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Translational Regulation in Response to Changes in Amino Acid Availability in *Neurospora crassa*

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We examined the regulation of *Neurospora crassa arg-2* and *cpc-1* in response to amino acid availability. *arg-2* encodes the small subunit of arginine-specific carbamoyl phosphate synthetase; it is subject to unique negative regulation by Arg and is positively regulated in response to limitation for many different amino acids through a mechanism known as cross-pathway control. *cpc-1* specifies a transcriptional activator important for cross-pathway control. Expression of these genes was compared with that of the cytochrome oxidase subunit V gene, *cox-5*. Analyses of mRNA levels, polypeptide pulse-labeling results, and the distribution of mRNA in polysomes indicated that Arg-specific negative regulation of *arg-2* affected the levels of both *arg-2* mRNA and *arg-2* mRNA translation. Negative translational effects on *arg-2* and positive translational effects on *cpc-1* were apparent soon after cells were provided with exogenous Arg. In cells limited for His, increased expression of *arg-2* and *cpc-1*, and decreased expression of *cox-5*, also had translational and transcriptional components. The *arg-2* and *cpc-1* transcripts contain upstream open reading frames (uORFs), as do their *Saccharomyces cerevisiae* homologs *CPA1* and *GCN4*. We examined the regulation of *arg-2–lacZ* reporter genes containing or lacking the uORF start codon; the capacity for *arg-2* uORF translation appeared critical for controlling gene expression.

Fungal amino acid biosynthetic genes and their products are subject to multiple levels of control (12, 26, 29, 30, 48). We examined translational and transcriptional modulation of the expression of two genes involved in *Neurospora crassa* amino acid biosynthesis, *arg-2* and *cpc-1*. The *arg-2* gene specifies the small subunit of arginine-specific carbamoyl phosphate synthetase (CPS-A) (41) and was among the first metabolic genes identified (58). *cpc-1* specifies a transcriptional activator of amino acid biosynthetic genes that positively regulates gene expression in response to amino acid starvation; it resembles *Saccharomyces cerevisiae GCN4* and mammalian c-*jun* (43, 48).

Control of *arg-2* expression regulates the flux of metabolites through the arginine (Arg) biosynthetic pathway under most growth conditions (12, 15). Three separate control mechanisms regulate *arg-2* expression. *arg-2* is the only component of the *N. crassa* Arg biosynthetic pathway known to be negatively regulated by Arg (12). Decreased activity is associated with a reduced level of Arg2p polypeptide in mitochondria (13). Growth with Arg also reduces the level of *arg-2* mRNA (41, 52). Second, *arg-2* is positively regulated in response to amino acid starvation, as are many genes involved in *N. crassa* amino acid metabolism (48). 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 (41, 52). Negative, Arg-specific regulation of *arg-2* is independent of cross-pathway control and does not require a functional *cpc-1* gene (52). Finally, *arg-2* is developmentally regulated in that the highest level of *arg-2* mRNA is found during spore germination and early exponential growth (52).

The *arg-2* homolog in *S. cerevisiae*, *CPA1*, is also subject to unique, Arg-specific negative regulation (12). In one study, Arg reduced *CPA1* transcription and *CPA1* transcript stability, as well as having an additional posttranscriptional effect (8). Other work identified the sites of *cis*-acting mutations that resulted in a loss of Arg-specific negative regulation of *CPA1*: these mutations were in a region of DNA specifying a 25-codon upstream open reading frame (uORF) and affected the formation of the predicted uORF peptide (65). In this study, growth with Arg was reported to reduce enzyme activity without reducing the level of *CPA1* transcript. Thus, genetic evidence has implicated a role for uORF translation in Arg-specific negative regulation of *CPA1* (16, 65). However, while translational control has been invoked to explain *CPA1* regulation by Arg, there has been no direct biochemical evidence for a translational component to regulation of *arg-2* or *CPA1*, although Arg has been shown to reduce the levels of *arg-2* and *CPA1* transcripts under steady-state conditions (8, 41, 52). As with *arg-2*, *CPA1* is also positively regulated in response to amino acid starvation (12). Positive regulation of *CPA1* at the transcript level in response to amino acid starvation requires the general control transcriptional activator *GCN4* (31).

The phenomenon of cross-pathway control of amino acid biosynthesis, in which starvation for one amino acid results in the induction of multiple amino acid biosynthetic genes, was first discovered in *N. crassa*; a similar phenomenon observed in *S. cerevisiae* has become well known as the general control of amino acid biosynthesis (26, 48). *N. crassa cpc-1* and *S. cerevisiae GCN4* encode similar bZIP polypeptides that appear to function as positive transcriptional activators of amino acid biosynthetic genes in the cross-pathway control/general control responses to amino acid starvation (26, 48). A transcriptional component to *cpc-1* regulation has been demonstrated (43, 52); a translational control component has been postulated on the basis of the presence of two uORFs (43), but there has been no direct evidence for translational control. There is also evidence for posttranslational modification of Cpc1p (19), suggesting the possibility of additional control mechanisms. Control of *S. cerevisiae GCN4* expression has transcriptional, translational, and posttranslational components (25, 33); it has been established that two of the four uORFs, uORF1 and uORF4,

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found in the 5′ region of the *GCN4* transcript are necessary for translational control (26).

Here we provide direct biochemical evidence for translational regulation of *arg-2* in response to Arg. The *arg-2* transcript contains a 24-codon uORF specifying a peptide similar to the *CPA1* uORF product (41). We also use *arg-2–lacZ* reporter genes targeted to the *N. crassa his-3* locus to show that eliminating the *arg-2* uORF translation initiation codon abolished Arg-specific negative regulation. We also examined the effects of histidine (His) limitation on *arg-2* and *cpc-1* by exposing cells to 3-amino-1,2,4-triazole (AT), a competitive inhibitor of imidazole glycerol phosphate dehydratase (4). When His was limited, levels of *cpc-1* and *arg-2* mRNAs increased, and these transcripts were found on larger polysomes than the *cox-5* mRNA control, indicating regulation at multiple levels in response to amino acid limitation in *N. crassa.*

MATERIALS AND METHODS

Growth of *N. crassa*. Wild-type strain 74-OR23-1VA (74A) was obtained from D. Perkins, Stanford University. Procedures described previously (52) were used to grow *N. crassa*, except that macroconidia were harvested with water to inoculate cultures for experimental analyses. Minimal (Min) growth medium was composed of 1× Vogel's N medium (63) and 2% sucrose. Min medium was supplemented prior to autoclaving with 0.5 mg of Arg or His per ml where indicated. Cells were starved for His by supplementing Min medium with 10 mM AT. AT was added from a fresh, filter-sterilized 0.1 M stock prepared in water after media were autoclaved and cooled. In typical experiments, 30 ml of medium in 125-ml Erlenmeyer flasks was inoculated with 2×10^7 conidia per ml, and cultures were grown at 34°C with orbital shaking (125 rpm). For experiments in which cells were switched to different media, cells were grown for 6.5 h and harvested by vacuum filtration onto Whatman no. 541 filters. The mycelial mat was peeled off the filter and resuspended by vigorous shaking in fresh medium.

Enzyme assays. CPS-A was assayed in preparations of purified *N. crassa* mitochondria as previously described (14). Cultures were grown in 700 ml of Min or Arg medium in 2-liter Erlenmeyer flasks for 24 h at 34°C with gentle agitation, harvested in cheesecloth, and washed with extraction buffer [10 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES; pH 7.5), 0.33 M sucrose, 0.3% bovine serum albumin (BSA), 1 mM EDTA]. To isolate mitochondria, cells were broken in a 250-ml Beadbeater (Biospec, Bartlesville, Okla.) with acid-washed 0.5-mm-diameter glass beads, using three successive 30-s bursts followed by 30-s rests on ice. The homogenate was filtered through cheesecloth and centrifuged for 6 min at 600 × *g* in a GSA rotor (Sorvall); the supernatant was removed and centrifuged for 20 min at 15,000 × *g* in a GSA rotor. The pellet was resuspended in 10 ml of extraction buffer containing 1 mM phenylmethylsulfonyl chloride, layered onto 20-ml sucrose step gradients (1.6 and 1.2 M sucrose in extraction buffer), and centrifuged for 20 min at 45,000 × *g* in an SS34 rotor (Sorvall). Pure mitochondria from the interphase between the gradient steps were resuspended in 1 ml of extraction buffer containing 1 mM phenylmethylsulfonyl fluoride 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 broken in a Mini-Beadbeater (Biospec) in 1.35 ml of extraction buffer and 1 g of 0.5-mm-diameter acid-washed glass beads for 60 s and centrifuged at 13,000 × *g* for 10 min, and the membrane-free mitochondrial extract was transferred to a fresh tube. Mitochondrial extracts (100 μl) were mixed with 400 μl of 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. Residual $^{14}CO_2$ was 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). The yields of reaction products were determined by scintillation counting. One relative unit of activity is defined as 1,000 cpm/mg of mitochondrial protein.

Fumarase was assayed as previously described (59). Briefly, 300 μl of purified mitochondria was added to 670 μl of reaction buffer [100 mM TES (pH 9.5), 100 mM $(NH_4)_2SO_4$, 30 μl of 0.5% Triton X-100], the reaction was initiated by addition of 30 μl of 10 mM potassium fumarate, and the decrease in A_{240} was monitored. Activity is defined as the change in absorbance per minute per milligram of protein.

Preparation of anti-Arg2p serum. The 1,828-bp *Kpn*I fragment of the *arg-2* gene includes the coding region for residues 68 to 453 of the predicted Arg2p polypeptide (41). A plasmid was constructed to fuse this region of Arg2p to glutathione *S*-transferase for production of recombinant protein in *Escherichia coli*. The *arg-2 Kpn*I fragment was first placed into the *Kpn*I site of pSP72 (Promega) to create pIG8; then the *Bam*HI-*Bgl*II fragment from pIG8 containing *arg-2* DNA was placed in the *Bam*HI site of pGEX-KG (23) in the appropriate orientation to generate pDM-1. *E. coli* DH5αF′ was transformed with pDM1, and production of the fusion protein induced with isopropylthiogalactopyranoside (IPTG). The glutathione *S*-transferase–Arg2p fusion protein was localized to inclusion bodies. Inclusion bodies were isolated and solubilized, and the fusion protein was purified by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (24). Polyclonal antiserum directed against the fusion protein was prepared by Pocono Rabbit Farms (Canadensis, Pa.), using a rabbit pretested for low initial reactivity against *N. crassa* polypeptides.

Polyclonal antisera directed against cytochrome oxidase subunit V and cytoplasmic leucyl-tRNA synthetase have been previously described (2, 50).

Immunoblot analyses. Cell extracts were prepared from freshly harvested mycelia. Mycelia (ca. 1 g [wet weight]) were added to 0.8 g of ice-cold acid-washed glass beads (0.5-mm diameter) in 2-ml screw-cap Eppendorf tubes (Sarstedt) that also contained 1 ml of breaking buffer (20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES]-NaOH [pH 7.9], 100 mM KCl, 2 mM EDTA, 10 mM dithiothreitol, 20% glycerol [51]). Tubes were filled completely with breaking buffer, and cells were broken in the cold room by two 1-min disruption cycles in a Mini-Beadbeater (Biospec) separated by a 2-min chill on ice. Extracts were clarified by centrifugation for 10 min at 16,000 × *g* at 4°C. Clarified whole cell extracts were transferred to a fresh tube, quick-frozen in liquid nitrogen, and stored at −80°C. Protein concentrations were determined by the Bradford assay, using BSA as the standard (3). For comparisons, equal amounts of total protein (20 to 40 μg) for each sample were adjusted to equal volumes with SDS sample buffer (50 mM Tris-HCl, [pH 6.8], 0.05% bromophenol blue, 1% SDS, 10% glycerol, 2% β-mercaptoethanol), boiled, and examined by SDS-PAGE in 10% polyacrylamide gels. Polypeptides were transferred to PolyScreen membranes (DuPont) by electroblotting according to the manufacturer's protocol. Membranes were incubated for 1 h in PBST (5% nonfat dry milk, 0.83 mM NaH_2PO_4, 8.1 mM Na_2HPO_4, 145 mM NaCl, 0.05% Tween 20). Antiserum (1:5,000 [vol/vol]) was added, and membranes were incubated with serum for 1 h. Membranes were washed three times (5 min each) in PBST and incubated for 1 h in PBST containing anti-rabbit immunoglobulin G-alkaline phosphatase conjugate (Promega). Membranes were again washed in PBST and air dried. Antigenically reactive polypeptides were visualized by using alkaline phosphatase-coupled antibody, 5-bromo-4-chloro-3-indolylphosphate toluidinium (BCIP), and nitroblue tetrazolium (53).

Pulse-labeling with [^{35}S]methionine and immunoprecipitation. The technique for labeling of *N. crassa* cells with [^{35}S]methionine and immunoprecipitation of newly labeled peptides was adapted from a procedure devised for *S. cerevisiae* (32). For a typical labeling reaction, 1 ml of culture was placed in a 50-ml Falcon tube; 100 μCi of [^{35}S]methionine (800 Ci/mmol; New England Nuclear) was added, and the culture incubated for 5 min at 34°C with gentle agitation. The amount of [^{35}S]Met was not limiting under these conditions, because incorporation of radioactivity into polypeptide doubled, as assayed by hot-trichloroacetic acid (TCA) precipitation, when twice the standard amount of cells was used.

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

To compare the relative rates of synthesis of different polypeptides under different growth conditions, equal amounts of pulse-labeled polypeptides (2×10^6 hot-TCA-precipitable cpm) were used for immunoprecipitation. That equal amounts of TCA-precipitable counts per minute represented equal amounts of labeled protein was confirmed by SDS-PAGE and autoradiography: densitometric analysis revealed less than 10% differences among samples. For immunoprecipitation, samples were diluted to 1 ml with IP (immunoprecipitation) buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.1 mM EDTA, 0.5% Tween 20, 1 mM phenylmethylsulfonyl fluoride) containing antiserum (2 μl) and incubated for 1 h at 4°C with gentle rotation. Protein A-Sepharose 6MB beads (30 μl of beads per 60 μl of IP buffer) were added; incubation with antiserum and beads was continued overnight at 4°C with gentle rotation. Beads were collected by centrifugation, and the supernatant was aspirated. Beads were washed three times with 1 ml of IP buffer and resuspended in 20 μl of SDS sample buffer. Beads were heated to 90°C for 8 min, and the supernatant was collected for SDS-PAGE (53). Gels were stained in Coomassie brilliant blue R-250, destained, treated with sodium salicylate (5), dried, and exposed to X-ray film. Control experiments in which immunoprecipitations were repeated with supernatants from the initial immunoprecipitations as starting material indicated that more than 90% of immunoprecipitable antigen was recovered in the first immunoprecipitation.

Preparation of RNA. Small-scale total RNA samples were prepared by breaking 50 to 100 mg of frozen mycelia in 2-ml screw-cap tubes with 0.7 g of acid-washed 0.5-mm-diameter glass beads, 840 μl of phenol-chloroform, 580 μl of extraction buffer (50 mM Tris-HCl [pH 7.5], 100 mM LiCl, 20 mM dithiothreitol), and 84 μl of 10% SDS for 40 s in a Mini-Beadbeater (Biospec). Tubes

were centrifuged in a microcentrifuge at 13,000 × *g* for 5 min. The aqueous phase was removed and extracted once with 840 μl of phenol-chloroform and once with 800 μl of chloroform. RNA was precipitated twice with ethanol and sodium acetate, dissolved in sterile diethyl pyrocarbonate (DEPC)-treated water, and stored at −80°C.

Large-scale total RNA samples were prepared by modification of a previously described method (52). Cells frozen in liquid nitrogen (0.5 to 1 g [wet weight]) were broken for 40 s in phenol (7.5 ml), chloroform (7.5 ml), 10% SDS (1.5 ml), and 15 ml of extraction buffer, using a 250-ml stainless steel Waring blender. The homogenate was transferred to 50-ml centrifuge tubes, and phases were separated by centrifugation. The aqueous phase was removed and extracted once with 15 ml of phenol-chloroform and once with 15 ml of chloroform. RNA was precipitated twice with ethanol and sodium acetate, dissolved in sterile DEPC-treated water, and stored at −80°C.

Poly(A) RNA was prepared from large-scale total RNA samples by oligo(dT)-cellulose chromatography as described previously (52).

Northern (RNA) blot analyses. Radioactive probes for Northern blot analyses were prepared by using gel-purified *N. crassa* DNA fragments obtained by digestion of recombinant plasmids with appropriate restriction enzymes. The *arg-2* probe was the 1.3-kb *Pvu*II-*Nco*I cDNA fragment of *arg-2* (41); the *cox-5* probe was the 0.77-kb *Eco*RI fragment from pSRCOX5 (49); the *cpc-1* probe was the 1.2-kb *Bgl*II-*Bam*HI fragment from pCPC-1-2 (43); the *leu-6* probe was the 1.1-kb *Bam*HI-*Pst*I genomic fragment (2, 6) from pBX1a; the *lacZ* probe was the 0.83-kb *Sac*I-*Eco*RV fragment from pDE2 (17). Probes were labeled with [α-^{32}P]dCTP by random priming (20). Unincorporated label was removed by spin chromatography through Sephadex G-50 columns (Boehringer Mannheim). The methods used for gel electrophoreses of RNA, membrane blotting, and probe hybridization were as described previously (52) except that dextran sulfate was omitted from the hybridization solution.

Polysome analyses. The procedures for polysome preparation and analyses were adapted from previously described procedures (7, 47). Cycloheximide (100 μg/ml) was added to cultures 5 min prior to harvesting. Mycelia (0.5 g [wet weight]) were harvested by vacuum filtration onto Whatman no. 541 filters, transferred to 2-ml screw-cap tubes containing ice-cold 0.5 g of acid-washed glass beads (0.5-mm diameter) and 1.5 ml of polysome extraction buffer (100 mM KCl, 20 mM HEPES-KOH [pH 7.5], 2 mM magnesium acetate, 15 mM 2-mercaptoethanol, 100 μg of cycloheximide per ml), and disrupted for 50 s in a Mini-Beadbeater at 4°C. Homogenates were centrifuged at 4°C for 5 min at full speed in a microcentrifuge. Supernatants (0.9 ml) were transferred to fresh 2-ml screw-cap tubes containing 100 μl of 50-mg/ml heparin and 250 μl of 50% glycerol, quick-frozen in liquid nitrogen, and stored at −80°C until further analysis.

For polysome analyses, 10 or 16.7 A_{260} units of homogenate, in a maximum volume of 400 μl, was layered on 12-ml linear sucrose gradients (15 to 50% [wt/wt] sucrose in 10 mM HEPES-KOH [pH 7.5]–70 mM ammonium acetate–4 mM magnesium acetate). Gradients were centrifuged in a Beckman SW41 rotor at 40,000 rpm for 2 h at 4°C. Twelve 1-ml fractions were collected from the bottom with a Hoefer gradient tube fractionator into screw-cap tubes containing 50 μl of 10% SDS; polysome profiles were generated by monitoring the A_{254} with an ISCO UA5 absorbance monitor. Fractions were quick-frozen in liquid nitrogen and stored at −80°C until further processing.

Polysomal RNA was extracted by mixing each fraction with 1 ml of phenol-chloroform (1:1) for 1 min in the Mini-Beadbeater. Phases were separated by centrifugation for 10 min at full speed in a microcentrifuge. The aqueous phase was transferred to a fresh tube containing 1 ml of chloroform; mixing and centrifugation were repeated. The aqueous phase was transferred to a fresh tube, and RNA was precipitated with sodium acetate (pH 5.5) and isopropanol. RNA was centrifuged, and the supernatant was 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.

Quantification. The values reported in Tables 2 and 3 represent averages for two independent growth experiments, each examined for duplicate samples. Differences among individual samples used for averaging were all less than 15%. The relative levels of polypeptides visualized by Western blotting (immunoblotting) were analyzed by scanning the blots with a Microtek MSF 300ZS scanner and analyzing the data with NIH Image version 1.53. The relative levels of mRNA detected by RNA blotting and the levels of pulse-labeled polypeptide detected following immunoprecipitation and SDS-PAGE were determined by densitometric analysis of film images, using a Bio-Rad model 620 Video Densitometer or by direct analysis with a Molecular Dynamics PhosphorImager. Quantitative methods were validated through reconstruction experiments using a dilution series of comparable samples.

Plasmid constructions for *lacZ* expression studies and *N. crassa* transformation. Plasmid pFo2 was generated by subcloning the 1.1-kb *Kpn*I-*Eco*RI fragment of pAE1 (41) into the corresponding sites of pUC119. This fragment of pAE1 contains the 5′ region of *arg-2* including the uORF. pFo2ΔAUG was generated by site-directed mutagenesis (39) of pFo2 with the oligonucleotide 5′-TTGTCG CAATCTGCCCTCgagAACGGGCGCCG-3′. This oligonucleotide replaces the ATG start codon of the *arg-2* uORF with GAG and generates a novel *Xho*I site in its place.

TABLE 1. Effects of growth in Arg on the levels of *arg-2*- and *arg-3*-dependent CPS-A activity

Strain	Activity[a]					
	arg-2-dependent CPS-A[b]		*arg-3*-dependent CPS-A[c]		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] Reported as relative units (see Materials and Methods). M, Min medium; R, Arg 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.

pZL601 was generated by first treating the 0.85-kb *Eco*RI-*Sty*I fragment from pFo2 with the Klenow fragment of DNA polymerase I and deoxynucleoside triphosphates to generate flush ends and then ligating this fragment into the *Sma*I site of pDE3, a vector designed for placing *lacZ* reporter genes at the *his-3* locus (17). This construct creates a translational fusion of the LacZ coding region with the Arg2p coding region at codon 10 of the predicted Arg2p polypeptide. pZL610 was generated by the corresponding ligation of the filled 0.85-kb *Eco*RI-*Sty*I fragment from pFo2ΔATG into the *Sma*I site of pDE3. The structures of these constructs were verified by DNA sequencing (54).

The *N. crassa his-3* strain (FGSC 6103) was transformed with plasmids pZL601, pZL610, and pDE1 (a plasmid similar to pDE3 [17] used as a control) as described previously (56), and transformants were obtained by selection for His prototrophy (51). The recipient strain was chosen because the *his-3* mutation that it contained mapped to the distal region of the gene (42). Prototrophic *N. crassa* homokaryons were obtained by microconidiation (18).

Southern blot analyses. Southern blot analysis (53) was accomplished by using *N. crassa* genomic DNA prepared by a modification of a previously described procedure (40); sodium trichloroacetate used in DNA isolation was obtained from Aldrich, St. Louis, Mo. The amount of DNA recovered was quantified by using Hoechst 33258 (35) and a Hoefer TKO-100 fluorometer as instructed by Hoefer. One microgram of DNA digested with an appropriate restriction enzyme was loaded per lane. The *E. coli lacZ* probe was the 0.83-kb *Sac*I-*Eco*RV fragment from pDE2 (17); the *arg-2* probe was the 0.85-kb *Eco*RI-*Sty*I fragment from pFo2ΔATG; the *his-3* probe was the 1.7-kb *Xho*I fragment from pDE2. Hybridization and washing conditions were the same as those used for Northern analyses.

β-Galactosidase assays. Cell extracts were prepared as described above for immunoblot analyses. β-Galactosidase activity was assayed by using 50 μg of protein as described previously (36, 51).

RESULTS

Prolonged exposure to Arg reduces CPS-A activity, the level of Arg2p, the rate of Arg2p synthesis, and the level of *arg-2* transcript. The *arg-2* (glutamine-dependent) and *arg-3* (ammonium-dependent) activities of CPS-A were measured to examine the effects of growth in Arg medium on enzyme activity. Wild-type *N. crassa* and the *arg-12*^{s} mutant were grown for 24 h in Min or Arg medium to generate sufficient material for enzyme assays. The *arg-12*^{s} mutant has a leaky block in ornithine transcarbamoylase and a reduced capacity to synthesize Arg in Min medium (11). *arg-2* is known to be highly expressed when the *arg-12*^{s} mutant is grown in Min medium, with increases in both enzyme and transcript levels (12, 52).

In wild-type *N. crassa*, the *arg-2*-dependent activity of the holoenzyme was reduced threefold when Arg was present, whereas the *arg-3*-dependent activity of the large subunit of CPS-A was not regulated by exogenous Arg (Table 1). In the *arg12*^{s} strain, *arg-2*-dependent CPS-A activity was fivefold greater in Min medium than observed for the wild type; in Arg medium, *arg-2*-dependent activity was lower than the wild-type activity. Overall, 35-fold modulation of *arg-2*-dependent activ-

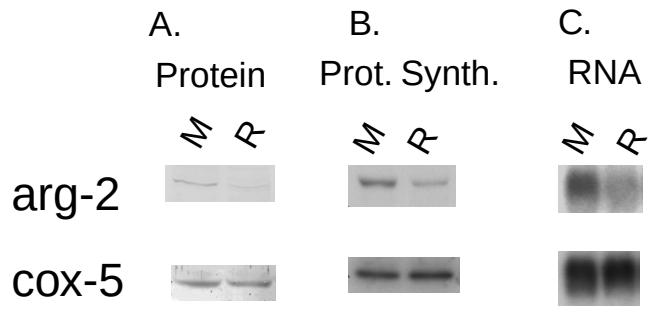

FIG. 1. Effects of prolonged exposure to Arg on the expression of *arg-2* and *cox-5*. Wild-type *N. crassa* cells were germinated for 6.5 h in Min (M) or Arg (R) medium. (A) levels of Arg2p and Cox5p examined by Western blot analysis; (B) protein synthesis (Prot. Synth.) rates of Arg2p and Cox5p examined by immunoprecipitation following a 5-min pulse-label with [^{35}S]methionine; (C) *arg-2* and *cox-5* transcript levels examined by Northern blot analysis.

ity was observed in the *arg-12^s* strain, compared with 1.7-fold modulation of *arg-3*-dependent activity. The mitochondria used for CPS-A assays were of similar quality in all samples, as judged by similarities in the levels of the mitochondrial matrix enzyme fumarase (Table 1).

The expression of *arg-2* was compared with the expression of *cox-5* in wild-type cells grown for 6.5 h in Min or Arg medium. The *cox-5* gene, which encodes cytochrome oxidase subunit V, was used as a control because it is not directly involved in amino acid metabolism. Exogenous Arg reduced the level of Arg2p and the rate of Arg2p synthesis but not the level of Cox5p or the rate of Cox5p synthesis (Fig. 1A and B). The level of *arg-2*, but not *cox-5*, transcript was also reduced by growth with Arg (Fig. 1C), as previously observed (41, 52). As judged from quantification of experimental data, including the results shown in Table 1 and Fig. 1, the observed reductions in the level of *arg-2*-dependent enzyme activity (3-fold), the cellular level of Arg2p (2.9-fold), the rate of Arg2p synthesis (3.3-fold), and the level of *arg-2* transcript (2.9-fold) were similar. Thus, within the precision of these measurements, the difference in the level of RNA appeared sufficient to account for most of the difference in the relative rate of Arg2p translation and the level of Arg2p polypeptide in wild-type cells, but effects at the level of translation could not be excluded.

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

Short-term exposure to Arg reduces the rate of Arg2p synthesis but not the level of *arg-2* transcript. The effect of short-term exposure to Arg was examined by switching cells grown for 6.5 h in Min medium to fresh Min or fresh Arg medium for 2, 10, or 30 min and comparing the expression levels of *arg-2* and *cox-5* (Fig. 3; Table 2). The levels of Arg2p and Cox5p were not affected by transfer of cells from Min to fresh Min

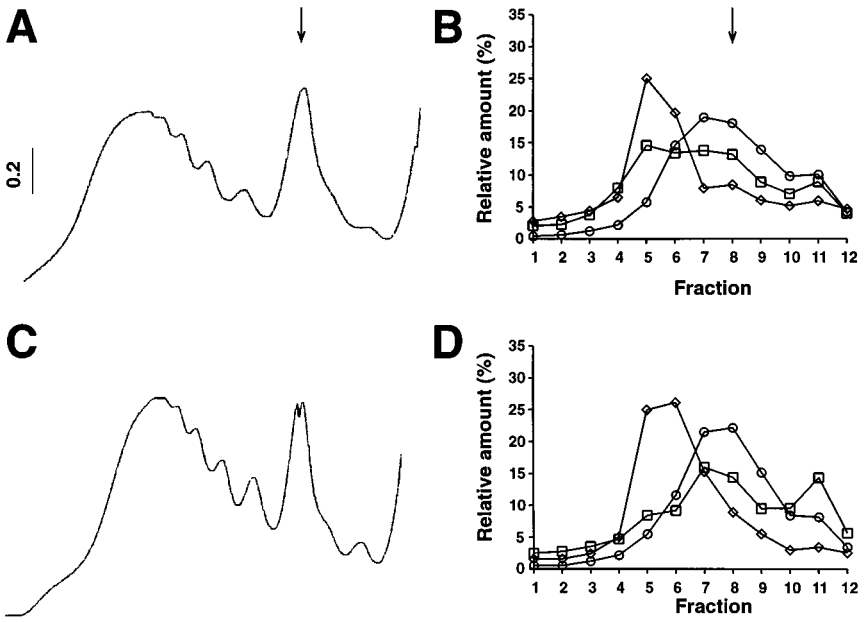

FIG. 2. Analysis of mRNA distribution in polysomes prepared from cells grown in Min and Arg media. Wild-type *N. crassa* cells were germinated for 6.5 h in Minimal or Arg medium. Extracts were prepared and separated through sucrose gradients. (A and C) A_{254} profiles of gradients from cells grown in Min and Arg media, 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} units was loaded. (B and D) Distribution of *arg-2* (squares), *cox-5* (diamonds), and *cpc-1* (circles) mRNA in gradient fractions from cells grown in Min and Arg media, 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 is the top. The position of the monosome peak is indicated with an arrow.

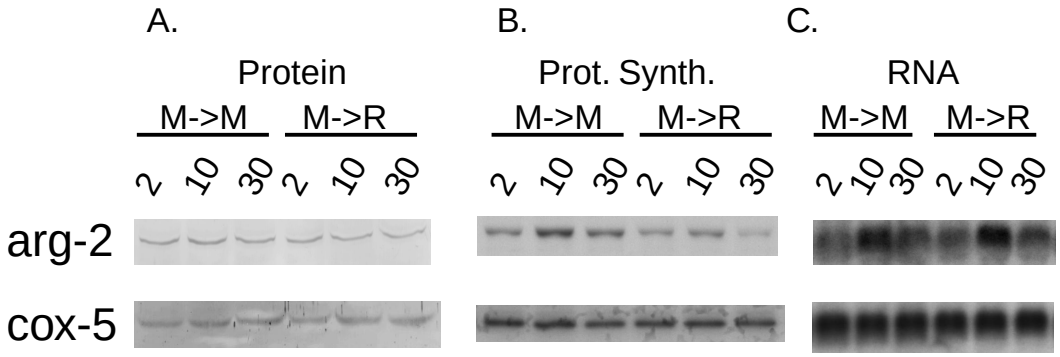

FIG. 3. Effects of short-term cell exposure to Arg on *arg-2* and *cox-5*. Wild-type cells were grown in Min medium (M) for 6.5 h and then switched to either fresh Min or Arg medium (R) for 2, 5, and 30 min, respectively. (A) Levels of Arg2p and Cox5p examined by Western blot analysis; (B) protein synthesis (Prot. Synth.) rates of Arg2p and Cox5p examined by immunoprecipitation following a 5-min pulse-label with [^{35}S]methionine; (C) transcript levels of *arg-2* and *cox-5* examined by Northern blot analysis.

medium for 2, 10, or 30 min (Fig. 3A). In this experiment, we observed a 35% reduction in the level of Arg2p but not Cox5p in cells transferred from Min to Arg medium. This reduction in the level of Arg2p after short-term exposure to Arg was not always observed (data not shown).

Pulse-labeling data showed that transfer to Arg instead of Min medium significantly reduced the rate of Arg2p synthesis but not Cox5p synthesis (Fig. 3B; Table 2). The level of *arg-2* transcript was not reduced during short-term exposure to Arg medium (Fig. 3C; Table 2); it was similar in cells switched to either Min or Arg medium, with a transient increase in *arg-2* transcript level observed 10 min after transfer to either medium (Fig. 3B; Table 2). Comparison of the level of Arg2p synthesis with the level of *arg-2* transcript following shifts to Min or Arg medium indicated that net synthesis of Arg2p per unit of *arg-2* transcript was reduced twofold within 10 min of transfer to Arg medium compared with transfer to Min medium (Table 2). The rate of Cox5p synthesis and the level of *cox-5* transcript were unaffected by switching to either fresh Min or Arg medium (Fig. 3B and C). These data indicate that following transfer to fresh Arg medium, the efficiency of Arg2p translation and/or the stability of newly synthesized Arg2p polypeptide were reduced.

In eukaryotes, polyadenylation of mRNA can play a role in its translatability (46). To determine whether the reduction of the rate of Arg2p synthesis was due to deadenylation of *arg-2* mRNA, we examined the levels of *arg-2* and *cox-5* transcripts in poly(A) mRNA prepared from cells that were switched from Min medium to either fresh Min medium or Arg medium for 30 min. Comparison of the relative amounts of *arg-2* and *cox-5* transcript in poly(A) preparations from cells grown in Min or Arg medium indicated that the level of *arg-2* mRNA containing poly(A) tails sufficient to bind to oligo(dT) was not changed by short-term exposure to Arg (Fig. 4).

The reduction in Arg2p synthesis rate observed after short-term exposure to Arg, without concomitant reduction in the level of *arg-2* transcript, suggested the possibility of regulation at the level of translation. Examination of polysomes from cells switched from Min to Min or from Min to Arg medium for 30 min revealed a reduction in the average size of polysomes translating *arg-2* in cells switched to Arg medium (Fig. 5), while short-term exposure to Arg did not affect the distribution of *cox-5* on polysomes (Fig. 5). Interestingly, short-term exposure to Arg resulted in the appearance of *cpc-1* transcript on larger polysomes, suggesting translational activation (Fig. 5) that was not observed after prolonged growth in Arg medium (Fig. 2).

Comparison of short-term changes in response to Arg or His availability. We examined short-term responses to limitation for His induced by AT in parallel with analyses of short-term exposure to Arg (Fig. 6). Exposure to Arg for 30 min reduced the level of Arg2p synthesis but not the level of *arg-2* RNA (Fig. 6; Table 3). A reproducible decrease in *leu-6* mRNA and Leu6p synthesis in response to fresh Arg medium compared with fresh Min medium was also observed (Fig. 6 and data not shown); the explanation for this is not known at present. The slight decrease in *cpc-1* transcript following transfer to Arg medium seen here did not always accompany the shift to Arg medium (data not shown).

His starvation is known to increase the levels of *arg-2*, *cpc-1*, and *leu-6* transcripts and to reduce the level of *cox-5* transcript

TABLE 2. Relative expression of *arg-2* and *cox-5* after short-term exposure to Arg[a]

Determination	Relative expression					
	M to M			M to R		
	2 min	10 min	30 min	2 min	10 min	30 min
Protein level	1.0	1.0	1.1	1.1	0.7	0.6
Protein synthesis rate	1.0	1.9	1.6	0.8	1.1	0.5
RNA level	1.0	2.1	1.3	1.1	2.1	1.1

[a] Cells were grown in Min medium (M) for 6.5 h and then transferred to Min or Arg (R) medium for 2, 10, or 30 min. Calculations were based on data as described in Materials and Methods. The relative amounts of *arg-2* compared with *cox-5* expression after transfer from Min medium to Min medium for 2 min were given the unit value of 1.0.

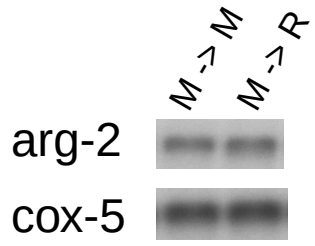

FIG. 4. Effects of short-term cell exposure to Arg on the *arg-2* and *cox-5* poly(A) mRNAs. Wild-type cells were grown for 6.5 h in Min medium (M) and then switched to either fresh Min medium or Arg medium (R) for 30 min. Poly(A) mRNA was prepared by oligo(dT)-cellulose chromatography, and the levels of *arg-2* and *cox-5* poly(A) mRNAs were examined by Northern blot analysis.

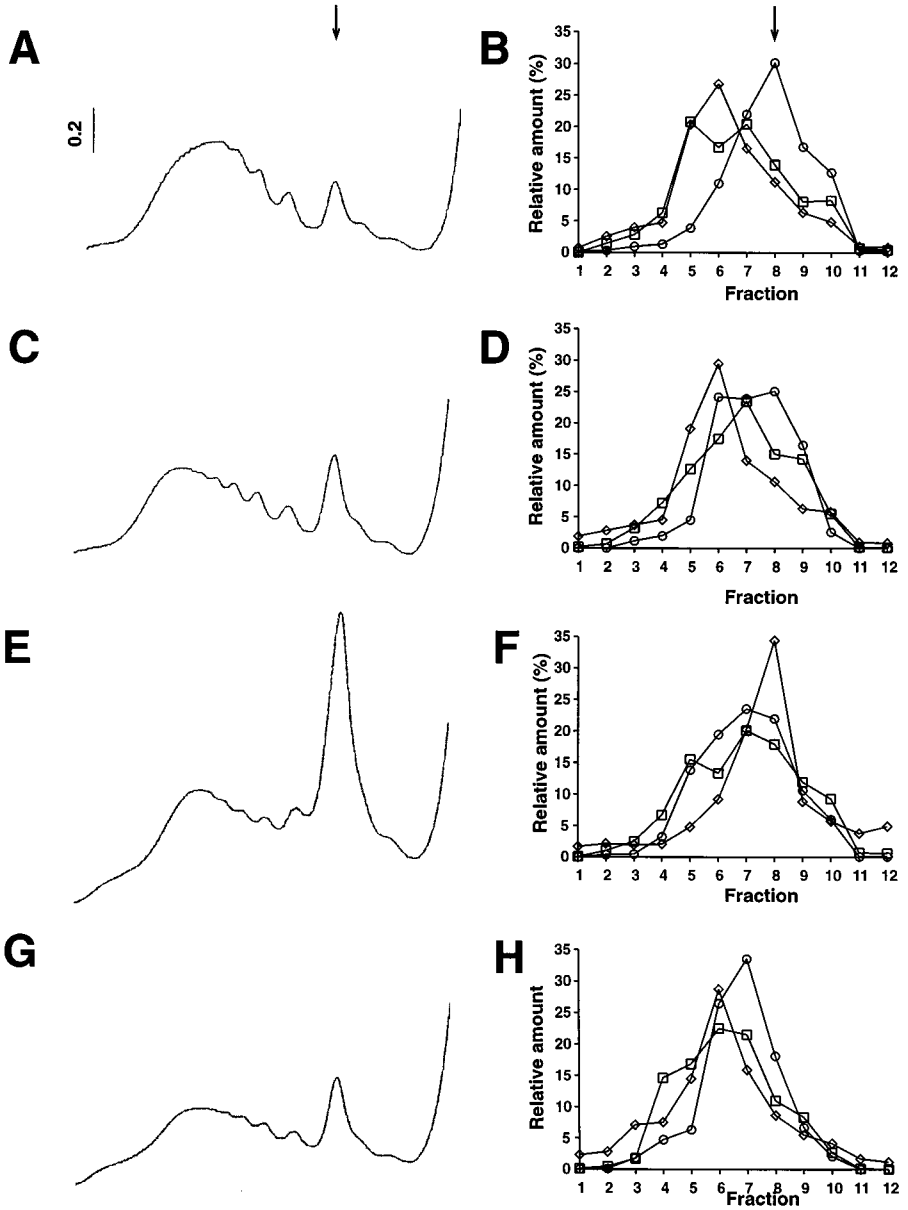

FIG. 5. Polysome profile analysis of cells switched from Min medium to Min, Arg, AT, or AT-His medium for 30 min. Wild-type *N. crassa* cells were germinated for 6.5 h in minimal medium and switched to Min (A and B), Arg (C and D), AT (E and F), or AT-His (G and H) medium for 30 min. Extracts were prepared and separated through sucrose gradients. (A, C, E, and G) 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} units was loaded. (B, D, F, and H) 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 is the top. The position of the monosome peak is indicated with an arrow.

(6, 41, 43, 52). In addition to these effects on transcript levels (Fig. 6), in AT-treated cells there were increased rates of Arg2p and Leu6p synthesis and a decreased rate of Cox5p synthesis (Fig. 6). There was slightly more synthesis of Arg2p than would be expected from the increase in transcript level alone (Table 3). The effects of AT were at least partially abrogated when His was added at the same time. However, His alone appeared to reduce both the level of Arg2p synthesis and the level of *arg-2* RNA, as well as affecting Cox5p synthesis (Fig. 6).

The effect of transfer to AT medium on translation could also be seen in analyses of polysomes (Fig. 5). Addition of AT to cells led to increased recovery of material in the monosome fraction in polysome preparations (compare Fig. 5A and E). In AT medium, relatively large amounts of *arg-2* and *cpc-1* transcripts were associated with polysomes, while the distribution of *cox-5* transcript shifted to monosomes and small polysomes. The effects of AT on translation were reduced when His was also added (Fig. 5).

Importance of the *arg-2* uORF in Arg-specific negative regulation. To assess the role of the *arg-2* uORF in gene expression, we constructed *arg-2–lacZ* fusion genes containing either the wild-type uORF (plasmid pZL601) or a mutated sequence lacking the uORF start codon (pZL610). These fusion genes

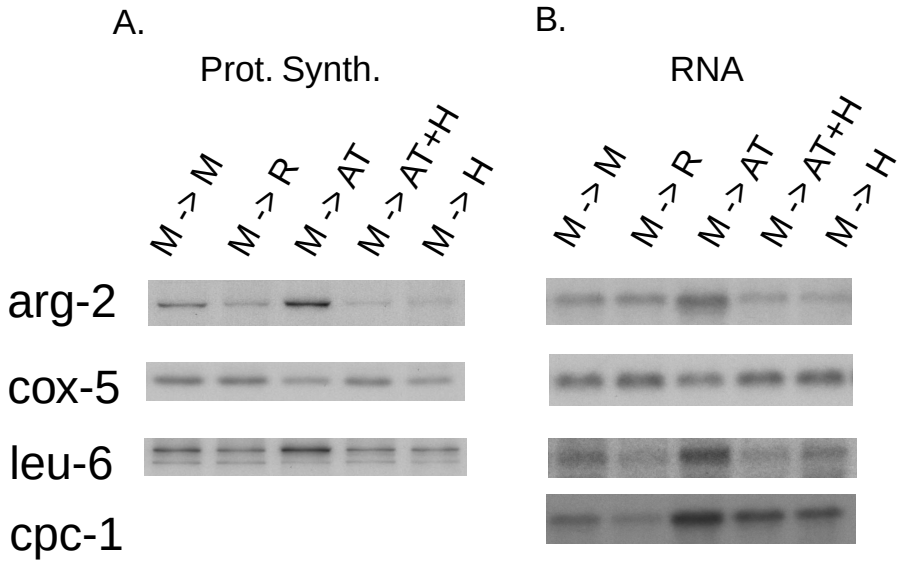

FIG. 6. Effects of amino acid availability on *arg-2*, *cox-5*, *leu-6*, and *cpc-1*. Wild-type cells were grown for 6.5 h in Min medium (M) and then transferred to fresh Min medium, Arg medium (R), AT-supplemented medium (AT), AT-His-supplemented medium (AT+H), and His-supplemented medium (H). (A) Arg2p, Cox5p, and Leu6p synthesis (Prot. Synth.) rates examined by immunoprecipitation following a 5-min pulse-label with [^{35}S]methionine; (B) *arg-2*, *cox-5*, *leu-6*, and *cpc-1* transcript levels examined by Northern blot analysis.

were placed in a vector that contained a truncated wild-type *his-3* gene which confers His prototrophy when it recombines with a mutant allele of *his-3* (51). The recombination will produce a wild-type, functional *his-3* gene and a truncated one flanking the *arg-2–lacZ* fusion gene (Fig. 7A).

Verification that constructs were integrated at *his-3* in recipient strains was accomplished by analyses of Southern blots of genomic DNA prepared from wild-type (74A), untransformed (His$^-$) recipient (6103), and transformed (His$^+$) recipient strains. Representative Southern blot analyses of *Hin*dIII-digested genomic DNA (Fig. 7B) showed a single, approximately 9.5-kb *Hin*dIII fragment that hybridized to both *lacZ* and *arg-2* probes in strains transformed with plasmid pZL601 or pZL610. The *lacZ* probe did not hybridize to the wild-type or untransformed recipient *N. crassa* strain, as expected (Fig. 7B). A transformant containing plasmid pDE1 contained a smaller fragment that hybridized to *lacZ* but not *arg-2*, as expected, because pDE1 has *lacZ* but not *arg-2* sequences (Fig. 7B). The *arg-2* probe also hybridized to a 2.3-kb *Hin*dIII fragment representing the endogenous *arg-2* in all strains. These results are what would be predicted from homologous integration of these plasmids at the *his-3* locus (Fig. 7A).

Further evidence confirming integration at *his-3* was obtained by using a *his-3* probe (Fig. 7C). As expected (Fig. 7A), an approximately 7.5-kb *Bgl*II fragment hybridized to the *his-3* probe in untransformed strains, and two fragments (a 9.5-kb fragment and a second fragment of 7.5 or 6.8 kb, depending on the integrating plasmid) hybridized to the probe in strains containing plasmids integrated at *his-3* (Fig. 7A). The sizes of these two fragments were consistent with integration of each of these plasmids at *his-3*. An additional, weakly hybridizing larger fragment was also detected in transformants; the size of this fragment appeared to be the sum of the sizes of two smaller fragments. Moreover, its intensity varied among repeated experiments with *Bgl*II-digested genomic DNA (data not shown). The appearance of this band is likely due to inefficient cutting at a *Bgl*II site, possibly because this enzyme is inhibited by substrate methylation, and methylation of *N. crassa* during vegetative growth near repeated sequences is known to occur (e.g., reference 57). Homologous recombination of pZL601, pZL610, and pDE1 in transformants was also confirmed by using other restriction enzymes, including *Dra*I and *Eco*RI (data not shown).

TABLE 3. Relative expression of *arg-2* and *cox-5* after short-term exposure to Arg, AT, AT-His, or His[a]

Determination	Relative expression				
	M to M	M to R	M to AT	M to AT-H	M to H
Protein level	1.0	1.0	0.8	0.8	0.7
Protein synthesis rate	1.0	0.4	3.9	0.3	0.5
RNA level	1.0	1.0	2.9	0.6	0.5

[a] Cells were grown in Min medium (M) for 6.5 h and then transferred to Min, Arg (R), AT-containing (AT), AT-His-containing (AT-H), and His-containing (H) media for 30 min. Calculations were based on data as described in Materials and Methods. The relative amounts of *arg-2* compared with *cox-5* expression after transfer from Min medium to Min medium for 30 min were given the unit value of 1.0.

Three independent transformants containing pZL601 or pZL610, two transformants containing pDE1, and wild-type *N. crassa* were used for further analyses. Measurements of β-galactosidase (Table 4) produced by strains containing the wild-type uORF in an *arg-2–lacZ* fusion gene (pZL601) showed that β-galactosidase activity was negatively regulated by Arg; the level of β-galactosidase was reduced in Arg medium to approximately 40% of the level observed in Min medium. In striking contrast, strains containing a mutant *arg-2–lacZ* gene lacking the uORF start codon (pZL610) showed higher levels of β-galactosidase in Min medium than strains containing the wild-type uORF, and enzyme activity increased slightly when cells were grown in Arg medium (Table 4). Strains containing plasmid pDE1, which lacked *arg-2* sequences, produced little detectable β-galactosidase above the endogenous activity of *N. crassa* β-galactosidase (Table 4). Thus, the capacity for translation of the *arg-2* uORF appeared to be critical for controlling the level of gene expression and the response of the gene to Arg.

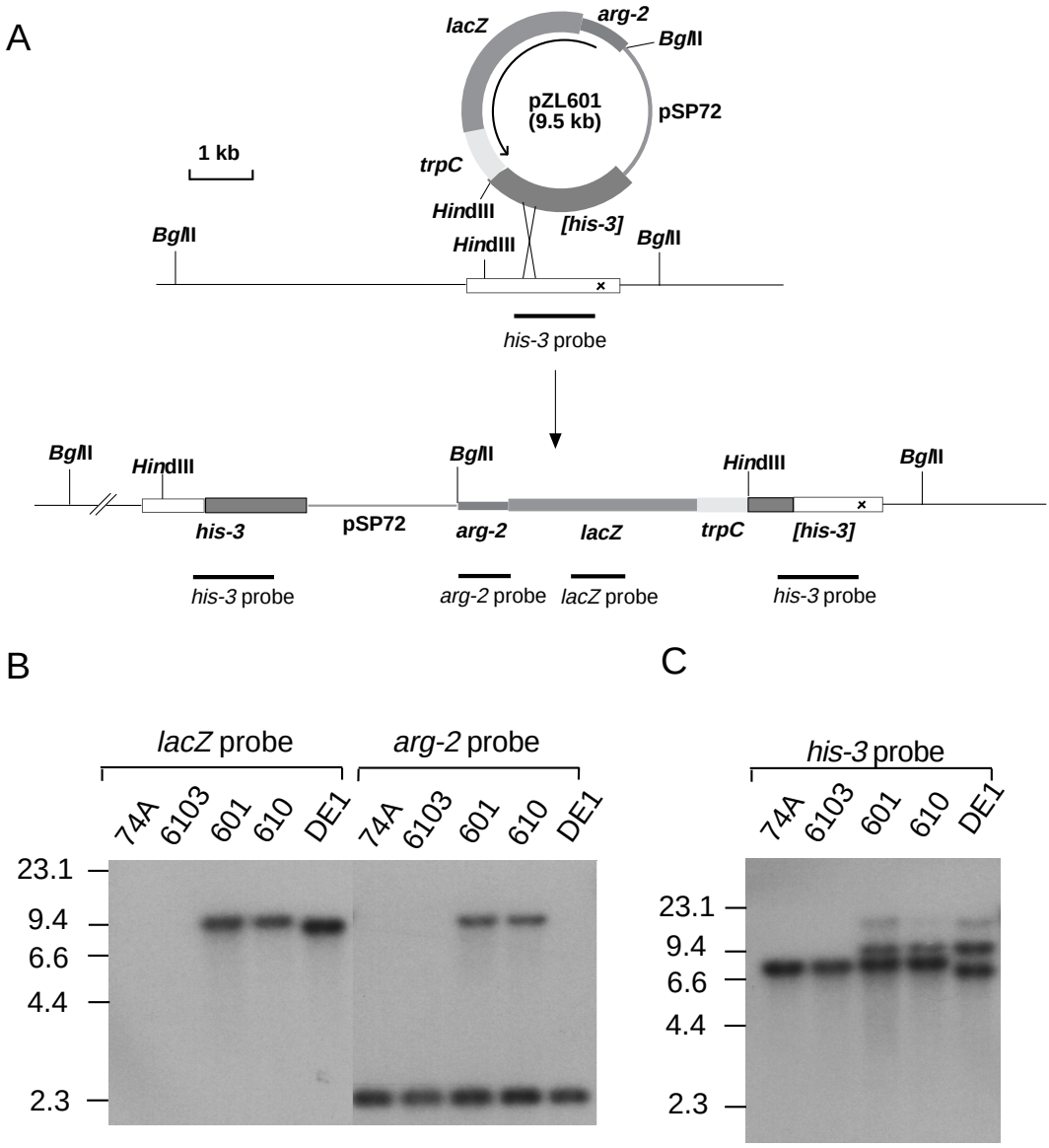

FIG. 7. Homologous recombination of transforming plasmids at the *his-3* locus. (A) Schematic diagram of pZL601 recombining at *his-3*. The origins of DNA sequences are indicated by different segment patterns and markings. *lacZ*, *E. coli lacZ* coding region; *arg-2*, *arg-2* 5′ region; *trpC*, *Aspergillus nidulans trpC* terminator region; pSP72, sequence from pSP72; *[his-3]*, *his-3* sequence with a truncated open reading frame; *his-3*, restored *his-3* sequence with wild-type function. The direction of *arg-2–lacZ* transcription is indicated with an arrow in the plasmid diagram. The recipient's *his-3* coding sequence is represented by an open rectangle, with the putative region of the inactivating mutation indicated by "x." Probes and restriction enzyme sites used in the Southern blots shown in panels B and C are indicated. (B) Southern blot analysis of *Hin*dIII-digested genomic DNA, using *lacZ* and *arg-2* probes. Lanes: 74A, wild-type *N. crassa*; 6103, the recipient strain used for transformation; 601, 610, and DE1, representative transformants containing plasmids pZL601, pZL610, and pDE1, respectively, integrated at *his-3*. (C) Southern blot analysis of *Bgl*II-digested genomic DNA from these strains, using the *his-3* probe. Sizes are indicated in kilobases.

It was important to examine *arg-2–lacZ* transcript levels to more fully evaluate how the presence of the uORF initiation codon affected gene expression. Compared with levels of *arg-2* and *cox-5*, the level of *arg-2–lacZ* mRNA was more difficult to measure because of its lower abundance. The mRNA was undetectable in our Northern blot analyses of total RNA, and thus poly(A) mRNA samples were used to visualize the approximately 3.7-kb transcript (Fig. 8).

In contrast to the reduction in the level of *arg-2* transcripts relative to *cox-5* transcripts observed in each transformant and the wild-type control strain (Fig. 8; Table 5), the levels of *arg-2–lacZ* transcripts (present only in transformants) relative to *cox-5* transcripts were unaffected by Arg (Fig. 8; Table 5). The levels of *arg-2–lacZ* mRNA for constructs containing or lacking a uORF initiator AUG were similar when expression of each was examined relative to expression of *cox-5* (Fig. 8; Table 5), in contrast to the increased LacZ enzyme activity in the strain lacking the uORF (Table 4). Thus, the effects of the uORF translation initiation codon on the expression of these genes were primarily posttranscriptional and did not affect relative levels of poly(A) transcript.

DISCUSSION

Multiple levels of regulation for *arg-2* and *cpc-1*. We examined mechanisms regulating *N. crassa arg-2*, a gene that is

TABLE 4. Effects of growth in Arg on expression of *arg-2–lacZ* reporter genes[a]

Plasmid	β-Galactosidase activity (Miller units/mg of protein)	
	M	R
pZL601	57	22
	63	24
	58	24
pZL610	131	133
	124	133
	170	180
pDE1	0.9	0.8
	0.8	0.7
No plasmid	0.5	0.3

[a] *N. crassa* containing the indicated plasmids integrated at *his-3* and wild-type *N. crassa* (no plasmid) were grown in parallel for 6.5 h in Min (M) or Arg (R) medium. Each line of data was obtained from an independent strain and represents the average of duplicate measurements which differed by less than 10%.

TABLE 5. Expression of *arg-2–lacZ* and *arg-2* transcripts relative to *cox-5*[a]

Transcript	Relative expression					
	74A		pZL601		pZL610	
	M	R	M	R	M	R
arg-2–lacZ	ND	ND	1.0	1.0	1.1	1.3
arg-2	1.0	0.3	1.1	0.4	1.2	0.4

[a] Cells were grown in Min (M) or Arg (R) medium for 6.5 h as described for Fig. 8. Data reported are averages from two independent growth experiments assayed in duplicate. ND, *arg-2–lacZ* transcripts were not detectable. Transcript levels are reported relative to the *cox-5* signal from a similar sample. The relative amounts of *arg-2–lacZ* compared with *cox-5* for pZL601 grown in Min medium were assigned the unit value of 1.0; the relative amounts of *arg-2* compared with *cox-5* for 74A grown in Min medium were assigned the unit value of 1.0.

negatively regulated by Arg and positively regulated by cross-pathway control, and mechanisms regulating *cpc-1*, a gene whose function is essential for cross-pathway control, in response to amino acid availability. The data indicate that multiple translational mechanisms, as well as transcriptional components, appear to affect the expression of *arg-2* and *cpc-1* relative to *cox-5* in response to changes in amino acid availability. In wild-type cells, short-term exposure to Arg reduced the rate of Arg2p synthesis and shifted *arg-2* transcript to smaller polysomes but did not affect the level of *arg-2* transcript, suggesting that a negative translational control mechanism is responsible for modulating Arg2p expression as an immediate response to Arg exposure. At the same time, the *cpc-1* transcript shifted to larger polysomes, indicating positive translational control. Exposure to AT, which creates His limitation, appeared to increase both *arg-2* and *cpc-1* expression at translational and transcriptional levels.

***arg-2* regulation by Arg.** The effect of Arg on the expression of *arg-2* in wild-type *N. crassa* is approximately threefold, a magnitude similar to that observed for Arg-specific regulation of *S. cerevisiae CPA1* (65). This effect is small but highly reproducible in experiments containing internal controls such as those presented here. Why should a cell bother to regulate a gene threefold in response to Arg availability? In fact, the amplitude of the regulatory response observed in wild-type *N. crassa* grown in Min compared with Arg medium is lower than the full range of regulation that is possible. Experiments in which the level of *arg-2*-dependent enzyme activity was measured in Arg auxotrophs grown under conditions in which Arg was available or Arg was depleted showed that *arg-2*-dependent activity could be modulated over a 100-fold range (13). We observed 35-fold regulation of *arg-2*-dependent CPS-A activity in an *arg-12*^{s} strain by Arg, compared with threefold regulation in the wild type (Table 1), largely because the expression of *arg-2* is greater in the *arg-12*^{s} strain in Min medium (9, 52). Intracellular Arg concentrations are 10-fold lower in the *arg-12*^{s} strain than in the wild type in Min medium (9). Presumably, both the translational effects described here and previously observed effects on transcript levels (52) account for the increased amplitude of regulation in *arg-12*^{s}. Thus, it seems likely that wild-type *N. crassa* growing in Min medium synthesizes sufficient Arg to partially engage mechanisms that limit *arg-2* expression and/or disengage mechanisms that stimulate expression in response to limitation.

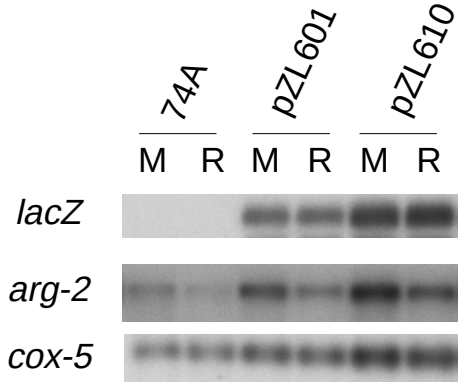

FIG. 8. Levels of *arg-2–lacZ*, *arg-2*, and *cox-5* mRNAs in poly(A) samples from strains grown in Min or Arg medium. *N. crassa* wild-type strain 74A and representative transformants containing pZL601 or pZL610 were grown at 34°C for 6.5 h in minimal (M) or Arg (R) medium. Poly(A) mRNA was prepared by oligo(dT)-cellulose chromatography, and the levels of *arg-2–lacZ*, *arg-2*, and *cox-5* poly(A) mRNA were examined by Northern blot analysis. Approximately 4-μg samples of poly(A) mRNA (determined by A_{260} measurement) were used to detect *arg-2–lacZ* mRNA, and approximately 1-μg samples of poly(A) mRNA were used for detecting *arg-2* and *cox-5* mRNA. The variation in the absolute intensities of signals in samples from different strains likely reflects differences in the overall recovery of poly(A) mRNA following a single round of chromatography.

The studies described here provide direct biochemical evidence for a translational component to *arg-2* regulation. The net rate of Arg2p synthesis was reduced in Arg medium, and reduced Arg2p synthesis was accompanied by a reduction in the fraction of *arg-2* mRNA associated with large polysomes. By analogy, *CPA1* could be expected to be similarly regulated. The uORFs in these genes have roles in Arg-specific negative regulation (Table 4) (16, 64).

The rapid reduction in the rate of Arg2p synthesis in response to Arg was sometimes accompanied by a rapid, smaller decrease in the cellular level of Arg2p. A rapid reduction in cellular Arg2p might result from reduced synthesis of new Arg2p and a relatively high rate of polypeptide degradation. Consistent with this hypothesis, the *arg-2*- and *CPA1*-specified small subunits of carbamoyl phosphate synthetase have been reported to be difficult to purify, possibly because of their instability (14, 44). The possibility that growth in Arg affects the stability of Arg2p will have to be addressed by more sensitive measurements, such as pulse-chase studies. Similar experiments have revealed that the stability of *S. cerevisiae* Gcn4p is regulated by amino acid availability (33) and that the stability of *N. crassa* ornithine decarboxylase is regulated by polyamines (1).

The importance of the uORF in negative regulation was revealed by using *arg-2–lacZ* fusion genes (Table 4). Independent *N. crassa* transformants containing the wild-type uORF proximal to *lacZ* showed negative regulation of β-galactosidase activity in response to Arg similar to the level of regulation observed for the endogenous *arg-2* gene product (Tables 1 and 4). For neither construct were *arg-2–lacZ* mRNA transcript levels reduced by Arg (Fig. 8; Table 5), indicating (i) that negative regulation by Arg of the construct containing the wild-type uORF was posttranscriptional and (ii) that the signals conferring regulation by Arg at the level of mRNA were either lacking or masked in these constructs. Another observation indicating the importance of the capacity to translate the uORF in controlling gene expression was that removal of the uORF initiation codon resulted in increased, constitutive expression of *lacZ* without affecting relative transcript levels.

One model consistent with the 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 data obtained concerning other uORFs whose sequences are important in negatively regulating gene expression, is that uORF translation stalls ribosomes, blocks subsequent rounds of ribosomal scanning from the mRNA 5′ end, and thus reduces translation (21). According to this model, stalling would increase when Arg is present.

It is possible that translational control of the wild-type *arg-2* gene has consequences for mRNA stability. The relative contributions of transcript synthesis and transcript stability to determining the net level of transcript are not known for *N. crassa arg-2*. In *S. cerevisiae*, Arg decreases *CPA1* transcription and the *CPA1* mRNA half-life each by a factor of 2 (8). Because *arg-2* and *CPA1* contain uORFs which have roles in Arg-specific negative regulation, it must be considered whether termination of translation in the mRNA 5′ regions could affect mRNA stability, possibly without affecting polyadenylation (38). There is also precedent that the uORF peptide could have a specific effect on transcript stability: the stability of β-tubulin mRNA is determined by mechanisms that require translation of the amino terminus of the β-tubulin polypeptide (66).

Effects of His limitation on gene expression. The *cpc-1* transcript becomes associated with larger polysomes when *N. crassa* is switched from Min to Arg or from Min to His-starvation medium (Fig. 5). In *S. cerevisiae* shifted from nutrient-rich to nutrient-poor medium, the *GCN4* transcript also becomes associated with larger polysomes (60). This effect on *GCN4* appears to be mediated at the level of translation initiation (27, 60). 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 (22, 37). Two uORFs are present in the *cpc-1* transcript (43, 48). The sequence of the 10 nucleotides following *cpc-1* uORF1 (based on the revised *cpc-1* sequence in GenBank) resembles sequences preferred for reinitiation following *GCN4* uORF1. There are also similarities in the regions downstream of *GCN4* uORF4 and *cpc-1* uORF2; specifically, the blocks of high G+C content important for *GCN4* uORF4 function appear to be conserved downstream of *cpc-1* uORF2. Thus, a model for translational regulation of *cpc-1*, based on current understanding of *GCN4* regulation (26, 27), would posit that translational control by amino acid availability regulates initiation at the uORF2 start codon versus the Cpc1p start codon.

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

Other aspects of translational and transcriptional regulation in *N. crassa*. Much remains to be determined concerning the mechanisms regulating translation and transcript metabolism in *N. crassa*. *N. crassa* mRNA is capped (55), and presumably initiation occurs after scanning (34) from the cap structure of the mRNA. Internal initiation, as sometimes seen in mammals (61), may also occur in *N. crassa* (62). As is typical in eukaryotes, most *N. crassa* mRNAs lack uORFs, although there are other examples of functional uORF-containing genes (45, 62) in addition to *arg-2* and *cpc-1*.

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 ancestors of *N. crassa* and *S. cerevisiae* diverged. It is interesting in this regard that *N. crassa arg-2*, but not *S. cerevisiae CPA1*, specifies a polypeptide targeted to mitochondria and that *arg-2* contains sequences that may function as intronic enhancers, features it shares with genes in mammals (12, 28, 41).

There is an increasing understanding of the importance of translational control mechanisms in the regulation of uORF-containing eukaryotic genes (10, 21), but few instances are known in which the stimuli eliciting translational control are well defined. The evidence indicates that *N. crassa arg-2* and *cpc-1* are examples of uORF-containing genes that are translationally controlled in response to amino acid availability. By a combination of genetic and biochemical approaches, such as described here, it should be possible to develop a greater understanding of the mechanisms responsible for eukaryotic translational control.

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