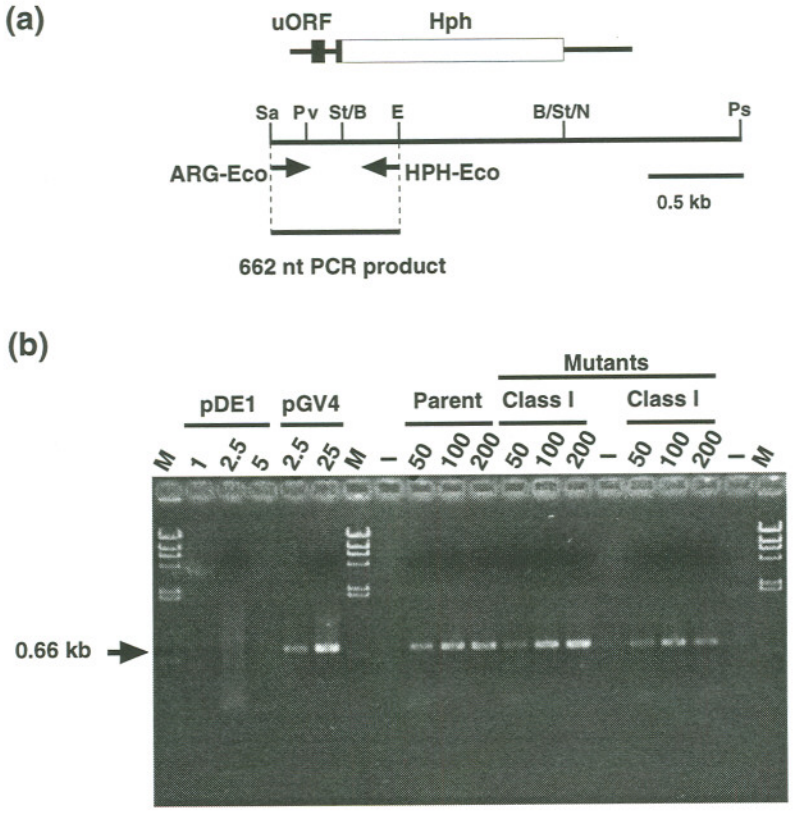

Figure 3.5: Recovery of wild type and mutated *arg-2–hph* 5' regions from transformed *N. crassa* strain by the PCR. (a) Partial restriction map of the *arg-2–hph* gene. When using primers ARG-Eco and HPH-Eco in PCR reactions, a 662 nt product is expected to be amplified from plasmid or *N. crassa* genomic DNA that contains the *arg-2–hph* reporter gene. Restriction enzymes sites are indicated (see Fig. 3.1 for key). (b) Amplified PCR products detected by agarose gel electrophoresis. A single 662 nt fragment was detected when genomic DNA from transformant MF13-3 (parent) or the class I mutant MF13-3-40 (mutant) were subjected to PCR. No discrete product was amplified from a plasmid lacking *arg-2–hph* sequences, pDE1. Size marker (M): phage lambda digested with *Hind*III (lane 1). Reactions contained 1, 2.5 or 5 ng of plasmid DNA or 50, 100 or 200 ng of genomic *N. crassa* DNA as template. Homokaryons of the parent and mutants were directly subjected to PCR and products digested with *Eco*RI, subcloned and analyzed by sequencing. Progeny of crosses of the wild type 74*A* to the parent MF13-3 and the class I mutant MF13-3-40 were similarly analyzed. At least three independent PCR products per strain were analyzed.

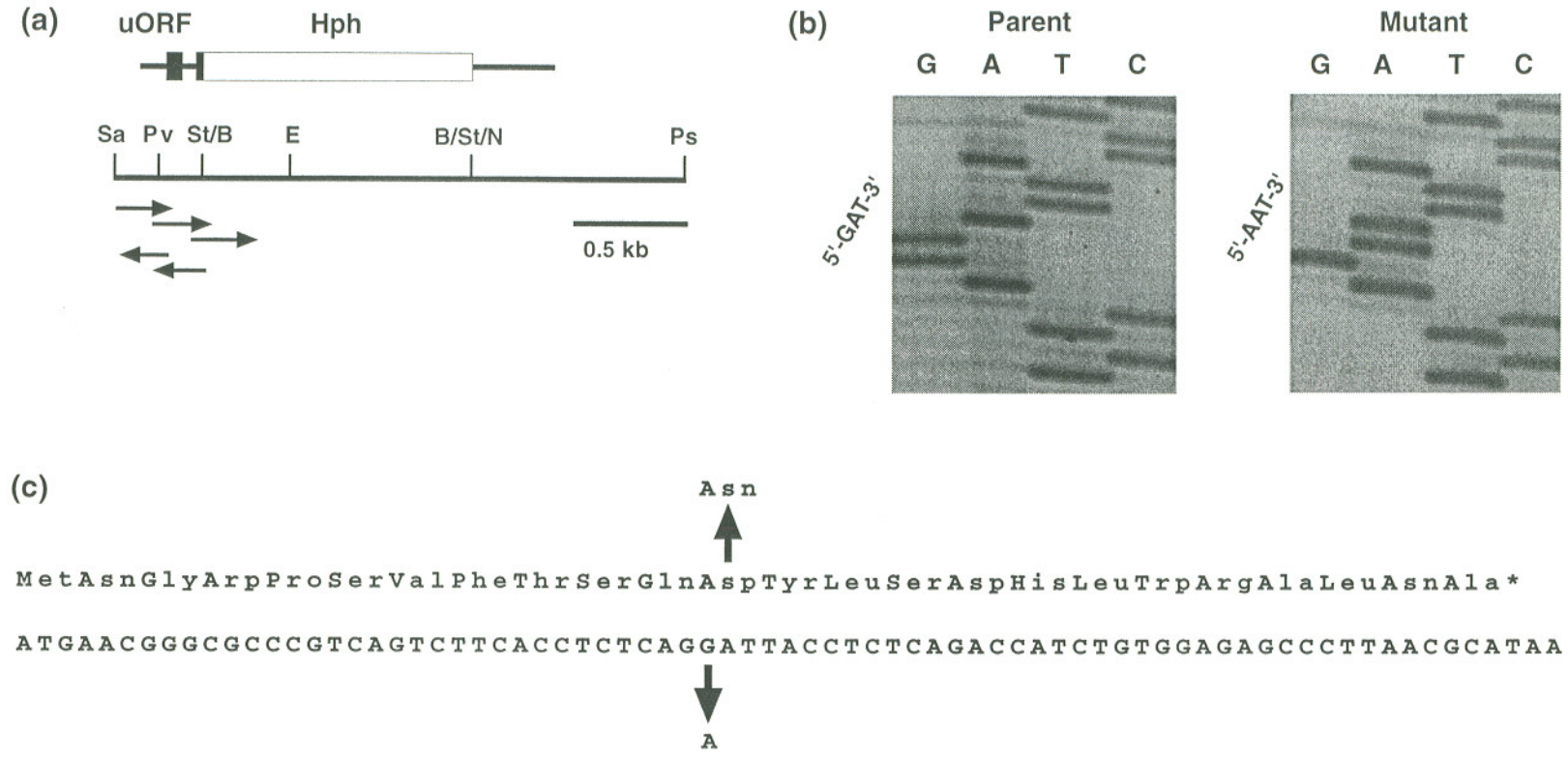

Figure 3.6: Characterization of the mutation in the class I mutant MF13-3-40 by sequence analyses. (a) Partial restriction map of the *arg-2–hph* gene. Arrows indicate locations of sequenced regions of PCR-amplified wild type and mutant *arg-2–hph* reporter genes. Restriction enzymes sites are indicated (see Fig. 3.1 for key). (b) Sequences of codon 10 to 14 of the *arg-2–hph* uORF top strands from the transformant MF13-3 (parent) and from the class I mutant MF13-3-40 (mutant); sequences read 5' to 3' from bottom to top. Codon 12 of the mutant contains a single G to A transition mutation. (c) Consequence of the G to A mutation for the predicted uORF peptide sequence. The Asp in codon 12 of the predicted uORF is changed into an Asn

Both strands of subcloned PCR fragments were sequenced (Fig. 3.6.a). Three subcloned fragments for each independent transformant and mutant strain were characterized to minimize the possibility for mutations introduced by the PCR to remain undetected. Additionally, PCRs were carried out with VentPolymerase, which is less error-prone than conventional *Taq* polymerase. Sequencing analyses of subcloned PCR products from pGV4, MF13-3 and the various mutants also confirmed that in all cases *arg-2* sequences were translationally fused to *hph* at codon 10 of *arg-2*.

Sequences obtained from the subcloned PCR products of the transformant MF13-3 or its backcrossed progeny were consistent with the corresponding previously published *arg-2* (Orbach, Sachs and Yanofsky 1990) and *hph* (Gritz and Davies 1983) sequences. In contrast, all sequences obtained from the subcloned PCR products of the class I mutant MF13-3-40 and its backcrossed progeny carried a single G to A transition mutation in the uORF coding sequence (Fig. 3.6.b). This single G to A transition would change the aspartic acid to an asparagine residue at codon 12 (D12N) of the predicted uORF peptide (Fig. 3.6.c). As described below, this mutation was sufficient to abolish Arg-specific negative regulation of the *arg-2–hph* reporter gene.

Two of the *arg-2–hph* mutants (MF13-3-12 and MF13-3-16) exhibited class I phenotypes and the mutations appeared genetically linked to the *arg-2–hph* locus, as assayed by growth of progeny on Hyg-containing media. However, sequence analyses of the 5' regulatory regions of the *arg-2–hph* gene in these mutants revealed no sequence changes. Moreover, the expression of the fusion gene, as determined by Hph activity assays and *hph* transcript levels appeared indistinguishable from wild type *arg-2–hph* genes (see sections 3.8.2 and 3.10). Why these two strains were Hyg^r on Hyg+Uri+Arg remains to be determined.

Sequences of subcloned PCR products of the putative class II mutants MF13-3-37, MF13-3-39, MF13-3-45 and MF13-3-47 were identical to the sequence of the corresponding wild type *arg-2–hph* fragment. This finding was consistent with genetic analyses, because class II mutations were expected to be unlinked to the *arg-2–hph* locus (see section 3.4).

3.7 The D12N mutation is sufficient to abrogate Arg-specific negative regulation of *arg-2–hph*

The effect of the D12N mutation on expression was examined by subcloning PCR fragments obtained from MF13-1 or the class I mutant MF13-3-40 into pMF2 and reintroduction into an *N. crassa his-3* strain by targeted integration at the *his-3* locus (Sachs

and Ebbole 1990). The 638 nt *Eco*RI fragments from pBS-derived shuttle vectors were each cloned into the unique *Eco*RI site of the *his-3*-targeting vector pMF2 (Fig. 3.7). Plasmid pMF11-wt contains the fragment derived from transformant MF13-3-1, whereas pMF11-D12N contains the corresponding fragment from class I mutant MF13-3-40-11; the only difference in nucleotide sequence between the two plasmids is the single G to A transition at codon 12 of the *arg-2* uORF of pMF11-D12N.

Plasmids pMF11-wt and pMF11-D12N were transformed into an auxotrophic *N. crassa his-3* strain. Homologous recombination at *his-3* resulted in prototrophic transformants. Transformants were grown on Min, Arg, Hyg and Hyg+Arg, at either 0.2 mg/ml or 2mg/ml Hyg. Strains transformed with pMF11-wt were Hyg^r at both concentrations on Hyg, but did not grow on Hyg+Arg at either concentration. In contrast, strains transformed with pMF11-D12N grew on all media. Transformants containing pMF1 or pMF11-37 (a plasmid with a subcloned PCR fragment from MF13-3-37) resembled strains transformed with pMF11-wt; as expected, strains transformed with pMF2, which contains a truncated *hph* gene, were not resistant to Hyg. These results indicated that the D12N mutation by itself was sufficient to confer resistance to Hyg that was no longer regulated by Arg.

Southern analyses were used to ascertain that pMF11-wt and pMF11-D12N had integrated at the *his-3* locus in single copy (Fig. 3.8). Genomic DNA obtained from wild type, the *his-3* mutant and selected transformants were digested with *Dra*I and probed with ^{32}P-labeled *his-3*, *arg-2* or *hph* DNA fragments. In wild type and the *his-3* mutant, only fragments of the expected sizes for the genomic *his-3* (19 kb) and *arg-2* genes (9.8 kb) were detected; *hph* probe detected no fragments, as anticipated (Fig. 3.8.b). In some *his-3* strains transformed with pMF11-wt or pMF11-D12N, two fragments hybridized to *his-3* (ca. 19 kb and 9.4 kb) and *arg-2* (9.8 kb and 9.4 kb); a single fragment (9.4 kb) was detected with the *hph* probe. These data indicated that, in these strains, plasmids had integrated in single copy at the *his-3* locus (Fig. 3.8.c). Several other transformants carried multiple copies of the transforming plasmids, either as tandem duplications at the *his-3* locus or as ectopic integrants (data not shown).

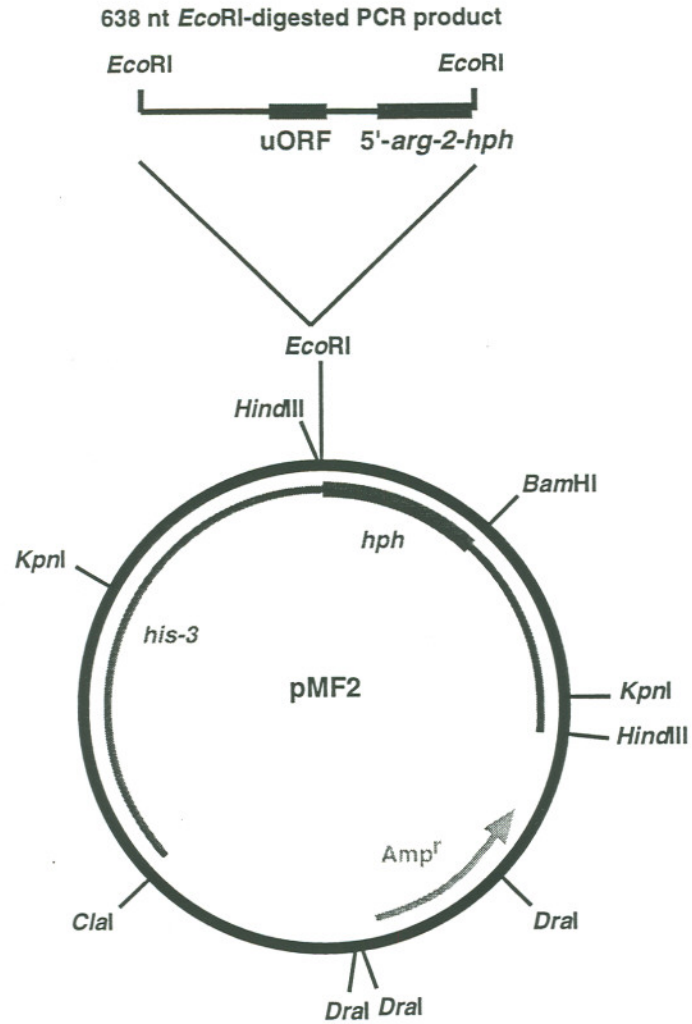

Figure 3.7: Structure of pMF2, a vector for targeted integration of DNA at the *N. crassa his-3* locus. Partial restriction map of pMF2. PCR fragments were digested with *Eco*RI and cloned into the unique *Eco*RI site in pMF2. The location of the *arg-2–hph* transcript, indicating the uORF and Hph are indicated by heavy grey boxes. Orientations were confirmed by restriction mapping.

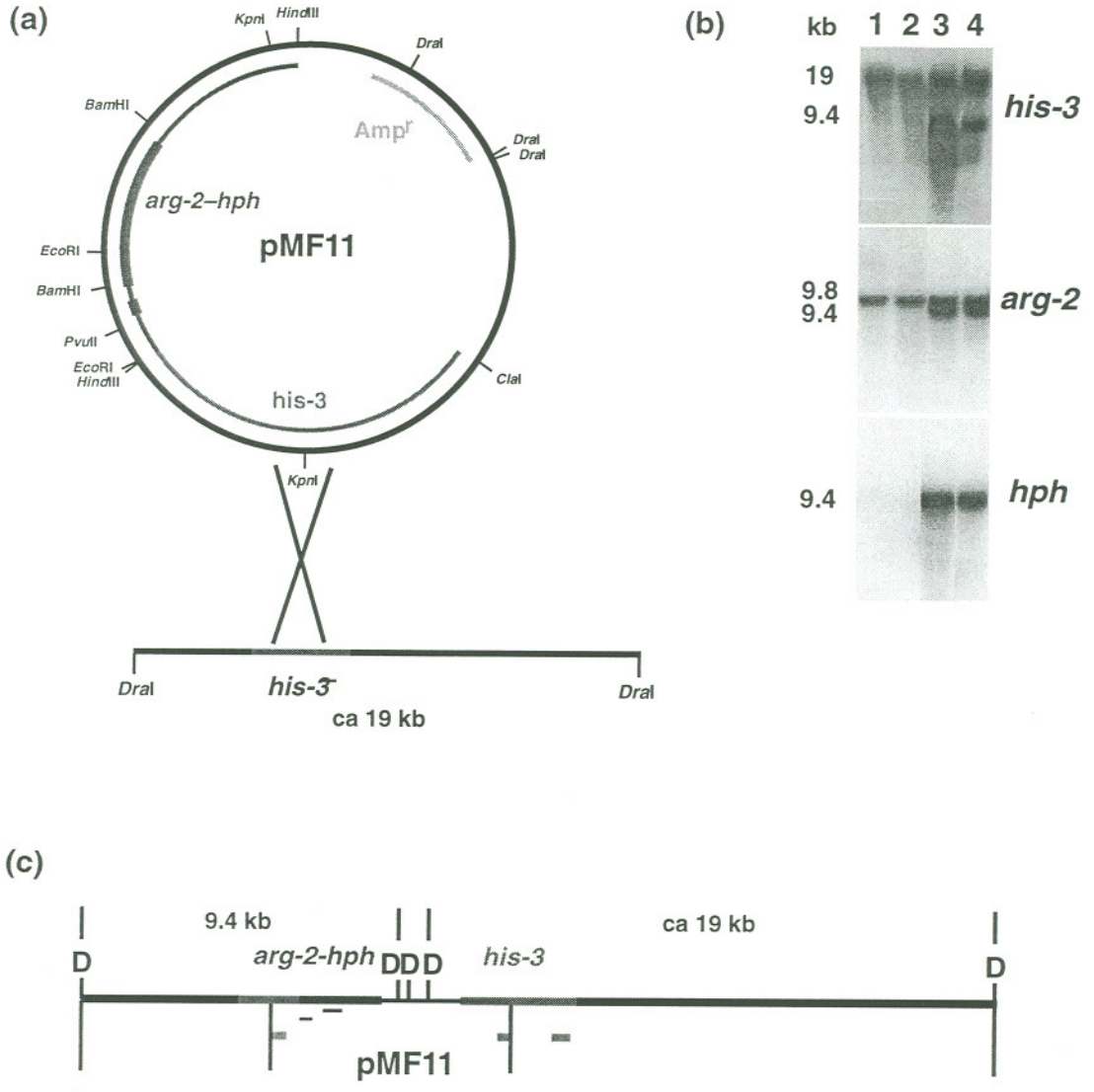

Figure 3.8: Targeted integration of *arg-2–hph* genes at the *N. crassa his-3* locus. (a) Structure and partial restriction map of pMF11-wt. Upon homologous recombination at *his-3*⁻ this vector can confer prototrophy to *his-3* mutants. Analagous integration experiments were performed with vectors that contained subcloned PCR fragments from the D12N mutant strain MF13-3-40 (pMF11-D12N). (b) Southern analyses confirmed that a single copy of *arg-2–hph* had integrated at *his-3*. Wild type (1) and *his-3* (2) genomic DNA digested with *Dra*I and probed with *his-3* or *arg-2* probes detected the endogenous genes (ca. 19kb and 9.8 kb), but failed to detect the *hph* gene, as expected. In strains transformed with pMF11-wt (3) or pMF11-D12N (4) one extra 9.4 kb fragment was detected with all probes, indicating that both transformants contained a single copy of the plasmid. (c) Schematic of the integration event. Radioactively labeled probes are indicated by thin lines and are black (*hph*), dark grey *arg-2*) or light grey (*his-3*). D = *Dra*I.

3.8 Biochemical characterization of wild type and mutant strains

3.8.1 Effect of Arg on CPS-A and fumarase activities and on amounts of Arg2p in wild type, *arg-12^S*, and *arg-12^S pyr-3 arg-2–hph* strains

Arg-specific carbamoyl phosphate synthetase (CPS-A) is a two subunit enzyme; *arg-2* encodes the small, glutamine-dependent activity that transfers an amino group to the large, carbamoyl phosphate synthetase subunit, encoded by *arg-3* (Davis 1986). The activity of the large subunit alone can be measured, if amino groups are supplied in the form of ammonia in the reaction (ammonium- or *arg-3*-dependent activity), whereas the activity of the holoenzyme can be measured, if glutamine is supplied (glutamine- or *arg-2*-dependent activity). Both *arg-2*- and *arg-3*-dependent activities were measured to examine the effects of growth in Arg on CPS-A activity. The wild type or *arg-12^S* mutant were grown for 24 h in Min or Arg to generate sufficient material for enzyme assays. The quality of the mitochondria used for CPS-A assays were similar in all samples as judged by similarities in the levels of the mitochondrial matrix enzyme fumarase (Table 3.3).

In wild type, the *arg-2*-dependent activity of the holoenzyme was reduced 3-fold when Arg was present, whereas the *arg-3*-dependent activity of the large subunit of CPS-A was not regulated by exogenous Arg (Table 3.3). In *arg12^S*, *arg-2*-dependent CPS-A activity was 5.8-fold greater in Min than observed for the wild type; in Arg medium, *arg-2*-dependent activity in the *arg-12^S* strain was 2-fold lower than in wild type, resulting in an overall 35-fold modulation of *arg-2*-dependent activity in *arg-12^S*, compared to a 1.7-fold modulation of *arg-3*-dependent activity.

In addition to measurements of CPS-A activity in wild type and *arg12^S* strains, the level of the small subunit of CPS-A, Arg2p, was determined by immunoblotting of whole cell extracts prepared from *arg-12^S pyr-3*, MF13-3 and several class I and class II mutants that were germinated for 6.5 h in Uri or Uri+Arg (Fig. 3.9). The amount of Arg2p appeared to be strongly regulated by Arg in all strains assayed, when equal amounts of whole cell protein (40 μg) were examined by immunoblotting (Fig. 3.9.a). The magnitude of regulation by Arg was more accurately determined by examining the intensity of bands on immunoblots that were obtained by loading different amounts of whole cell protein as determined by Bradford assay (Fig. 3.9.b). The amount of Arg2p detected by immunoblotting of 10 μg of whole cell protein from Uri-grown cells was approximately 2.5-fold higher than the amount detected from 100 μg of whole cell protein from cells grown in Uri+Arg in *arg-12^S pyr-3*, MF13-3 and the class I mutant MF13-3-40 (Fig. 3.9.b), which indicated approximately 25-fold negative regulation of Arg2p by Arg in these strains. The amount of Arg2p detected by immunoblotting was almost identical when 5 μg

Table 3.3: Effect of growth in Arg on the levels of *arg-2* and *arg-3*-dependent CPS-A activity[a]

Strain	*arg-2*-dependent[a] CPS-A		*arg-3*-dependent[b] CPS-A		Fumarase	
	M	R	M	R	M	R
wild type	12	4	19	18	3.6	4.2
arg-12^{s}	69	2	44	26	3.7	4.1

[a]Activities are reported as relative units (see Materials and Methods). M: minimal medium; R: arginine-containing medium. All data are averages of triplicates from one experiment, the differences between individual measurements were less than 15 %. Experiments were repeated with similar results.

[b]Glutamine-dependent activity, which represents the combined activities of both *arg-2*- and *arg-3*-specified CPS-A subunits.

[c]NH_4-dependent activity, which represents the activity of the *arg-3*-encoded subunit only.

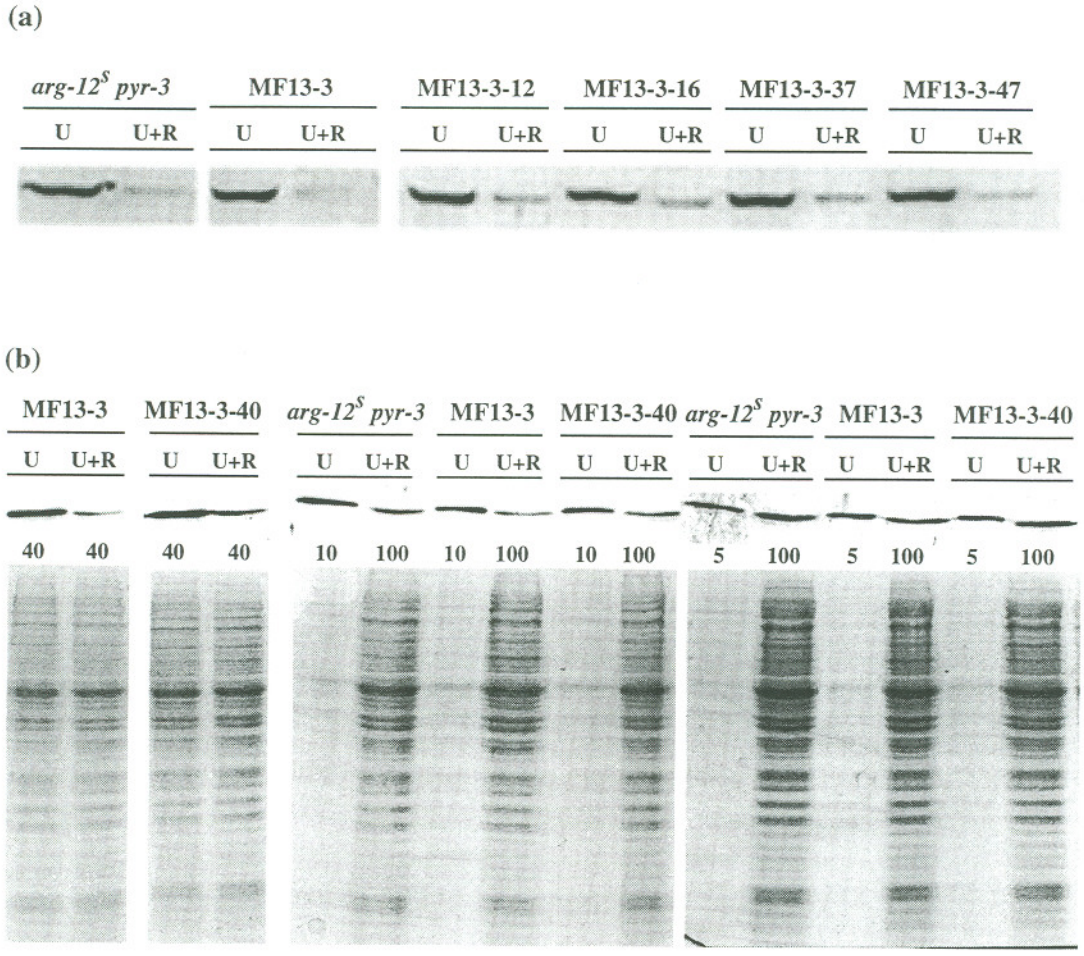

Figure 3.9: Levels of Arg2p in *arg-12^S pyr-3* and *arg-12^S pyr-3 arg-2–hph* strains. (a) Equal amounts of whole cell protein (40μg) were loaded onto 10% SDS/PAGE gels and examined by immunoblotting with anti-Arg2p sera. (b) Various amounts of whole cell protein (as indicated) were loaded to more accurately determine the magnitude of Arg-specific regulation of Arg2p. Strains: MF13-3 = parent; MF13-3-40 = D12N mutant; MF13-3-12 and MF13-3-16 = class I mutants; MF13-3-37 and MF13-3-47 = class II mutants. U = minimal medium supplemented with 0.5 mg/ml uridine; U+R = minimal medium supplemented with 0.5 mg/ml uridine and 0.5 mg/ml arginine.

of whole cell protein of Uri-grown cells were compared to 100 µg of whole cell protein of Uri+Arg-grown cells, which indicated approximately 20-fold negative regulation by Arg. The overall regulation of the amount of Arg2p by Arg in these strains was estimated to be 20- to 25-fold, as quantified by direct analyses of scanned western blots with the IPLab Gel software. These results showed that the D12N mutation carried in MF13-3-40 does not affect the expression of *arg-2*, when assayed in comparison to the *arg-12^S pyr-3* strain and the parent transformant MF13-3. Similar results were obtained with other class I mutants (MF13-3-12 and MF13-3-16; Fig. 3.9.a). The fact that the putative class II mutants assayed in these experiments (MF13-3-37 and MF13-3-47) were not affected in the expression of *arg-2* (Fig. 3.9.a), seemed to indicate that mutations in these strains may not be directly involved in the regulation of *arg-2* expression.

3.8.2 Effect of the D12N uORF mutation on hygromycin B phosphotransferase activity

The expression of the *hph* gene was directly measured by adapting a previously described hygromycin B phosphotransferase (Hph) dot blot assay (Sørensen *et al.* 1992) to measure Hph activity in whole cell extracts from *N. crassa* (Freitag and Sachs 1995). Hph activity was assayed by quantifying the amount of weakly positively charged ^{32}P-labeled Hyg bound to negatively charged P81 phosphocellulose paper with a phosphorimager system. Total Hph activities vary with different experimental conditions. Strains were examined in parallel and at least one reference strain was used in every assay to compare Hph activity in various strains; assays were repeated at least three times in duplicate with four different concentrations of total protein.

As expected, wild type lacked Hph activity, while an *N. crassa* control strain transformed with plasmid tgCMV/HyTK (Lupton *et al.* 1991) showed Hph activity that was not regulated by Arg (Fig. 3.10; Table 3.4). Hph activity in the transformant MF13-3, which contains the wild type *arg-2–hph* reporter gene, was regulated approximately 3-fold by Arg. Arg-specific regulation of Hph was abolished in the class I mutant MF13-3-40 which contained the D12N mutation in the uORF (Fig. 3.10; Table 3.4). Interestingly, Hph activity in the mutant MF13-3-40 was reproducibly 2-fold higher in Uri, and thus almost 7-fold higher in Uri+Arg, when compared to Hph activitiy in the original transformant (Table 3.4).

Hph activity was also measured in *N. crassa his-3* strains that contained *arg-2–hph* genes integrated at the *his-3* locus. Strains transformed with pMF11-wt were regulated approximately 3-fold by Arg. In contrast, *his-3* strains transformed with pMF11-D12N showed no Arg-specific Hph regulation (Fig. 3.10; Table 3.5). These data indicated that

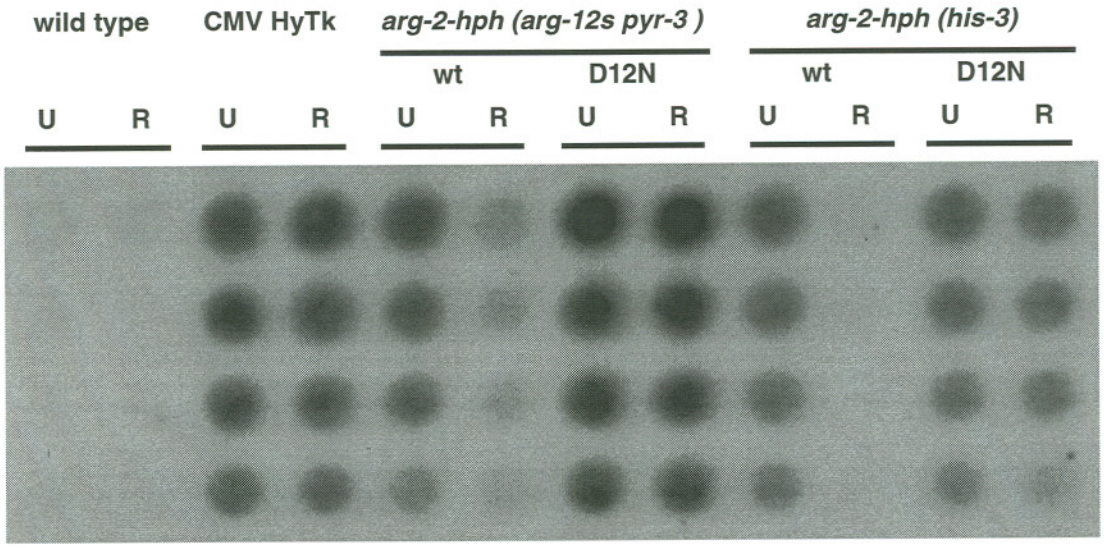

Figure 3.10: Hygromycin phosphotransferase activity assays of whole cell extracts of wild type or mutant *N. crassa* strains. (a) Phosphorimager analyses of Hph dot blot assay. No Hph activity was detected in an untransformed wild type strain (wild type), whereas Hph activity was not Arg-specifically regulated in an *arg-12^S pyr-3* strain transformed with an *hph-tk* fusion gene under the control of a human cytomegalovirus promoter (CMV HyTk). Hph activity was Arg-specifically regulated in *arg-12^S pyr-3 arg-2–hph* strains containing a wt uORF (wt), whereas strains containing a D12N mutation in the *arg-2–hph* uORF were no longer regulated by Arg (D12N). Similar results were obtained when *his-3-* strains were transformed to prototrophy with vectors containing wt *arg-2–hph* (wt) or *arg-2–hph* genes with a uORF D12N mutation (D12N). (b) Quantitation of relative Hph expression in the absence or presence of Arg. Cells were germinated in minimal medium supplemented with 0.5 mg/ml uridine (Uri) or minimal medium supplemented with 0.5 mg/ml of both uridine and arginine (Uri+Arg). Assays were performed and analyzed as described in Materials and Methods. Values are based on triplicate assays, each performed in duplicate at four different protein concentrations of whole cell protein. Ratio indicates realtive amounts of Hph activity in cells grown in Uri versus Uri+Arg medium.

Table 3.4: Relative Hph expression of *N. crassa* strains after 6.5 h growth in Uri or Uri+Arg medium[a]

	wild type[b]	CMV-HyTk	MF13-3	MF13-3-40	MF11-wt	MF11-D12N
Uri	3,600 ± 490	42,200 ± 3,300	45,500 ± 2,600	83,000 ± 5,200	47,300 ± 4,100	56,600 ± 4,200
Uri+Arg	3,500 ± 1,100	37,000 ± 2,400	11,100 ± 3,000	82,200 ± 4,600	16,300 ± 2,100	58,400 ± 5,500
Ratio[b]	N/A	1.1	4.1	1.0	2.9	1.0

[a]Cells were grown in uridine (0.5 mg/ml; Uri) or in uridine and arginine (both 0.5 mg/ml; Uri+Arg) medium for 6.5 hr. Assays were performed and analyzed as described in the Materials and Methods. Data are based on triplicate assays, each performed in duplicate and at four different concentrations of total protein. [b]RSignal detected for wild type is due to small amounts of label which does not filter through the phosphocellulose paper; values were only 3-5% higher than background values. [c]Ratio indicates relative amounts of Hph activity in cells grown in Uri versus Uri+Arg medium.

the single D12N missense mutation in the *arg-2–hph* uORF was sufficient to abrogate Arg-specific negative regulation of *arg-2–hph*.

3.9 Effects of Arg and 3AT on the distribution of selected mRNAs in polysome gradients from wild type or *arg-12^S pyr-3 arg-2–hph N. crassa* cells

Efficiently translated mRNAs are expected to be associated with a larger number of ribosomes at a given time. Once a block in translation initiation or elongation is established, or when an mRNA is translated less efficiently for other reasons, the number of polysomes associated with that message decreases (Hershey 1991; Hill and Morris 1993).

The distribution of *arg-2*, *arg-2–hph*, *cpc-1* and *cox-5* transcripts on polysomes prepared from wild type cells grown in Min and Arg or from *arg-12^S pyr-3 arg-2–hph* cells grown in Uri and Uri+Arg was examined. The *cox-5* gene encodes the *N. crassa* gene for cytochrome oxidase subunit V and is not directly involved in Arg metabolism (Luo, Freitag and Sachs 1995; Sachs and Yanofsky 1991). The *cpc-1* gene is homologous to the *S. cerevisiae GCN4* gene and encodes the *N. crassa* cross-pathway transcriptional activator, Cpc1p (Ebbole *et al.* 1991; Paluh *et al.* 1988).

The amount of RNA loaded, measured as A_{260} units, can vary widely from gradient to gradient. Therefore, *cox-5* mRNA, whose level is not significantly affected by Arg, was used as an internal standard to assess changes in the amounts of *arg-2*, *cpc-1* and *arg-2–hph* mRNAs loaded on polysomes. Additionally, *cox-5* and *cpc-1* transcripts proved to be good controls to assess translational efficiency, because *cox-5* transcripts were relatively well-translated under all conditions and were associated with larger polysomes than the *cpc-1* transcripts. The amount of *arg-2–hph*, *arg-2* or *cpc-1* were expressed relative to the amount of *cox-5* mRNA loaded onto polysomes in Min (wild type) or Uri (*arg-12^S pyr-3 arg-2–hph*).

3.9.1 Long-term effects of Arg on the distribution of selected mRNAs in polysomes gradients from wild type

Prolonged exposure of wild type to Arg did not affect the overall distribution of ribosomes between polysomes and monosomes, as indicated by the similarity of polysome profiles (Fig. 3.11). In both Min and Arg, the *cox-5* transcript was found on larger polysomes (fractions 5 and 6), on average, than *cpc-1* transcript (fractions 7 and 8), indicating that overall *cox-5* transcripts were more efficiently translated than *cpc-1* mRNAs

(Fig. 3.11). There was little difference between cells grown in Min or Arg in the amounts of *cox-5* and *cpc-1* transcripts loaded on polysomes. Taken together, these results suggested that neither *cox-5* nor *cpc-1* transcripts were translationally regulated by long-term Arg exposure.

In contrast, the average size of polysomes associated with *arg-2* transcripts was reduced in cells grown in Arg (Fig. 3.11). The peak of *arg-2* mRNA moved from fraction 5 in Min to fraction 7 in Arg; monosomes are located in fraction 8 under both conditions (Fig. 3.11). Additionally, there was less *arg-2* transcript in the fractions collected from cells grown in Arg compared to cells grown in Min, consistent with the 3-fold reduced level of *arg-2* RNA in total RNA pools (see section 3.11; (Luo, Freitag and Sachs 1995; Orbach, Sachs and Yanofsky 1990; Sachs and Yanofsky 1991)). These findings suggested 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*.

3.9.2 Short-term effects of Arg on the distribution of selected mRNAs in polysome gradients from wild type

The approximately 3-fold reduction in Arg2p synthesis rate observed after short-term exposure to Arg, without concomitant reduction in the level of *arg-2* transcript (Luo, Freitag and Sachs 1995), also suggested the possibility of *arg-2* regulation at the level of translation. Examination of polysomes from cells switched from Min to Min or from Min to Arg for 30 min revealed a reduction in the average size of polysomes translating *arg-2* in cells switched from Min to Arg (from fractions 5-7 in Min to fraction 7 in Arg; monosomes were located in fraction 8 under both growth conditions), while this short term exposure to Arg did not affect the distribution of *cox-5* on polysomes (Fig. 3.12). These results suggested that a reduction in translational efficiency of *arg-2* occurs rapidly after the addition of Arg. Interestingly, short-term exposure to Arg also resulted in the appearance of *cpc-1* transcript on larger polysomes (fractions 6 and 7) when compared to a switch from Min to Min (fraction 8), suggesting translational activation by short-term amino acid imbalance (Fig. 3.12) that was not observed after prolonged growth in Arg (Fig. 3.11).

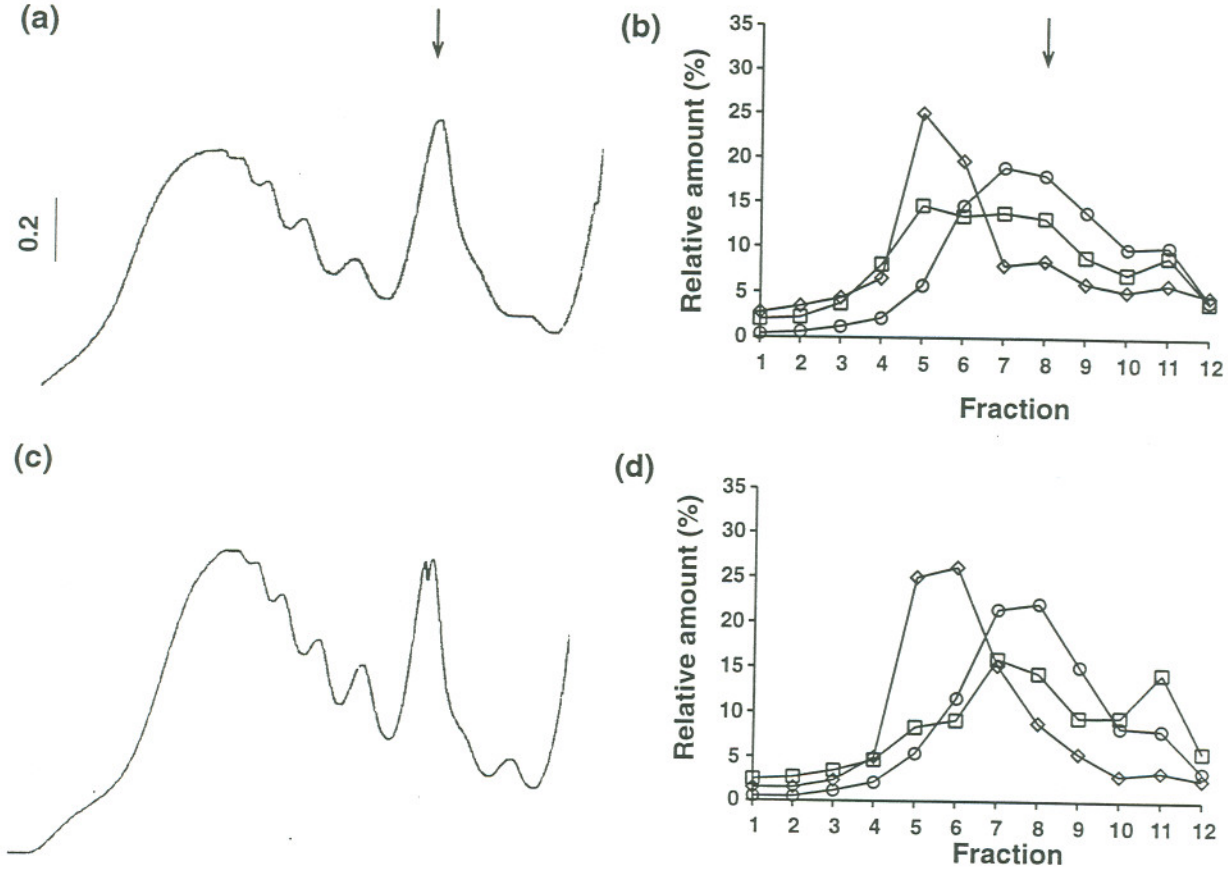

Fig. 3.11: Analysis of mRNA distribution in polysomes prepared from wild type cells. Wild type *N. crassa* were germinated for 6.5 hours in minimal medium (Min) or arginine-supplemented medium (Arg). Extracts were prepared and separated through sucrose gradients. (a, c): A_{254} absorbance profiles of 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 A_{260} 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.

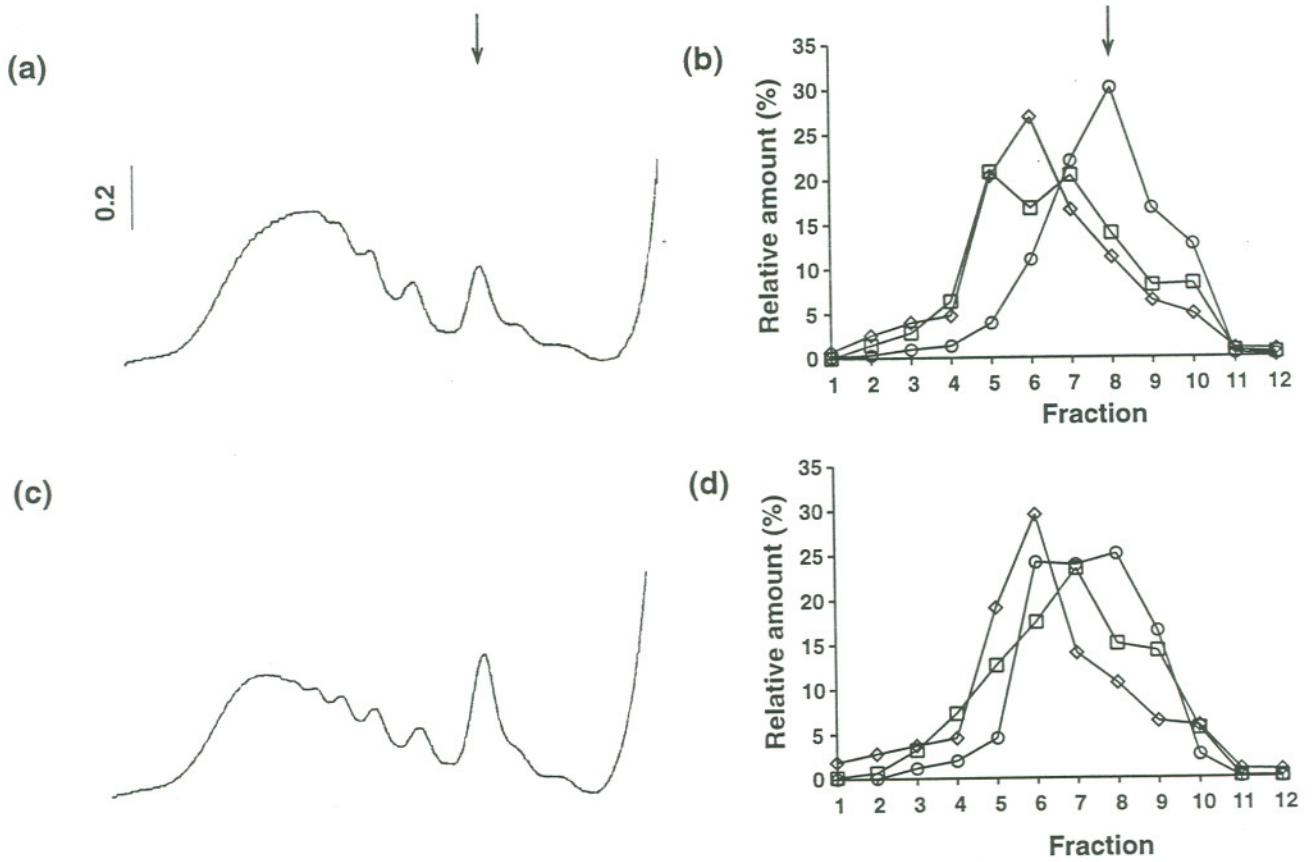

Fig. 3.12: Polysome profile analysis of cells switched from minimal medium to minimal or arginine-containing medium for 30 min. Wild type cells were germinated for 6.5 hours in minimal medium and switched to Min (a, b) or Arg (c, d) media for 30 min. Extracts were prepared and separated through sucrose gradients. (a, c): A_{254} 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 A_{260} were loaded. (b, d): distribution of *arg-2* (squares), *cox-5* (diamonds) and *cpc-1* (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.

3.9.3 Effects of 3AT on the distribution of selected mRNAs in polysomes gradients from wild type

Short-term responses to His limitation, induced by 3AT, on the distribution of *arg-2*, *cox-5* and *cpc-1* messages in polysome gradients were analyzed in parallel with analyses of short-term exposure to Arg. Exposure to Arg for 30 min reduced the level of Arg2p synthesis but not the level of *arg-2* RNA (Luo, Freitag and Sachs 1995). His starvation is known to increase the levels of *arg-2* and *cpc-1* transcripts, and reduce the level of *cox-5* transcript (Orbach, Sachs and Yanofsky 1990; Paluh *et al.* 1988; Sachs and Yanofsky 1991). 3AT increased the level of *arg-2* and *cpc-1* transcript in total RNA (Luo, Freitag and Sachs 1995) and the amount of *cpc-1* mRNA loaded on polysomes, while *cox-5* transcript levels were decreased.

One effect of transfer to 3AT medium on translation was revealed in analyses of polysomes from wild type (Fig. 3.13). Addition of 3AT to cells for 30 min resulted in increased recovery of material in the monosome fractions (Fig. 3.13). Compared to Min (Fig. 3.12), increased amounts of *cpc-1* transcripts were associated with polysomes (fractions 5 to 7) after the shift to 3AT, while the distribution of *cox-5* transcript shifted to monosomes (fraction 8) and small polysomes; the distribution of *arg-2* mRNAs was not significantly altered by the addition of 3AT (Fig. 3.13). The effects of 3AT on translation were reduced when His was added at the same time as 3AT. Whereas *arg-2* transcripts remained associated with large polysomes in His-3AT, *cpc-1* transcripts were associated with smaller polysomes and monosomes and *cox-5* transcripts were associated with larger polysomes (Fig. 3.13), comparable to the distribution in Min (Fig. 3.12). The polysome analyses of experiments with 3AT indicated that *arg-2* translation was not reduced by 3AT, in contrast to *cox-5* translation. The behavior of *arg-2* transcripts could be distinguished from that of *cpc-1* transcripts, which appeared to be shifted to larger polysomes in 3AT.

3.9.4 Long-term effects of Arg on the distribution of selected mRNAs in polysomes gradients from *arg-12^s pyr-3 arg-2–hph* strains

The distribution of *arg-2–hph*, *arg-2*, *cox-5* and *cpc-1* transcripts on polysomes from *arg-12^s pyr-3 arg-2–hph* cells grown in Uri or Uri+Arg for 6.5 hrs was examined (Fig. 3.14). The *cox-5* transcript was used as an internal control for quantification of translational regulatory effects on the *arg-2–hph*, *arg-2* and *cpc-1* transcripts; as in experiments with wild type cells, the amounts of *arg-2–hph*, *arg-2* or *cpc-1* mRNA loaded on polysomes in Uri or Uri+Arg are expressed relative to the amounts of *cox-5* mRNA loaded on polysomes in Uri.

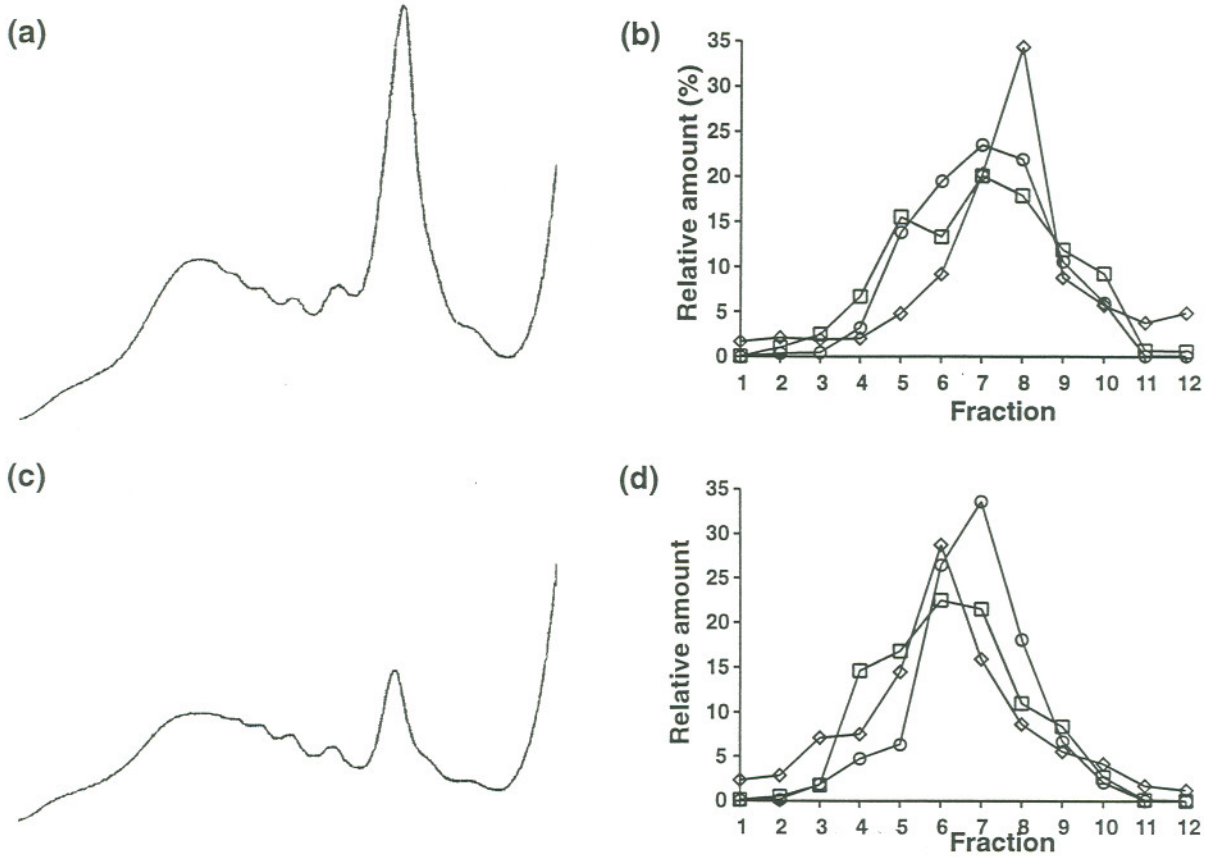

Fig. 3.13: Polysome profile analysis of cells switched from minimal medium to 3-AT or 3-AT+His medium for 30 min. Wild type cells were germinated for 6.5 hours in minimal medium and switched to amino triazole (3AT) (a, b) or 3AT+His (c, d) media for 30 min. Extracts were prepared and separated through sucrose gradients. (a, c): A_{254} 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 A_{260} were loaded. (b, d): distribution of *arg-2* (squares), *cox-5* (diamonds) and *cpc-1* (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.

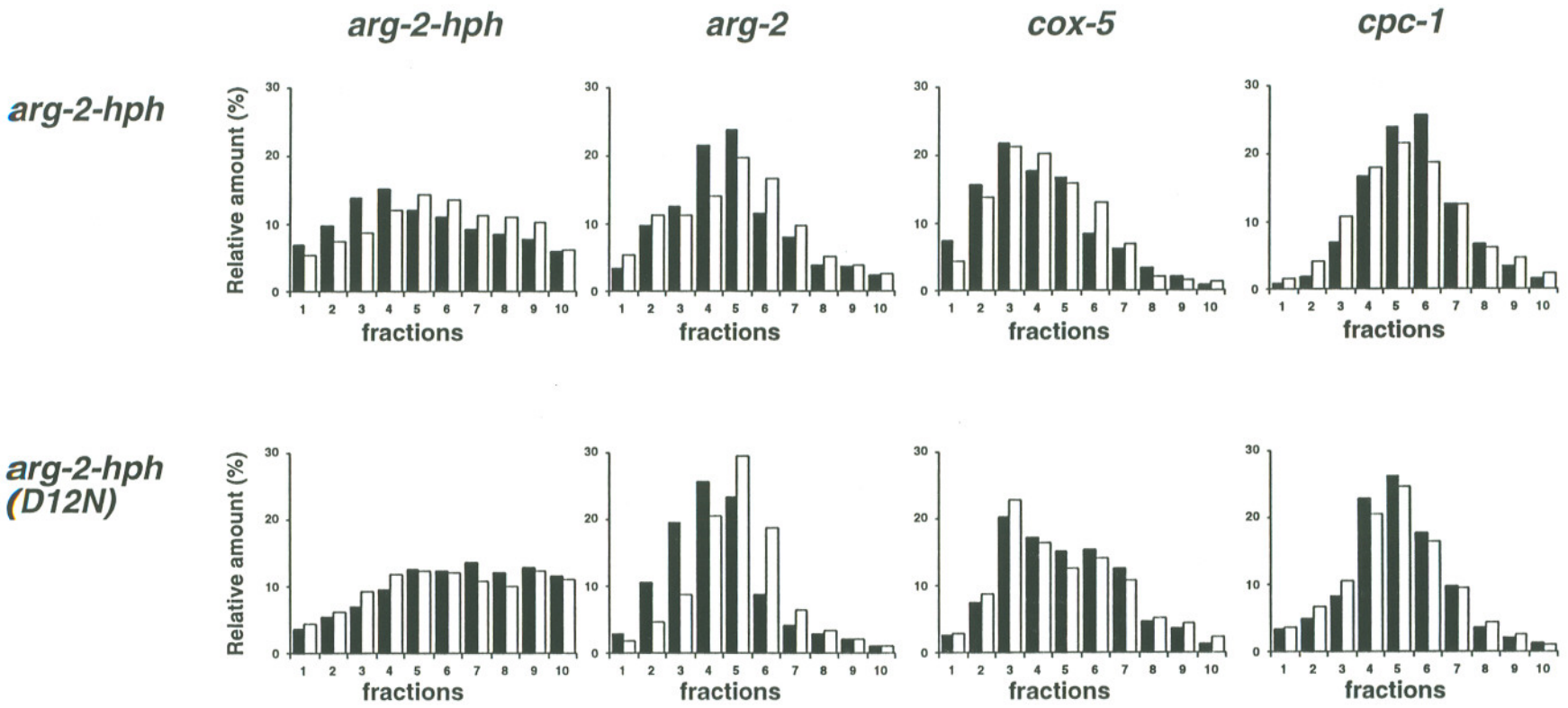

Figure 3.14: The effect of Arg on the distribution of *arg-2–hph*, *arg-2*, *cox-5* and *cpc-1* transcripts in polysome profiles. The *N. crassa* strain MF13-3, transformed with *arg-2–hph* (*arg-2–hph*), or the class I mutant MF13-3-40 were germinated for 6.5 hrs in minimal medium supplemented with 0.5 mg/ml uridine (Uri; solid bars) or 0.5 mg/ml of both uridine and arginine (Uri+Arg; open bars). Extracts were prepared and separated by sucrose gradient centrifugation as described in Materials and Methods. Equal volumes of each fraction were examined by northern blot and phosphorimager analyses; 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 10 is the top. Monosomes were located in fractions 6 and 7.

Similar to results obtained with wild type cells, the distribution of *cox-5* and *cpc-1* mRNAs in polysome profiles of either the transformant MF13-3 or class I mutant MF13-3-40 showed that there was essentially no difference in distribution of *cox-5* and *cpc-1* transcripts between cells grown in minimal or Arg-containing medium (Fig. 3.14), indicating the absence of Arg-specific effects on translation efficiency of both transcripts. In comparison to *cox-5* messages, *cpc-1* transcripts appeared to be less efficiently translated, as indicated by their association with predominantly smaller polysomes and monosomes (Fig. 3.14).

The distribution of *arg-2* mRNAs in the same polysome gradients indicated that *arg-2* translation in both strains was Arg-specifically controlled. In both strains, *arg-2* transcripts shifted towards smaller polysomes in cells grown in Uri+Arg (Fig. 3.14). These findings were consistent with results obtained from experiments in which the distribution of *arg-2* message in polysome profiles from wild type *N. crassa* were examined (Fig. 3.12). These results also indicated that the *arg-2–hph* wild type uORF peptide or the *arg-2–hph* uORF which contained the D12N missense mutation had no *trans*-effect on the translational regulation of *arg-2*.

The distribution of *arg-2–hph* transcripts in polysome profiles from the *arg-2–hph* transformant MF13-3 grown in Uri or Uri+Arg, revealed a shift of *arg-2–hph* mRNAs from larger to smaller polysomes, indicating that *arg-2–hph* was translationally controlled by Arg in the transformed strain (Fig. 3.14). In contrast, examination of the distribution of *arg-2–hph* mRNAs on polysomes extracted from the class I mutant MF13-3-40, which carries the D12N missense mutation, revealed no such shift. Instead, *arg-2–hph* mRNAs appeared to be slightly more efficiently translated in cells that are grown in Uri+Arg (Fig. 3.14). These results strongly indicated that the G to A mutation which causes a predicted missense uORF peptide was sufficient to abolish translational control of the *arg-2–hph* reporter gene.

3.10 Effect of the D12N uORF mutation on *arg-2* and *arg–hph* transcript levels in *arg-12^S pyr-3* strains

Examination of message distribution in polysome profiles established a translational control element for the wild type *arg-2–hph* gene which was absent in the gene with the D12N mutation in the uORF. To assess regulatory effects of the D12N missense mutation at the transcript level, total RNA preparations from wild type, *arg-12^S pyr-3*, MF13-3 and the class I mutant MF13-3-40 were examined by northern blot analyses. Cells were

germinated for 6.5 hrs in Uri or Uri+Arg. The levels of *arg-2–hph* and *arg-2* transcript were compared to the *cox-5* transcript level.

As anticipated, no *arg-2–hph* transcript was detectable in wild type or *arg-12^S pyr-3* strains, which lacked the reporter gene (data not shown). In the *arg-2–hph* transformant MF13-3, *arg-2–hph* transcripts were readily detectable; the level of *arg-2–hph* transcript was 2- to 3-fold higher when cells were grown in Uri compared to Uri+Arg (Fig. 3.15; Table 3.5). Thus, Arg-specific negative regulation of *arg-2–hph* transcript levels appeared less pronounced than the 5- to 7-fold regulation of the endogenous *arg-2* transcripts. Arg-specific negative regulation of the *arg-2–hph* transcripts was not observed in the class I mutant MF13-3-40 (Fig. 3.15; Table 3.5).

In wild type *N. crassa*, *arg-2* transcripts were approximately 3-fold negatively regulated by Arg, relative to the level of *cox-5* transcript (Table 3.5); this finding corresponded to results from earlier studies (Luo, Freitag and Sachs 1995; Orbach, Sachs and Yanofsky 1990; Sachs and Yanofsky 1991). Arg-specific negative regulation of *arg-2* transcript levels in the *arg-12^S pyr-3* strain, the *arg-2–hph* transformant MF13-3 and the class I mutant MF13-3-40 was approximately 5- to 7-fold (Fig. 3.15; Table 3.5). *arg-2* transcript levels in four of the seven class II mutants examined (MF13-3-37; MF13-3-11, MF13-3-44, MF13-3-45) were regulated as in the parental *arg-12^S pyr-3* strain, whereas Arg-specific regulation of *arg-2* transcript was reduced to wild type levels in three of the seven class II mutants analyzed (MF13-3-47; MF13-3-39, MF13-3-43; data not shown). As expected (Luo, Freitag and Sachs 1995; Orbach, Sachs and Yanofsky 1990; Sachs and Yanofsky 1991), the level of *cox-5* transcripts was not significantly affected by Arg in any of the strains analyzed (Fig. 3.15).

3.11 Preliminary analyses of class II mutants

Class II mutants appeared affected in the expression of both the *arg-2–hph* and the *arg-2* genes because these strains grew on all Arg-containing media. Genetic analyses revealed that mutations were unlinked to the *arg-2–hph* gene, but Hph activities in all class II mutants analyzed to date were negatively regulated by Arg, as were *arg-2* and *hph* transcript levels. Class II mutants could potentially be affected in the uptake or intracellular compartmentation of Arg. A reduction of Arg influx or compartmentation of Arg in the vacuole would lead to reduced inhibitory effect on Hph expression and thus survival on Arg-containing media. This possibility was addressed by examining growth phenotypes of all class II mutants on media containing thialysine or DFMO. *N. crassa* wild type cells are sensitive to specific concentrations of both compounds, but mutants that are defective in

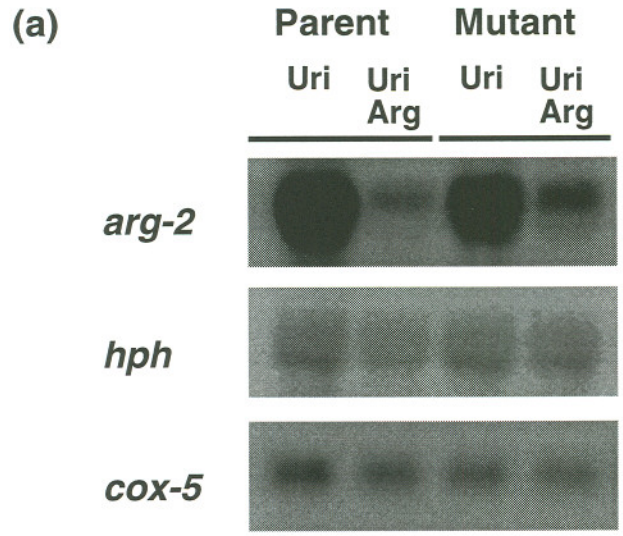

Figure 3.15: The effect of Arg on transcript levels of *arg-2*, *cox-5* and *hph*. (a) Northern blot analyses of *arg-2*, *arg-2–hph* and *cox-5* transcripts in the transformant MF13-3 (parent) and class I mutant MF13-3-40 (mutant). Cells were germinated for 6.5 hrs in minimal medium supplemented with either 0.5 mg/ml uridine (Uri) or 0.5 mg/ml of both uridine and arginine (Uri+Arg).

Northern blots were analyzed with a phosphorimager system as described in Materials and Methods. The amounts of *arg-2–hph* or *arg-2* relative to *cox-5* in Uri were given the unit value 1.0; values are the relative expression of *arg-2–hph* and *arg-2* in Uri+Arg. ND = no *arg-2–hph* message was detected in untransformed wild type and *arg-12^S pyr-3* strains.

Table 3.5: Expression of *arg-2–hph* and *arg-2* relative to *cox-5* in response to long-term growth in the presence of Arg[a]

	wild type	*arg-12s pyr-3*	MF13-3	MF13-3-40
arg-2–hph	ND[b]	ND	2.5	1.5
arg-2	2.5	5.5	7.5	6.3

[a]Cells were grown in uridine(0.5 mg/ml; Uri) or in uridine and arginine (both 0.5 mg/ml; Uri+Arg) medium for 6.5 hr. Analyses of northern blots were performed as described in the Materials and Methods. The relative amounts of *arg-2–hph* or *arg-2* compared to *cox-5* expression in Uri were given the unit value of 1.0; values are the relative expression of *arg-2–hph* and *arg-2* in Uri+Arg. [b]ND = no *arg-2–hph* message was detected.

amino acid uptake are resistant (Davis, Lieu and Ristow 1994). The effect of DFMO and thialysine on wild type, *arg-12^{S} pyr-3*, MF13-3, MF13-3-40 and selected class II mutants was examined, but these analyses were hampered by the fact that different *N. crassa* strains showed different levels of sensitivity to either compound. Wild type was more sensitive to DFMO than to thialysine, while the *arg-12^{S} pyr-3* strain and the transformants and mutants derived from it were more sensitive to thialysine than DFMO. These complications notwithstanding, at least three class II mutants (MF13-3-11, MF13-3-39 and MF13-3-47) were resistant to high concentrations of both DFMO and thialysine, indicating that they may carry mutations in the basic amino acid transporter, *pmb*.

Chapter 4

Discussion

Fungal amino acid biosynthetic genes and their products are subject to regulation at the level of transcription, translation or protein degradation (Davis 1986; Kornitzer *et al.* 1994; Luo, Freitag and Sachs 1995; Paluh *et al.* 1988; Sachs and Yanofsky 1991; Williams *et al.* 1992). Under most growth conditions, expression of the *N. crassa arg-2* gene, which encodes the small subunit of Arg-specific carbamoyl phosphate synthetase, regulates the flux of metabolites through the Arg biosynthetic pathway (Davis and Weiss 1988). *arg-2* is subject to multiple control mechanisms that regulate its expression during the asexual development of *N. crassa* (Davis 1986; Luo, Freitag and Sachs 1995; Sachs and Yanofsky 1991). The *arg-2* transcript level is positively regulated in response to amino acid starvation or imbalance, a response mediated by the cross pathway control transcriptional activator Cpc1p (Ebbole *et al.* 1991; Sachs in press; Sachs and Yanofsky 1991). *arg-2* is the only gene of the *N. crassa* Arg biosynthetic pathway known to be under negative control by Arg (Davis 1986). Decreased CPS-A activity is associated with a reduced level of Arg2p polypeptide in mitochondria (Davis, Ristow and Ginsburgh 1981).

Long-term growth in Arg-containing medium reduces the level of *arg-2* mRNA, indicating a transcriptional component to Arg-specific negative control of *arg-2* (Luo, Freitag and Sachs 1995; Orbach, Sachs and Yanofsky 1990; Sachs and Yanofsky 1991). In this study, the use of *arg-2–hph* reporter genes for the isolation of mutants that were altered in their response to Arg was examined. Mutants with potential defects in Arg-specific regulation were obtained and one mutant that was deficient in Arg-specific negative regulation of *arg-2–hph* was characterized extensively.

4.1 Isolation of *N. crassa* mutants affected in their responses to arginine

A combination of classical and reverse genetic analyses with biochemical methods was used to isolate *N. crassa* mutants involved in Arg-specific negative regulation. Whereas selection for the loss of the Uri requirement in the *S. cerevisiae ura2C* strain led to the characterization of mutants that were altered in Arg-specific regulation (Thuriaux *et al.* 1972; Werner *et al.* 1987), no such mutants of the *N. crassa arg-12^{S} pyr-3* double mutant had been found in earlier studies, even after extensive searches (Davis 1986; Perkins *et al.* 1982). Therefore, a different approach to isolate *N. crassa* mutants that appeared changed in their responses to Arg in the growth medium was taken. A selection for altered expression of the *arg-2–hph* reporter gene after UV mutagenesis allowed for selection and identification of two classes of mutant phenotypes. One class appeared primarily affected in the expression of the *arg-2–hph* reporter gene, as indicated by growth on Hyg+Uri+Arg. In the second class, both the reporter gene and *arg-2* expression appeared affected because mutants grew on Arg, Hyg+Arg and Hyg+Uri+Arg.

Genetic analyses of progeny from crosses of class I mutants to wild type identified strains (MF13-3-12, -16 and -40) that carried mutations which appeared tightly linked to *arg-2–hph*. The genetic analyses of progeny from crosses of class II mutants to wild type was complicated by the fact that *arg-12^{+} pyr-3^{+} arg-2–hph* progeny, as expected, exhibited the same phenotype as class II mutants (*arg-12^{S} pyr-3 arg-2–hph X^{mut}*); such strains grew on all test media. Therefore, the unmutagenized parent MF13-3, the class I mutant MF13-3-40 and selected class II mutants should be further backcrossed to an *arg-12^{S} pyr-3 A* strain. Ideally, none of the Hyg^{r} progeny from a cross of MF13-3 to *arg-12^{S} pyr-3 A* that grow on Hyg and Hyg+Uri should grow on Arg, Hyg+Arg or Hyg+Uri+Arg. All Hyg^{r} progeny of a cross of MF13-3-40 to *arg-12^{S} pyr-3 A* should grow on Hyg, Hyg+Uri and Hyg+Uri+Arg, but not on Arg or Hyg+Arg medium, confirming that the MF13-3-40 strain carries a mutation that is tightly linked to the *arg-2–hph* locus. In contrast, approximately 50% of the progeny from a cross of class II mutants to *arg-12^{S}pyr-3 A* that grow on Hyg, Hyg+Uri or Hyg+Uri+Arg, should grow on Arg and 25% should grow on Hyg+Arg. Such a result would demonstrate that class II mutants carry mutations that are unlinked to *arg-2–hph* and affect the expression of both *arg-2–hph* and *arg-2*.

4.2 Meiotic stability of the *arg-2–hph* reporter gene

Mutants that contained *arg-2–hph* reporter genes were analyzed by classical genetic methods. These analyses indicated that class I mutations were tightly linked to the *arg-2–hph* gene, whereas class II mutations were unlinked to the reporter gene. The fact that

these genetic analyses could be accomplished with mutants containing this particular reporter gene is of importance, because *N. crassa* possesses an efficient system that detects duplicated sequences at a premeiotic step in the life cycle and introduces numerous G to A and C to T point mutations in both sequences. This process is known as repeat induced point mutation (RIP) and can result in inactivation of both copies of the duplicated sequence (Selker 1990; Selker *et al.* 1993). RIPing works best on long stretches (> 2 kb) of duplicated, unlinked DNA or on DNA sequences that are present as tandem duplications (> 0.4 kb; Stadler, Macleod and Dillon 1991; E. Selker and M. Singer, pers. commun.).

The *arg-2–hph* gene contains sequences that are identical to endogenous *arg-2* sequences. Therefore, both genes could potentially be inactivated by RIP. Other than an apparent attenuation of expression after multiple backcrosses, which resulted in somewhat decreased Hyg^r, there was no phenotypic evidence for extensive RIPing in the progeny of a total of 25 Hyg^r x Hyg^s crosses that were analyzed. If extensive RIP had occurred, a much lower percentage of viable ascospores would have been expected in crosses of parent and mutant strains to wild type, due to the presence of Arg-requiring ascospores among the progeny. Extensively RIPed progeny from crosses of parent and mutant strains to *arg-12^s pyr-3 A* would also be expected to require Arg, thus skewing the distribution of progeny towards a new class that would only able to grow on Uri+Arg or Hyg+Uri+Arg, but unable to grow on Min, Uri, Hyg and Hyg+Uri. Such progeny were not observed. In these experiments, ascospores were germinated on Uri+Arg medium, a permissive medium, even in the case of extensive RIPing of both *arg-2–hph* and *arg-2*. Approximately 90% of ascospores isolated formed colonies on Uri+Arg medium and all of these progeny grew on Min and Uri, indicating that none of the ascosores isolated on Uri+Arg were extensively ripped. The lack of linkage of the fusion gene to *arg-2*, the small size of the non-contiguous *arg-2* 5' and 3' regions in the *arg-2–hph* gene (403 nt and 971 nt, respectively) and the fact that the 5' *arg-2* region of the reporter gene was intronless presumably helped to shield the reporter gene from RIPing. These results indicate that similar studies involving other *N. crassa* genes are feasible.

Progeny of second generation backcrosses of parent and mutant strains to wild type and progeny of backcrosses of parent and mutant strains to *arg-12^s pyr-3 A* were found to grow more slowly on Hyg-containing media then the original isolates when the Hyg concentration was 2mg/ml. Therefore, progeny was also tested at lower Hyg concentrations (0.2 and 1 mg/ml) to confirm the results obtained in initial spot tests. It is conceivable that the expression of reporter genes in *N. crassa* attenuates after single or multiple meioses. Attenuation of reporter gene expression in strains containing multiple copies of reporter genes has been observed during the asexual cycle; this phenomenon has

been called "quelling" (Pandit and Russo 1992; Romano and Macino 1992). However, in the same studies, quelling of gene expression of single copy reporter genes, such as present in strain MF13-3, has not been observed during the asexual cycle. Another explanation for the attenuation of the Hyg^r phenotype after several backcrosses would be the occurrence of light RIP that would result in diminished, but not abolished *arg-2–hph* expression.

4.3 The expression of *arg-2* in wild type and *arg-12^S pyr-3* strains is regulated at multiple levels

The effects of Arg on the regulation of translation and transcript levels of *arg-2* were examined in wild type and transformed and untransformed *arg-12^S pyr-3* strains. Translational efficiency and transcript levels of *arg-2* were compared to those of *cox-5* (Sachs *et al.* 1989) and *cpc-1*, the *N. crassa* homologue of *S. cerevisiae GCN4* (Ebbole *et al.* 1991; Paluh *et al.* 1988).

The long-term negative effect of Arg on the expression of *arg-2* in wild type *N. crassa* is approximately three-fold, a magnitude similar to that observed for Arg-specific regulation of *S. cerevisiae CPA1* (Luo, Freitag and Sachs 1995; Orbach, Sachs and Yanofsky 1990; Sachs and Yanofsky 1991; Werner *et al.* 1987). This effect is small compared to regulation observed, for example, in the *GCN4* system (Hinnebusch 1984; Hinnebusch 1993), but highly reproducible in experiments containing internal controls, such as those presented in this study. In such experiments, the level of *arg-2*-dependent enzyme activity, the cellular level of Arg2p, the rate of Arg2p synthesis and the level of *arg-2* transcript were each reduced approximately 3-fold in wild type (this study; (Luo, Freitag and Sachs 1995). 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 polysome analyses a shift of *arg-2* transcripts from larger to smaller polysomes was observed, indicating that even under long-term exposure to Arg, translation of *arg-2* transcripts is regulated. When wild type cells were exposed to Arg for brief periods (2-30 min), no reduction in transcript level was observed, but the synthesis of Arg2p decreased by about 3-fold (Luo, Freitag and Sachs 1995) and *arg-2* transcript shifted from larger to smaller polysomes. These results indicated that a rapid response to Arg occurs at the translational level.

arg-2 transcript levels in the *arg-12^S pyr-3* and *arg-12^S pyr-3 arg-2–hph* strains tested were approximately 5- to 7-fold negatively regulated when strains were grown in Uri compared to Uri+Arg. Differences between the transcript level of *arg-2* and *arg-2–hph* that

were observed were presumably due to the lack of transcriptional response elements in the *arg-2–hph* 5' region; for example, all four TGACTC response elements, which are required for the binding of the cross pathway control transcriptional activator Cpc1p to *arg-2*, have been removed in the *arg-2–hph* construct. In addition to affecting *arg-2* transcript levels, Arg had a negative effect on translation of *arg-2* in *arg-12^S pyr-3* strains. The magnitude of translational regulation, as indicated by polysome analyses, was comparable to that observed in earlier experiments with wild type cells (Luo, Freitag and Sachs 1995).

Wild type *N. crassa* growing in Min appears to synthesize sufficient Arg to partially engage mechanisms that limit *arg-2* expression or disengage mechanisms that stimulate expression in response to limitation. In contrast, the amount of Arg2p in *arg-12^S pyr-3* strains was approximately 25-fold lower in cells grown in Uri+Arg versus cells grown in Uri. Thirty-five-fold negative regulation of *arg-2*-dependent CPS-A activity by Arg, as shown here, had been previously observed in the *arg-12^S* strain when cells were grown in minimal versus Arg-containing medium, largely because the expression of *arg-2* is greater in *arg-12^S* strains in minimal medium (Cybis and Davis 1975; Luo, Freitag and Sachs 1995; Sachs and Yanofsky 1991).

The 25-fold regulation of Arg2p levels and the 35-fold regulation of CPS-A activity in *arg-12^S pyr-3* strains could be explained if the regulatory effects on transcript levels and translation are multiplicative. Additionally, *arg-2* transcript stability in *arg-12^S* and *arg-12^S pyr-3* strains following switches from Min to Arg or Uri to Uri+Arg, respectively, was decreased when compared to *arg-2* transcripts in wild type (Z. Luo, unpublished results and data not shown). It is also possible that the stability of Arg2p itself may be altered in *arg-12^S* and *arg-12^S pyr-3* strains. More detailed biochemical studies to examine transcript stability and direct evaluation of translation elongation are clearly needed to settle the question of how much of the observed *arg-2* regulation in *arg-12^S pyr-3* strains is attributable solely to transcriptional or translational control components.

4.4 Translational control of *arg-2* and *cpc-1* in wild type

Through the analyses of the distribution of transcripts in polysome gradients this study provided direct biochemical evidence for a translational component to *arg-2* regulation. In wild type cells, long-term exposure to Arg shifted *arg-2* transcript to smaller polysomes, with a concomitant reduction of the amount of *arg-2* transcript loaded onto polysomes and an approximately 3-fold reduction of *arg-2* transcript level detected in total RNA preparations. These data suggested that a negative translational control mechanism was acting even under conditions of long-term exposure to Arg. Under conditions of

short-term exposure to Arg, a similar, but less extensive shift of *arg-2* transcripts into smaller polysomes was detected. These results indicated that a translational control mechanism was responsible for modulating Arg2p expression as an immediate response to Arg exposure, because under these conditions the net rate of Arg2p synthesis, assayed in parallel (Luo, Freitag and Sachs 1995), was reduced in Arg medium.

In additon to changes in expression of *arg-2*, transcript levels and translational efficiency of *cpc-1* in response to Arg and 3AT were examined. *cpc-1* and *S. cerevisiae GCN4* encode similar bZIP polypeptides that appear to function as positive transcriptional activators of amino acid biosynthetic genes in response to amino acid starvation (Hinnebusch 1992; Sachs in press). A transcriptional component to *cpc-1* regulation has been demonstrated (Paluh and Yanofsky 1991; Sachs and Yanofsky 1991) and a translational control component has been postulated on the basis of the presence of two uORFs (Paluh *et al.* 1988). There is also evidence for post-translational modification of Cpc1p (Ebbole *et al.* 1991), suggesting the possibility of additional control mechanisms. Control of *S. cerevisiae GCN4* expression has transcriptional, translational and post-translational components (Hinnebusch 1984; Kornitzer *et al.* 1994) . It has been well established that two of the four uORFs, uORF1 and uORF4, in the 5' region of the *GCN4* transcript are necessary for translational control via a reinitiation mechanism (Abastado *et al.* 1991; Hinnebusch 1994).

The response of *arg-2*, *cox-5* and *cpc-1* to His-limitation induced by 3-amino-1,2,4-triazole (3AT), a competitive inhibitor of imidazole glycerol phosphate dehydratase (Carsiotis, Jones and Wesseling 1974), were examined. When His was limited, the *cpc-1* and *arg-2* mRNAs were found on larger polysomes than the *cox-5* mRNA control, indicating a translational control mechanism for the expression of *cpc-1* and an additional translational component to regulation by amino acid limitation for *arg-2*. The response of *cpc-1* is similar to that observed in studies with *S. cerevisiae GCN4*. When *S. cerevisiae* was shifted from nutrient-rich to nutrient-poor media, the *GCN4* transcript also became associated with larger polysomes (Tzamarias, Roussou and Thireos 1989). This effect appeared to be mediated at the level of translation initiation (Hinnebusch 1994; Tzamarias, Roussou and Thireos 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-1* transcript (Paluh *et al.* 1988). There are similarities in the region downstream of *GCN4* uORF4 and *cpc-1* uORF2, based on the revised *cpc-1* sequence in Genbank. Particularly, regions of high G+C content

important for *GCN4* uORF4 function appear to be conserved downstream of *cpc-1* uORF2. Thus one model for translational regulation of *cpc-1*, based on current understanding of *GCN4* regulation (Hinnebusch 1993; Hinnebusch 1994) would predict that translational control by amino acid availability regulates initiation at the uORF2 start codon versus the Cpc1p start codon.

4.5 Translational regulation of the *arg-2–hph* reporter gene is abolished by a uORF missense mutation

Results from polysome analyses in strains transformed with *arg-2–hph* reporter genes indicated that the D12N missense mutation in the *arg-2* uORF peptide coding sequence was sufficient to abolish Arg-specific translational control of the *arg-2–hph* gene. Whereas the shifts of *arg-2–hph* wild type and *arg-2* transcripts into monosomes and disomes were comparable to and consistent with the shift found in experiments in wild type, *arg-2–hph* transcripts that carried the D12N mutation in the uORF did not shift into smaller polysomes in Uri+Arg. Slight differences in the polysome distributions may be accounted for by differences in the polysome profiles between studies. The observed shift effects were small but reproducible. As in other systems (Hill and Morris 1993; Ruan *et al.* 1994), *arg-2* and *arg-2–hph* transcripts that become less efficiently translated in the presence of Arg do not shift completely into the monosome fractions.

4.6 The uORF peptide is *cis*-acting

Arg-specific translational regulation of the endogenous *arg-2* gene in the class I mutant MF13-3-40 was observed, while translational regulation of *arg-2–hph* was abolished. This finding indicated *cis*-activity for the *arg-2* uORF small peptide. Similarly, the mutants found to alter the *CPA1* uORF acted only in *cis* (Werner *et al.* 1987) and no indication of *trans* activity of the wild type uORF peptide has been found in co-transfection studies on AdoMetDC (Hill and Morris 1993) or cytomegalovirus gp48 (Degnin *et al.* 1993).

There are at least three explanations for apparent *cis*-activity of small leader peptides. First, only partial translation of the leader peptide may be necessary to exert regulatory effects. Secondly, the nascent uORF peptide may affect translation by binding to nearby sites on the translational machinery or the downstream ORF to inhibit further translation. Finally, even if the leader peptide functioned after leaving the vicinity of the translating ribosome, its specific inhibitory effect on other messages would be impossible

to gauge at very low peptide concentration. The uORF peptide has yet to be detected in *N. crassa* extracts. This is a difficult task, because the free peptide concentration is expected to be extremely low, even under inhibitory conditions.

4.7 The *arg-2* uORF is similar to other uORFs whose sequence is essential for translational control

The 24-codon uORF in the *arg-2* transcript encodes a peptide that is similar to the peptide specified by the 25-codon *S. cerevisiae CPA1* uORF (Werner *et al.* 1987; Orbach, Sachs and Yanofsky 1990); 9 of the 15 central residues are identical and 4 are conservative substitutions (Fig. 4.1). The nucleotide sequences of the *arg-2* and *CPA1* uORFs are also conserved in this 45 nt region: of 16 differences, 5 affect the codon wobble position, 8 result in 5 conservative substitutions and the remaining 3 differences lead to a non-conservative substitution (Nyunoya and Lusty 1984; Orbach, Sachs and Yanofsky 1990; Werner, Feller and Piérard 1985). Codons specified by the nucleotides in this stretch of *CPA1* are not rare (Werner, Feller and Piérard 1985), whereas at least the second Arg codon in the *arg-2* uORF sequence is rare, compared to the overall *N. crassa* codon bias (Edelman and Staben 1994). Sequences surrounding the uORFs of *arg-2* and *CPA1* are different; *CPA1* sequences 5' and 3' of the uORF are fairly AT-rich, while *arg-2* sequences have a higher GC percentage. Although most silent mutations introduced into the *CPA1* uORF singly or in multiple combinations (up to 19 nucleotides at a time) were not in the most conserved regions between the *arg-2* and *CPA1* uORFs, and would therefore presumably most easily be tolerated, regulation of the downstream *CPA1* uORF was not affected by such mutations; the same was true when sequences 5' or 3' of the uORF were deleted or inserted (Delbecq *et al.* 1994). However, possible roles of nucleotide secondary structures in regulation of translation of *arg-2* cannot be excluded at this time.

Characterization of the 5' regulatory sequences of the *arg-2–hph* reporter gene from the class I mutant analyzed here revealed a point mutation that changed the sequence of the predicted uORF peptide at codon 12 from Asp to Asn (D12N). Interestingly, two independently isolated *cis*-acting *S. cerevisiae CPA1* mutants with abolished Arg-specific regulation of *CPA1* carried homologous mutations (D13N) in the uORF (the third missense mutation in the *CPA1* uORF was a change from Cys to Tyr at codon 11; Werner *et al.* 1987). Comparisons of peptide sequences of uORFs that exert translational control on downstream ORFs revealed a group of at least six uORFs that contain an Asp residue (Fig. 4.1). Moreover, when the Asp residue in five of these uORFs was altered, regulation by the uORF peptide was abolished (this study; Gu *et al.* 1994a; Gu *et al.* 1994b; Hill and

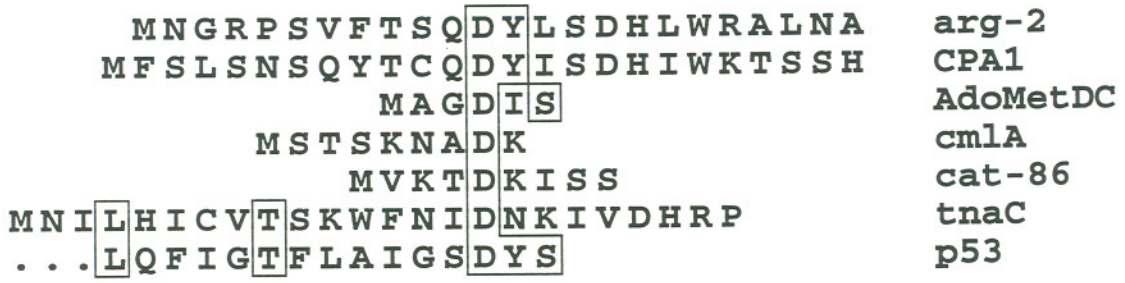

Figure 4.1: Sequence-dependent uORF peptides from prokaryotes and eukaryotes. The predicted peptide sequences of uORFs in *N. crassa arg-2* (Orbach, Sachs and Yanofsky 1990), *S cerevisiae CPA1* (Werner *et al.* 1987), human AdoMetDC (Hill and Morris 1992), bacterial transposon Tn*1696 cmlA* (Stokes and Hall 1991), *Bacillus pumulis cat-86* (Ambulos *et al.* 1985), *E. coli tnaC* (Stewart and Yanofsky 1985) and the carboxyl terminus of a predicted uORF conserved in mouse and rat *p53* (Bienz-Tadmor *et al.* 1985) are aligned at an aspartic acid residue (boxed) known to be important for the function of the top five of the uORFs shown.

Morris 1993; Werner *et al.* 1987)). Currently, no data about the importance of the Asp residue in *tnaC*, which modulates the expression of tryptophanase in *E. coli*, are available. The region of the uORF containing Asp, however, seems conserved among different bacterial species (Gish and Yanofsky 1991). Finally, the carboxyl-terminus of a predicted uORF in the first exon of mouse and rat *p53* (Bienz-Tadmor *et al.* 1985), a gene critical for mammalian growth control, bears some resemblance to uORFs with known roles in translational control (Fig. 4.1). It would be interesting to see, whether the *p53* gene is also subject to translational control mediated by a sequence-dependent uORF and whether the Asp residue in the *p53* uORF is important for function.

Mutations that removed the translation initiator codon of the *arg-2* or *CPA1* uORFs abolished Arg-specific regulation (Luo, Freitag and Sachs submitted; Werner *et al.* 1987), as did *CPA1* uORFs that were shortened by nonsense mutations changing Gln at codon 8 or Trp at codon 20 to stop codons (Werner *et al.* 1987). The importance of the uORF in negative regulation was also studied using *arg-2-lacZ* fusion genes (Luo, Freitag and Sachs 1995; Luo and Sachs submitted). Independent *N. crassa* transformants which contained the wild type uORF proximal to *E. coli lacZ* showed approximately 2.5-fold regulation of ß-galactosidase activity in response to Arg. Elimination of the uORF initiation codon resulted in increased and unregulated expression of ß-galactosidase. These data indicated that uORF translation is important for Arg-specific regulation.

The mere presence of a uORF, while required, does not by itself confer specific regulation of *arg-2* (Luo, Freitag and Sachs 1995; Luo and Sachs submitted). The same has been found in studies using missense uORFs in *CPA1*, human AdoMetDC and human cytomegalovirus *gp48* (Cao and Geballe 1994; Hill and Morris 1993; Werner *et al.* 1987). Mutations that resulted in a loss of the second uORF of cytomegalovirus gp48 or the small peptide of AdoMetDC led to a loss of regulation (Hill and Morris 1993; Schleiss, Degnin and Geballe 1991). Similarly, mutations in the stop codons of AdoMetDC or uORF2 of gp48 resulted in loss of inhibition (Degnin *et al.* 1993; Hill and Morris 1993; Schleiss, Degnin and Geballe 1991). Silent mutations in *CPA1* (Delbecq *et al.* 1994) or in codons 2 to 6 of the hexapeptide MAGDIS, encoded by the uORF of *S*-adenosylmethionine decarboxylase (AdoMetDC), did not affect translational regulation (Hill and Morris 1993).

4.8 Translation attenuation of *arg-2* by the *arg-2* uORF: stalled ribosome models in prokaryotes and eukaryotes

One class of models consistent with data obtained from uORF mutagenesis and from analyses of the distribution of the native *arg-2* transcript in polysomes of cells grown with or without Arg, and consistent with findings with other uORFs whose sequences are important in negatively regulating gene expression (see Fig. 1.5 and Fig. 4.1) would predict that uORF translation stalls ribosomes scanning from the 5' end of the mRNA more efficiently when Arg is present. Such ribosome stalling models have been proposed for both prokaryotic and eukaryotic systems.

Small peptides encoded by uORFs in the chloramphenicol resistance genes *cat-86* of *Bacillus subtilis* or *cmlA* of *E. coli* result in translation attenuation by inhibiting peptidyl transferase activity (Gu *et al.* 1994a; Gu *et al.* 1994b; Gu, Rogers and Lovett 1993; Lovett 1994; Moffat, Tate and Lovett 1994; Rogers and Lovett 1994). This inhibitory effect was abolished when residues in these peptides were changed. A model predicting ribosome pausing and stalling has been proposed from these studies (Moffat, Tate and Lovett 1994).

Whether or how the *arg-2* uORF peptide interacts with the translational machinery is presently unclear. One may speculate that Arg can have a direct effect on the ability of the uORF peptide to bind to the peptidyl transferase site of the large ribosomal subunit; a similar idea has been put forward for the interaction of the small peptide of *cat-86* with the inducer, chloramphenicol, in ribosomal stalling and autoinduction of *cat-86* expression (Moffat, Tate and Lovett 1994). Binding of chloramphenicol to ribosomes has been shown; the antibiotic acts at the level of translation elongation (Lovett 1990; Lovett 1994). It is difficult, however, to envision a direct role of Arg in ribosome or translation factor binding.

At least two stalled ribosome models can be applied to *arg-2* uORF peptide function. A partially translated uORF peptide may interfere with transpeptidation; alternatively, a fully translated uORF peptide may inhibit translation termination events (Fig. 4.2). Ribosomes would stall on the uORF and would thus be unable to proceed to the Arg2p start codon. Both models are consistent with the finding that *arg-2* transcripts from cultures grown in Arg-containing medium sedimented predominantly in the smaller polysome or monosome fractions. The same differential distribution of transcripts has been found for AdoMetDC or gp48 transcripts under non-inhibitory or inhibitory conditions (Degnin *et al.* 1993; Hill and Morris 1993; Schleiss, Degnin and Geballe 1991). Stalled ribosome models have been proposed previously to explain the regulation of expression of eukaryotic *CPA1* and AdoMetDC (Geballe and Morris 1994; Hill and Morris 1993; Werner *et al.* 1987).

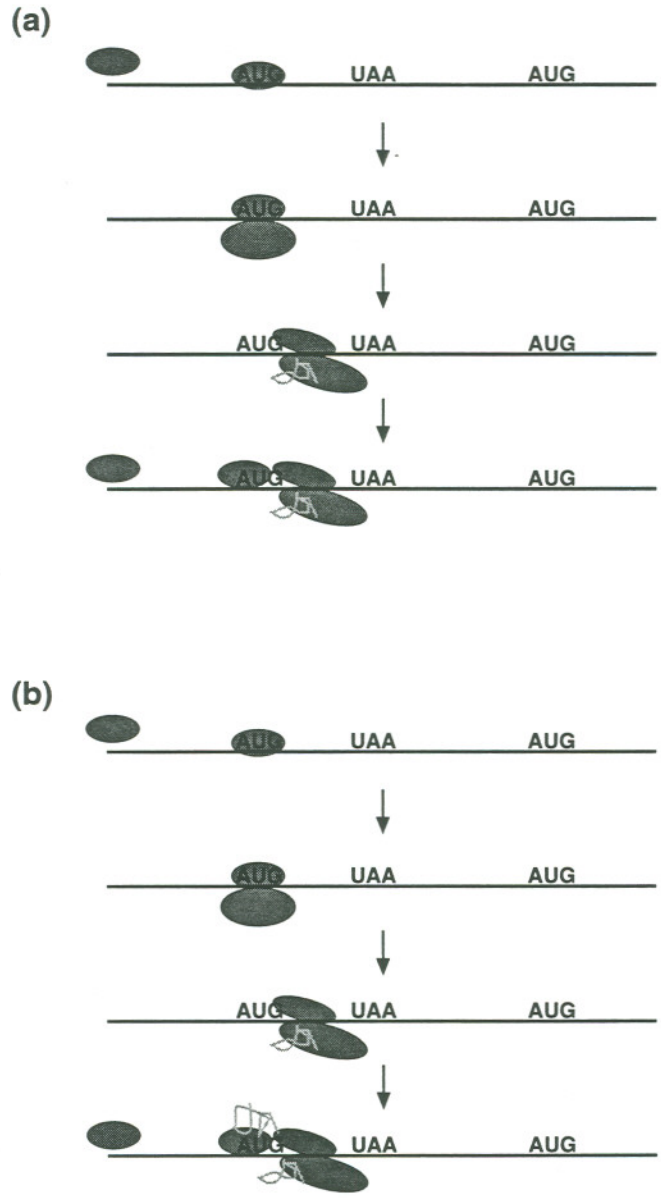

Figure 4.2: A ribosome stalling model can explain the effects of the *arg-2* uORF peptide. (a) elongation or termination of the uORF peptide is inhibited by its own synthesis; (b) the nascent uORF peptide inhibits the translational machinery (see text for details).

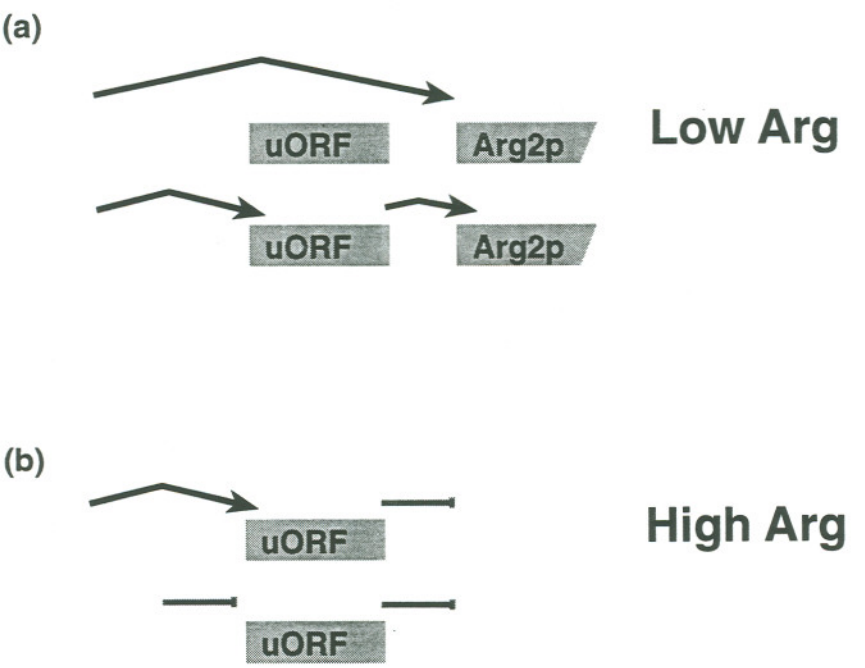

Figure 4.3: Models for the effects of arginine on the translation of the *arg-2* uORF and Arg2p. (a) under conditions of low arginine availability the uORF AUG may be inefficiently recognized and ribosomes initiate at the AUG of Arg2p. Alternatively, reinitiation at the Arg2p start codon may still be efficient, even though the uORF is translated. (b) under conditions of high arginine availability, the uORF AUG may become more efficiently recognized, leading to much enhanced uORF translation and subsequent stalling of ribosomes on the uORF. Alternatively, AUG recognition of both the uORF and the Arg2p AUG become so inefficient that initiation is abolished.

Stalled ribosome models can explain the action of the uORF peptide once it is translated. At present it is not clear exactly how the cell regulates uORF translation depending on the concentration of available Arg. A general model would posit that under conditions of low Arg availability, the uORF AUG would be inefficiently recognized; alternatively, reinitiation at the Arg2p start codon would be efficient, even though the uORF is translated (Fig. 4.3). Under conditions of high Arg availability, the uORF AUG may become more efficiently recognized, leading to enhanced uORF translation and subsequent stalling of ribosomes on the uORF. Alternatively, AUG recognition of both the uORF AUG and the Arg2p AUG may become very inefficient.

Ribosome stalling induced by encounter of the translational machinery of a rare codon, and therefore limited charged tRNA, appear inconsistent with the *CPA1* system, because the codons specified in the *CPA1* uORF differ not significantly from the codon usage in the downstream ORF (Werner, Feller and Piérard 1985). This possibility is not excluded in the *Neurospora arg-2* system, because the second Arg codon is rare compared to the overall codon bias found in *N. crassa* genes (Edelman and Staben 1994).

Other potential models have not been excluded. For example, translation of the uORF peptide could be involved in the destabilization of Arg2p or Cpa1p in the presence of Arg. Translation of the uORF would be required because the uORF peptide would serve as a cofactor for transcript degradation. Clearly, more detailed experiments addressing the mechanism of uORF peptide action and AUG selection under both conditions are needed before a conclusive model for *arg-2* translational regulation can be proposed.

4.9 The D12N mutation resulted in elevated *arg-2–hph* transcript levels in the presence of Arg

Compared to *arg-2–hph* transcript levels from reporter genes containing the wild type uORF, *arg-2–hph* transcript levels in strains carrying the D12N uORF missense mutation were increased when cells were grown in Uri+Arg. This relative increase in transcript level suggests the possibility that translation of the uORF affects mRNA stability. Complete or partial translation of the wild type uORF accompanied by stalling of ribosomes may destabilize the *arg-2–hph* transcript and result in preferential degradation. A missense uORF peptide may not result in stalled ribosomes and therefore result in stabilized transcripts and an apparent increase in *arg-2–hph* transcript. There is precedent for similar mechanisms (reviewed in (Decker and Parker 1994; McCarthy and Kollmus 1995; Muhlrad, Decker and Parker 1995). The relative contributions of transcript synthesis and transcript stability to the net transcript level have yet to be determined for *N. crassa arg-2–*

hph or *arg-2*. In *S. cerevisiae*, Arg decreases *CPA1* transcription and the half-life of *CPA1* mRNA each by a factor of two (Crabeel, Lavalle and Glansdorff 1990). It would not be far-fetched to assume that similar decreases in transcription rate and transcript half-life occur in the *arg-2* system. Taken together, these decreases would result in a 3- to 4-fold apparent reduction of transcript level in the presence of Arg.

4.10 What are the class II mutants?

Mutants of a second class that were able to grow on all Arg-containing media, indicating that the expression of both the *arg-2–hph* and the *arg-2* gene were affected. Genetic analyses revealed that several class II mutations were unlinked to *arg-2–hph*. Hph activities in all class II mutants analyzed to date were negatively regulated by Arg, as were *arg-2* and *hph* transcript levels. There are at least two possibilities to explain these results. First, expression of the *arg-2–hph* reporter gene may be increased, while Arg-specific regulation is maintained. Secondly, the phenotypic assay in plates or tubes asked for colony formation on plates or sporulation in tubes in the presence of Hyg. This was in contrast to Hph activity assays or northern blot analyses which had to be performed on germinated conidia in the early logarithmic growth phase in the absence of Hyg. Therefore, the cultures assayed for *arg-2–hph* transcript level and Hph enzyme activity were in vastly different growth stages when compared to cultures grown continuously on Hyg-containing medium. Expression of *arg-2–hph* may differ from one to the other stage.

It is possible that class II mutants may be affected in the uptake or intracellular compartmentation of Arg. Three of the tested class II mutants were resistant to both DFMO and thialysine, suggesting the possibility that amino acid uptake or localization were affected. There is precedence for the isolation of such mutants. Previously isolated *pmb* (*bat*) mutants enabled *arg-12^{S} pyr-3* strains to grow in the presence of Arg (Perkins *et al.* 1982). Thialysine has been used previously to isolate mutants that were affected in amino acid import in *N. crassa* (Sanchez, Martinez and Mora 1972), *Schizosaccharomyces pombe* (Sychrová *et al.* 1992) and *Candida albicans* (Sychrová and Chevallier 1993). DFMO has similarly been used to isolate mutants in the basic amino acid import (Davis, Lieu and Ristow 1994).

Alternatively, mutations may have affected the import of polypeptides into mitochondria and thus resulted in increased cross-feeding of carbamoyl phosphate from the cytosol into the nuclear pyrimidine pathway. The *arg-2–hph* reporter gene includes the first 10 codons of Arg2p, which are part of the mitochondrial signal sequence (Orbach, Sachs and Yanofsky 1990). Mutations in factors affecting mitochondrial import of Arg2-Hphp

would also be responsible for import of Arg2p and Arg3p, as well as other proteins, thus resulting in increased CPS-A activity in the cytosol and susequent cross-feeding from the cytosol to the nucleus. This type of mutant would be expected to be leaky and its phenotype quite dependent on the concentration of Arg and Hyg used.

Class II mutants may also carry defects directly affecting translation. *S. cerevisiae gcd* mutants, which are resistant to amino acid analogs (including thialysine), increase the translation of the uORF-containing *GCN4* gene because they alter components of the translational machinery and affect translation initiation (Hinnebusch 1992; Sachs in press).

4.11 Conclusions

The understanding of translational control mechanisms that are important contributors in the regulation of uORF-containing eukaryotic genes is increasing rapidly. Few examples are known in which the stimuli responsible for translational control mechanisms are as well-defined as the negative regulation of *arg-2* or *CPA1* by Arg. One such example is the translational regulation of the *S. cervisiae* general control transcriptional activator, Gcn4p by the action of two short uORFs, triggered by amino acid starvation or imbalance (Hinnebusch 1993; Hinnebusch 1994). Importantly, the *GCN4* regulatory system differs from the systems discussed here in that the *GCN4* uORFs do not act in a peptide sequence-dependent manner.

The evidence presented here, indicates that *N. crassa arg-2* and *cpc-1* are examples of uORF-containing genes that are translationally controlled in response to amino acid availability. By combining reverse and classical genetic approaches, such as those that defined the importance of the *arg-2* uORF in Arg-specific negative regulation, with biochemical approaches such as studies of distribution of transcripts on polysomes and studies of protein synthesis rates, a better understanding of the mechanisms responsible for *arg-2* regulation has been achieved. Multiple levels of control, acting under short-term or long-term exposure of cells to Arg, are present to finely tune the activity of *arg-2* and therefore Arg metabolism in *N. crassa*. A translational control mechanism appears to be primarily responsible for the 3-fold decrease in Arg2p following short-term exposure of cells to Arg, whereas both changes in transcript level and translational efficiency were observed under conditions of long-term exposure to Arg. The mutational analyses described here were successful in that one mutation was characterized that directly affected the sequence of the uORF peptide. With the tools developed here, namely reporter plasmids, shuttle plasmids for retransformation of subcloned PCR fragments, Hph activity and polysome assays, it should be feasible to not only find more *in vivo* mutants (if so

desired), but also to facilitate studies of *in vitro* mutagenized translational fusions of selected 5' leader sequences of *arg-2* to *hph*.

Overall, similarities between the organization of *arg-2* and *CPA1*, and *cpc-1* and *GCN4*, suggest that the mechanisms that regulate amino acid biosynthesis were in place before the *N. crassa* and *S. cerevisiae* ancestors diverged. It is interesting to note 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, Sachs and Yanofsky 1990).

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). Presumably initiation occurs most often after scanning from the cap structure of the mRNA to the first AUG in favorable context; however, internal initiation, as sometimes seen in viruses and mammals, may also occur in *N. crassa* (Vittorioso *et al.* 1994). 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-1*. It is difficult to assess, how widespread translational control by sequence-dependent uORFs is in a single organism, let alone among fungi.

In conclusion: (1) multiple levels of Arg-specific negative control of *arg-2* and *arg-2–hph* genes in transformed *N. crassa arg-12^{S} pyr-3* strains have been demonstrated; (2) a novel approach to isolate regulatory mutants in *N. crassa* by making use of reverse genetics to engineer reporter genes and analyses of mutations by classical genetic methods has been described; (3) the *arg-2* uORF has been shown to affect translation of *arg-2–hph* in an Arg-specific and sequence-dependent manner, because a mutation that was predicted to alter the uORF peptide sequence eliminated regulation of *arg-2–hph* translation, as assayed by polysomal distribution of *arg-2–hph*, regulation of Hph activity and altered *arg-2–hph* transcript levels; (4) these results strongly indicate that the *cis*-acting *arg-2* uORF peptide is important in Arg-specific regulation of *arg-2* via inhibition of translation of the downstream ORF.

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Biographical Sketch

Michael Freitag was born in Hannover, Germany, on July 2, 1961. He has been living in and around Corvallis, Oregon, since 1987, the last seven years with his wife Camille and his two stepsons Nathan and Colin.

After attending high school in Hameln, Michael enrolled in the Forstliche Fakultät der Georg-August-Universität Göttingen to study forestry in the fall of 1980. He finished his studies in 1986 with a master's degree in forestry (Diplom-Forstwirt). During his studies in Göttingen, Michael received two IAESTE travel grants that allowed him to work, study and travel for six months in both Turkey (1984) and Tasmania, Australia (1987).

In 1987, Michael enrolled in the College of Forestry at Oregon State University in Corvallis in the Department of Forest Products. Under the supervision of Dr. Jeffrey J. Morrell, he earned a master's degree in Forest Products (1990). His thesis work focussed on biological control of wood decay and stain fungi on lumber. During his studies at OSU, Michael received a South Santiam Graduate Student Fellowship awarded by the College of Forestry.

In 1990, Michael started his Ph. D. research in the laboratory of Dr. Matthew S. Sachs in the Department of Chemistry, Biochemistry and Molecular Biology at the Oregon Graduate Institute of Science & Technology. He earned his Ph. D. in Biochemistry and Molecular Biology in 1996. Michael received a travel grant to the Annual Meeting of the Mycological Society of America in 1995; at the same meeting he received an award for the best oral presentation of research performed by a graduate student. In 1995 he also won the 2nd Annual Outstanding Graduate Student Award of the Oregon Graduate Institute of Science & Technology.

Fungi in general and their evolution and speciation in particular are the focus of Michael's professional interests. He is especially interested in the regulation of fungal genes and sexual and asexual development of fungi. He now works as a postdoctoral research associate with Dr. Eric U. Selker at the University of Oregon, Eugene, on the comtrol of DNA methylation in the ascomycete *Neurospora crassa*.