JOURNAL OF BACTERIOLOGY, Apr. 1996, p. 2172–2177 Vol. 178, No. 8
0021-9193/96/$04.00+0

Role of an Upstream Open Reading Frame in Mediating Arginine-Specific Translational Control in *Neurospora crassa*

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The *Neurospora crassa arg-2* transcript contains an upstream open reading frame (uORF) specifying a 24-residue leader peptide and is subject to a novel form of negative translational regulation in response to arginine. The role of the *arg-2* uORF in arginine-specific negative regulation was investigated by using translational fusions of wild-type and mutant *arg-2* sequences to the *Escherichia coli lacZ* reporter gene specifying β-galactosidase. The wild-type uORF conferred Arg-specific regulation on the reporter gene in *N. crassa*, but mutated or truncated uORFs did not, as determined by measurements of β-galactosidase activity produced in *N. crassa* strains expressing *arg-2–lacZ* fusion genes. All effects on reporter gene expression were posttranscriptional, as determined by measurement of RNA levels. Both sequence-dependent and sequence-independent effects of uORFs were observed. Genes containing the wild-type uORF or a 21-codon mutated uORF showed reduced translation in comparison with that of a gene lacking a uORF. Both uORF-containing transcripts showed reduced association with polysomes relative to transcripts lacking a uORF, but only the transcript with the wild-type uORF showed a reduced average number of ribosomes associated with it in response to arginine addition. Direct translational fusions between uORF sequences and *lacZ* sequences indicated that the uORF is translated. Overlapping the uORF with the *lacZ* initiation codon indicated that ribosome reinitiation at a downstream start codon is not integral to uORF-mediated, Arg-specific translational regulation. These studies provide direct biochemical evidence for *arg-2* uORF function in translational control.

Microbial amino acid biosynthetic enzymes are regulated in many ways in response to amino acid availability (19, 24). Repressors and activators regulate transcription (1, 17, 36). At the level of translation, upstream open reading frames (uORFs) that specify short leader peptides in the 5′ regions of mRNAs regulate gene expression in response to charged-tRNA availability (18, 21).

The functions of eukaryotic uORFs are of considerable interest because uORFs are present in the mRNAs for many genes involved in controlling cell growth (13, 14). The mechanism of translational control via uORFs is best understood for *Saccharomyces cerevisiae GCN4*, which specifies a transcriptional activator of amino acid biosynthetic genes that is related to *Neurospora crassa cpc-1* and to mammalian *jun* (17, 28). Modulation of initiation factor activity in response to charged-tRNA availability affects the ability of ribosomes to reinitiate translation at the Gcn4p polypeptide start codon following uORF translation (18). A related translational mechanism may regulate *cpc-1* (22).

N. crassa arg-2 and *S. cerevisiae CPA1* are homologous genes (25, 26, 34) specifying the small subunit of arginine-specific carbamoyl phosphate synthetase. Each is subject to Arg-specific negative regulation (6). In *N. crassa*, *arg-2* is the only Arg biosynthetic gene known to be negatively regulated by Arg; the level of Arg2 polypeptide (the small subunit of carbamoyl phosphate synthetase) controls Arg synthesis under most growth conditions (6, 7). There is evidence for transcriptional and posttranscriptional control of both *arg-2* and *CPA1* expression (3, 4, 22, 23, 26, 29, 33). Direct biochemical evidence for translational control of *arg-2* in response to Arg was obtained by comparison of mRNA levels and polypeptide synthesis rates, and by examination of the distribution of *arg-2* mRNA in polysomes in cells grown in the presence or absence of Arg (22).

The *arg-2* and *CPA1* genes each contain a single uORF in their transcripts, and the primary amino acid sequences of the predicted leader peptides are similar (26, 33). Analyses of *S. cerevisiae CPA1* mutants with altered regulation, and of *S. cerevisiae* and *N. crassa* strains producing β-galactosidase (β-gal) from reporter genes containing *lacZ* fused to *CPA1* or *arg-2* sequences, respectively, indicated that the translation of these leader peptides is important for Arg-specific regulation (8, 12, 22, 33).

In this study, we used a series of *arg-2–lacZ* fusion genes that lack Arg-specific regulation at the transcript level to show that the *arg-2* uORF has both sequence-dependent and -independent effects on translation. Our data indicate that both uORF translation and the predicted primary sequence of the leader peptide are important for Arg-specific translational control and that ribosome reinitiation does not appear to be required for Arg-specific translational control.

MATERIALS AND METHODS

Plasmid constructions. Standard procedures were used for plasmid constructions unless otherwise indicated (30). *Escherichia coli* XL-1 Blue (32) was used as the bacterial host. Plasmids that contained nested deletions of the *arg-2* 5′ sequence were constructed by exonuclease III and S1 nuclease treatment (16) of plasmid pFo2 (22) following linearization of the plasmid with *Bam*HI and *Pst*I. DNA sequences at deletion junctions were confirmed by the chain termination method (31) by using Sequenase (U.S. Biochemicals). Deletion plasmids included pUCZL1 (nucleotides [nt] 634 to 928 of *arg-2* [26]), pUCZL2 (nt 634 to 1270), pUCZL5 (nt 634 to 1359), pUCZL6 (nt 634 to 1373), pUCZL7 (nt 634 to 1405), and pUCZL8 (nt 634 to 1587).

Plasmids containing *arg-2–lacZ* genes were constructed by subcloning appropriate *arg-2* fragments into phosphatase-treated, *Sma*I-digested vectors pDE1, pDE2, and pDE3, which are vectors designed to target fusion genes to the *N.*

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crassa his-3 locus (9). All *arg-2* fragments were made flush ended by using the Klenow fragment of DNA polymerase I and nucleotide triphosphates to prepare them for subcloning. The structures of the gene fusions were confirmed by DNA sequencing.

Plasmids pZL601 and pZL610, which contain the wild-type uORF and a uORF lacking an initiation codon, respectively, in the 5′ regions of the *arg-2–lacZ* transcripts, have been described previously (22). Plasmid pZL613, containing a 21-codon uORF with an altered primary sequence (MNGPVSLHLSGL PLQPSVESP), was constructed in the following way. First, pZL304 was generated by subcloning the 0.97-kb *Eco*RI-*Hin*dIII fragment from pUCZL8 into the corresponding sites of pBS-SK+ (32). pZL304 then was cut with *Nar*I (a unique site in this plasmid, positioned at uORF codon 4), the overhanging ends were trimmed with mung bean nuclease, and the plasmid was recircularized by using T4 DNA ligase to generate pZL305. pZL613 was obtained by subcloning the 0.85-kb *Eco*RI-*Sty*I fragment from pZL305 into pDE3.

Additional plasmids for *N. crassa* expression were constructed as follows: pZL1 was generated by subcloning the 0.31-kb *Eco*RI-*Sph*I fragment from pUCZL1 into pDE1, pZL6a was generated by subcloning the 0.75-kb *Eco*RI-*Sph*I fragment from pUCZL6 into pDE1, pZL6b was generated by subcloning the 0.75-kb *Eco*RI-*Hin*dIII fragment from pUCZL6 into pDE3, pZL6fs was generated by subcloning the 0.75-kb *Eco*RI-*Sph*I fragment from pUCZL6 into pDE3, pZL7 was generated by subcloning the 0.79-kb *Eco*RI-*Hin*dIII fragment from pUCZL7 into pDE3, pZL2 was generated by subcloning the 0.63-kb *Eco*RI-*Sph*I fragment from pUCZL2 into pDE3, and pZL5 was generated by subcloning the 0.73-kb *Eco*RI-*Sph*I fragment from pUCZL5 into pDE3.

Construction and growth of *N. crassa* strains containing *arg-2–lacZ* integrated at the *his-3* locus. Procedures for constructing single-copy integrants of *arg-2–lacZ* genes at *his-3* have been previously described (22). The *N. crassa his-3(1-234-723)* strain FGSC 6103 (Fungal Genetics Stock Center, University of Kansas Medical Center) was transformed with plasmids by selection for His prototrophy. Prototrophic *N. crassa* homokaryons were obtained by microconidiation (10); Southern analyses were used to identify transformants containing a single copy of *arg-2–lacZ* integrated at *his-3*. Cells were grown in Vogel's minimal medium–2% sucrose (Min medium) or in minimal medium supplemented with 0.5 mg of arginine per ml (Arg medium) as described elsewhere (22).

β-gal assays. At least two independent transformants that contained a single copy of each construct integrated at *his-3* were initially examined. Cell extracts were prepared from cells grown in Min or Arg medium, and β-gal activity was assayed as described elsewhere (22). In every case, the levels of β-gal activity and the magnitude of regulation by Arg were similar in independent transformants representing a given construct. The data reported here represent one of two growth experiments in which representative transformants were grown and analyzed in parallel.

Northern (RNA) blot and polysome analyses. Preparation of poly(A) RNA from total RNA samples by oligo(dT) cellulose chromatography, preparation of *arg-2*, *cox-5*, and *lacZ* probes, and procedures for Northern analyses were described previously (22). Approximately 1.5 μg of poly(A) RNA was used to detect *lacZ* transcripts, and 0.5 μg of poly(A) RNA was used to detect *arg-2* and *cox-5* transcripts; the amounts of each sample were adjusted so that the *cox-5* probe detected similar amounts of material in each. Polysome analyses were performed as described elsewhere (22), except that 6 A_{260} units was loaded for each sample.

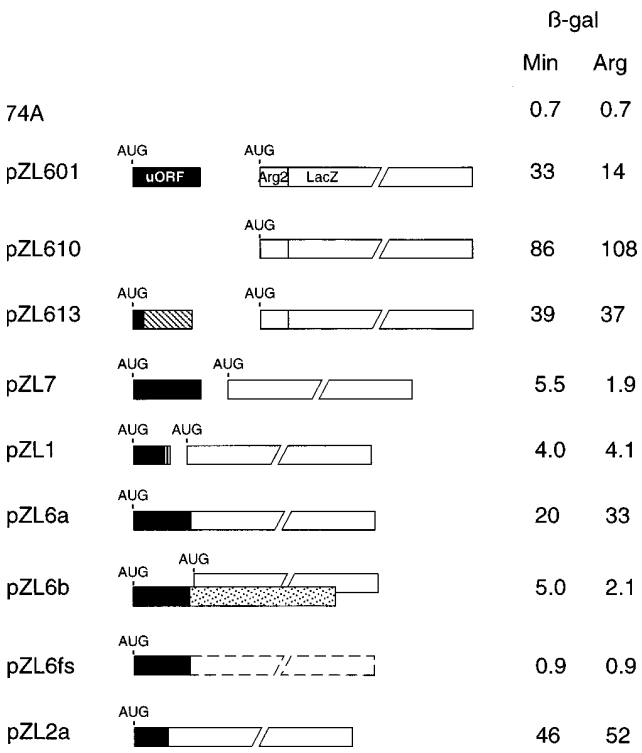

FIG. 1. Effect of growth in Arg on the level of β-gal activity expressed from *arg-2–lacZ* reporter genes. Strains were grown at 34°C for 6.5 h in Min or Arg medium. Crude cell extracts were prepared, and β-gal activity was assayed (see Materials and Methods). The structures of the uORF and *lacZ* coding regions of the *arg-2–lacZ* reporter construct present in each strain are diagrammed schematically. β-gal activities (Miller units per milligram of protein) measured for strains containing each construct grown in Min or Arg medium are as indicated. The wild type (74A) lacks any *arg-2–lacZ* reporter construct. Black boxes indicate the wild-type uORF leader peptide coding sequence, gray boxes indicate the wild-type Arg2 coding sequences to which *lacZ* coding sequences were translationally fused, and the white boxes indicate *lacZ* coding sequences. Predicted AUG initiation codons are indicated. The hatched boxes (pZL613 and pZL1) and stippled box (pZL6b) indicate novel uORF peptide coding sequences. The dashed lines in pZL6fs indicate that *lacZ* is fused out of frame to the uORF. The absolute levels of β-gal produced from constructs pZL601, pZL610, and pZL613 can be directly compared with each other, because in each case the context of the predicted initiation codon for LacZ and the N terminus of LacZ are the same; this does not hold for the other constructs, because contexts of initiation codons and N-terminal sequences differ among constructs.

RESULTS

Negative regulation of the *N. crassa arg-2* gene by Arg affects the level of *arg-2* mRNA and the translation of the *arg-2* mRNA. The *arg-2* transcript contains a uORF specifying a 24-residue leader peptide. Translation of this peptide is predicted to terminate 63 nt upstream of the predicted translation initiation site for the product of the *arg-2* gene, Arg2, which is the small subunit of arginine-specific carbamoyl phosphate synthetase (26). The role of this uORF in Arg-specific regulation was investigated by mutational analyses. Genes in which the *E. coli lacZ* polypeptide coding region was fused to *arg-2* 5′ sequences were constructed and introduced into the *N. crassa* genome by homologous integration at the *his-3* locus (22). Gene expression was monitored by measurements of (i) levels of β-gal activity, (ii) levels of *lacZ* poly(A) mRNA, and (iii) levels of translation as assessed by examination of the distribution of mRNAs in polysome gradients.

Effects of altering *arg-2* 5′ sequences on the expression of β-gal. In wild-type *N. crassa*, the level of Arg2p is reduced approximately 2.5-fold when cells are provided with Arg (22). Strains containing pZL601, an *arg-2–lacZ* construct containing the wild-type uORF, produced substantial levels of β-gal in comparison with the background levels detected in wild-type *N. crassa*, which lacks the *lacZ* gene (Fig. 1). The level of β-gal activity was 2.4-fold higher in Min than in Arg medium (Fig. 1). Thus, as previously observed, negative regulation of the fusion gene containing the wild-type uORF was similar to the level of regulation of the endogenous *arg-2* gene (22).

While the construct containing the wild-type uORF retained regulation, constructs in which the predicted translation initiation site for the uORF was eliminated by site-specific mutagenesis (pZL610) or in which the predicted primary amino acid sequence of the uORF was altered (pZL613) lost Arg-specific negative regulation (Fig. 1). Removal of the uORF start codon (pZL610) resulted in a higher level of β-gal expression in comparison with that of either of two constructs that contained a uORF (pZL601 and pZL613).

In contrast to the loss of regulation observed when the uORF was changed, shortening the distance between the uORF stop codon and the *lacZ* start codon from 63 to 20 nt and altering the context of the *lacZ* start codon did not affect

Arg-specific regulation (pZL7) (Fig. 1). However, shortening the uORF to 13 codons caused a loss of Arg-specific negative regulation (pZL1) (Fig. 1).

The effects of creating direct fusions of *lacZ* to the uORF coding region and of creating overlapping reading frames for the uORF peptide and LacZ were different. Construct pZL6a, in which *lacZ* was translationally fused to the 20th codon of the *arg-2* uORF, produced substantial β-gal activity that was not reduced by growth in Arg rather than in Min medium (Fig. 1). Instead, β-gal activity increased slightly in Arg medium. Construct pZL6b contained an *arg-2–lacZ* gene specifying nested open reading frames (Fig. 1), in which the uORF peptide should initiate normally and a second downstream AUG should initiate translation of the *lacZ* gene in a second reading frame that begins at an overlap with codon 20 of the uORF. Thus, two overlapping polypeptides are predicted to be specified by this construct: LacZ and a 72-residue uORF product in which the first 21 amino acid residues match the wild-type uORF peptide sequence. Expression of this gene was also negatively regulated by Arg (Fig. 1).

The production of β-gal from pZL6a indicated that the predicted reading frame of the uORF was translated. As a control to rule out other possibilities, we constructed pZL6fs, which was similar to pZL6a except that *lacZ* was out of frame with respect to the uORF and that there was no in-frame AUG available to initiate translation of *lacZ* until codon 106 of the *lacZ* coding region. This construct produced very little β-gal activity (Fig. 1). The slight increase in β-gal activity (approximately 0.2 U/mg of protein) above background observed in this transformant appeared to be due to synthesis of LacZ polypeptide since it could be immunoprecipitated with monoclonal antibody directed against LacZ (data not shown), whereas the antibody did not immunoprecipitate detectable β-gal from strains lacking *lacZ* sequences (<0.02 U/mg). The observation that a high level of β-gal was produced when *lacZ* was fused in frame with the uORF initiator (pZL6a) but not when fused out of frame (pZL6fs) indicates that the uORF was translated in vivo.

Additional data were also consistent with translation of the uORF: constitutive β-gal expression was also observed to occur in transformants containing construct pZL2a or pZL5a, in which *lacZ* was translationally fused to the 12th or 18th codon of the *arg-2* uORF, respectively (Fig. 1 and data not shown).

All constructs produced similar levels of *arg-2–lacZ* mRNA that were not subject to negative regulation by Arg. We examined *arg-2–lacZ* transcripts by Northern blotting in order to determine whether exogenous Arg affected the expression of *arg-2–lacZ* fusion genes at the mRNA level. Since levels of *arg-2–lacZ* mRNA in transformants were quite low, we analyzed poly(A) RNA rather than total RNA. As controls, we examined expression of the endogenous *arg-2* gene, whose transcript level decreases following prolonged growth in Arg medium, and the *cox-5* gene, whose transcript is not regulated by Arg (22, 26, 29). *lacZ* mRNA was not detectable in the wild-type strain but was detectable in all transformants analyzed (Fig. 2). No decrease in *arg-2–lacZ* transcript levels in Arg medium was observed with any of the transformants analyzed. In contrast, the level of *arg-2* mRNA was reduced by growth in Arg medium for all transformants, unlike the level of *cox-5*, which was unaffected by growth in Arg (Fig. 2 and data not shown). The levels of *arg-2–lacZ* and *arg-2* mRNAs relative to the level of *cox-5* mRNA were similar in all transformants.

The *arg-2* uORF is important for negative regulation of *arg-2–lacZ* by Arg at the translational level. The negative regulation of *arg-2–lacZ* expression at the enzyme level (Fig. 1) but not the transcript level (Fig. 2) indicated posttranscriptional control. To assess the importance of the uORF for translational regulation, we examined the distribution of ribosomes on *arg-2–lacZ*, *arg-2*, *cox-5*, and *cpc-1* transcripts using polysome gradients obtained from wild-type (74A) and transformant strains containing pZL601, pZL610, or pZL613 that were grown in Min or Arg medium (Fig. 3). We showed previously (22) that translational control of *arg-2* could be detected by the shift of *arg-2* transcripts in polysome gradients in cells exposed to Arg. In each of the strains examined, a smaller fraction of *arg-2* mRNA was found in polysomes from cells grown in Arg medium (Fig. 3). Neither *cox-5* nor *cpc-1* transcripts shifted in polysomes as a result of growth with Arg; the *cox-5* transcript was associated with larger polysomes than was *cpc-1* in cells grown in either Min or Arg medium (Fig. 3). The distributions of ribosomes on *arg-2*, *cox-5*, and *cpc-1* mRNAs were thus similar in each strain examined and were consistent with previous observations (22).

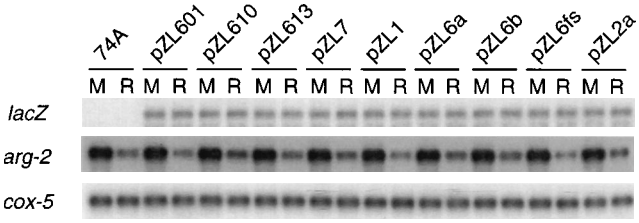

FIG. 2. Effect of growth in Arg medium on the levels of *arg-2–lacZ*, *arg-2*, and *cox-5* mRNA. Poly(A) mRNA was prepared from wild-type *N. crassa* (74A) and transformants containing *arg-2–lacZ* reporter genes grown in Min (M) or Arg (R) medium as described in the legend to Fig. 1. Poly(A) mRNA was examined by Northern analysis with *arg-2–lacZ*, *arg-2*, and *cox-5* probes as described in Materials and Methods. Blots probed with *lacZ* were exposed to film with an intensifying screen for 96 h, blots probed with *arg-2* were exposed for 10 h with a screen, and blots probed with *cox-5* were exposed for 4 h without a screen.

In transformants containing pZL601, *arg-2–lacZ* transcripts were on larger polysomes in cells grown in Min than they were in cells grown in Arg medium. This indicates that, like the endogenous *arg-2* transcript, the *arg-2–lacZ* gene containing the wild-type uORF was translationally regulated by Arg (Fig. 3). In contrast, *arg-2–lacZ* transcripts from cells containing pZL610, in which the uORF start codon was deleted, or pZL613, in which the uORF had a different primary sequence, were not shifted to smaller polysomes following growth in Arg medium (Fig. 3). This indicates that both uORF translation and the primary sequence of the uORF were important for Arg-specific translational control.

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

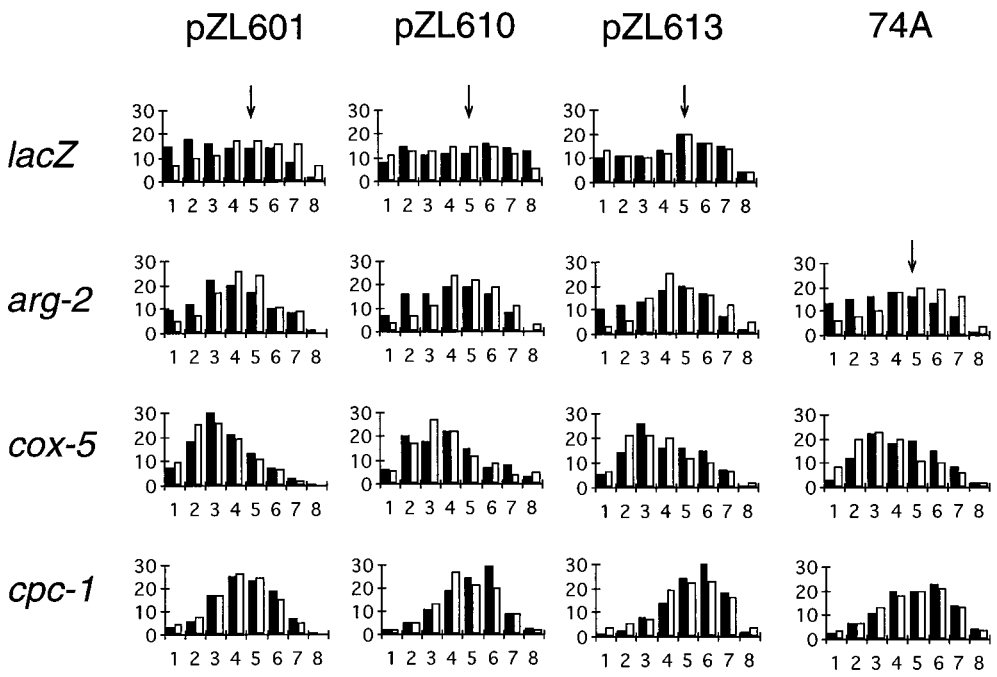

FIG. 3. Effect of Arg on the distribution of *arg-2–lacZ*, *arg-2*, *cox-5*, and *cpc-1* mRNA in polysomes. Transformants containing pZL601, pZL610, and pZL613 and wild-type *N. crassa* were grown for 6.5 h in Min or Arg medium. Extracts were prepared, and 6 A_{260} units of each extract was separated through sucrose gradients. The bar graphs represent data from one of two independent experiments that gave similar results. The *x* axis indicates the sucrose gradient fractions collected for each profile, with the sucrose concentration decreasing from fractions 1 to 8 (the four most dense fractions contained insignificant amounts of these transcripts and are not shown). Fractions 1 to 4 represent polysomal material; fraction 5 represents the monosome peak (arrow). Equal volumes of each fraction were examined by Northern blotting with *lacZ*, *arg-2*, *cox-5*, and *cpc-1* probes as indicated; Phosphorimager analysis was used to determine the relative amount of mRNA in each fraction, calculated as the percentage of the total amount of each transcript in all of the fractions (*y* axis). Bars represent the relative amounts of *lacZ*, *arg-2*, *cox-5*, and *cpc-1* transcripts in each fraction as indicated. Black bars represent samples from cells grown in Min medium; open bars represent samples from cells grown in Arg medium.

DISCUSSION

Defining the role of the *arg-2* uORF in translational control. Using *arg-2–lacZ* fusion genes, we obtained direct evidence that the *arg-2* uORF acts to regulate gene expression in response to Arg by reducing translation. The wild-type uORF specifying a 24-residue leader peptide and a frameshifted uORF specifying a 21-residue leader peptide both reduced gene expression at the level of translation, but only the wild-type uORF conferred Arg-specific negative translational regulation (Fig. 1 and 3).

Several distinct possibilities for uORF functions are suggested by these data. First, different uORFs might by a common mechanism reduce the pool of mRNA that is translated from the downstream AUG at a given time and thus reduce gene expression, e.g., by preemptive initiation. This is consistent with our observation that the amount of *arg-2–lacZ* mRNA associated with polysomes is reduced in both Min and Arg media when the mRNA contains either of two uORFs instead of lacking an uORF. Second, the wild-type *arg-2* uORF leader peptide could have additional sequence-specific effects on mRNA translation, i.e., by affecting transpeptidation or ribosome release (13, 15).

Is the *arg-2* uORF translated? Although we obtained no direct biochemical evidence for the leader peptide product, in-frame translational fusions of the *lacZ* coding region directly to the uORF coding region produced β-gal, while out-of-frame fusions did not, indicating that the predicted uORF was translated. Although the sequence context of the *arg-2* uORF translation initiation codon, CUU AUG A, appears to be a poor match to the consensus sequences surrounding *N. crassa* initiation sites (11, 26, 35), at least two other eukaryotic uORFs appear to have translational effects on gene expression despite poor initiation contexts (2, 27).

Our results are consistent with the scanning model (20) in that the presence of translatable uORFs had the effect of reducing expression from a downstream AUG. Our results contrast with the observation that removal of the AUG in the *S. cerevisiae CPA1* uORF results in the loss of Arg-specific negative regulation without increased constitutive expression (33). This could represent a difference between the *N. crassa* and *S. cerevisiae* systems or could be a consequence of additional negative regulatory factors acting to reduce *CPA1* expression (e.g., transcriptional or posttranscriptional control of RNA levels [4]) that were not acting on *N. crassa arg-2–lacZ* reporters.

Interestingly, the levels of *arg-2–lacZ* mRNA were not affected by prolonged growth in Arg medium, whereas the level of *arg-2* mRNA was reduced in our experiments (Fig. 2). It remains to be determined whether the fusion genes we used lacked sequences important for regulation of mRNA levels (e.g., the 3′ region of the *arg-2* transcript is absent in these constructs) or if the heterologous *lacZ* or *Aspergillus nidulans trpC* sequences present in the reporter genes interfered with regulation. *N. crassa* reporter constructs containing the *E. coli* hygromycin phosphotransferase gene instead of *lacZ* and retaining the 5′ and 3′ regions of *arg-2* retain Arg-specific negative regulation at the transcript level (12), consistent with these possibilities.

Comparison of the *arg-2* uORF and the *CPA1* uORF. The sequences for three fungal carbamoyl phosphate synthetase small-subunit genes, *N. crassa arg-2* (26), *S. cerevisiae CPA1* (33), and *Magnaporthe grisea arg-2* (31a), reveal that each con-

tains a highly conserved uORF in a similar position in the transcript. The amino acid sequences specified by the *arg-2* and *CPA1* uORFs appear to be important for Arg-specific regulation. All of the *cis*-acting mutations selected in vivo for constitutive *CPA1* expression affect the predicted leader peptide sequence (33). Multiple mutations in the *CPA1* uORF that leave the predicted amino acid sequence of the uORF unchanged have no effect on Arg-specific regulation of *CPA1* expression (8). A frameshift mutation that changes the *arg-2* uORF primary sequence, or truncation of the *arg-2* uORF, eliminates Arg-specific regulation (this work). Single missense mutations at codon 11 or 13 of the *CPA1* uORF and a similar mutation at one of these sites in the *N. crassa* uORF abolish Arg-specific regulation (12, 33).

The carboxyl-terminal regions of the predicted *arg-2* and *CPA1* leader peptides also appear important for regulation. The lack of Arg-specific regulation of the *arg-2* uORF–*lacZ* fusion gene in pZL6a (Fig. 1), which contrasts with the retention of regulation of a *CPA1* uORF-*lacZ* fusion containing several additional uORF codons (8), might also be a result of sequence changes that eliminate necessary functions of this region of the uORF. Consistent with this, a shortened *CPA1* uORF containing only the first 19 amino acid residues does not confer Arg-specific regulation (33). Since the region surrounding the stop codon has proven to be important in regulation by other eukaryotic uORFs (13), why this region of the *arg-2* uORF is required is an important question for further investigation.

Could ribosome stalling be involved in Arg-specific regulation? With both *arg-2* and *CPA1*, shortening the intercistronic distance between the uORF and the downstream open reading frame does not affect regulation, nor does lengthening the distance in *CPA1* (this work and reference 8). Furthermore, Arg-specific regulation is retained when the *arg-2* uORF and *lacZ* reading frames overlap (pZL6b) (Fig. 1). In *S. cerevisiae*, direct fusions of the *CPA1* uORF with *lacZ* retain Arg-specific regulation (8). The data indicate that ribosome reinitiation following termination of uORF translation is not an integral component of Arg-specific translational regulation through the *arg-2* and *CPA1* uORFs, in contrast to the translational response of *GCN4* to amino acid limitation (18). One possibility consistent with these data is that the wild-type *arg-2* uORF causes ribosomes to stall. Increased ribosome stalling mediated by the *arg-2* uORF in cells grown in Arg medium could explain the sequence-specific function of the *arg-2* uORF to reduce the average number of ribosomes associated with the mRNA in Arg medium.

Ribosome stalling has been proposed as a possible mechanism by which several eukaryotic uORFs control translation (14). In prokaryotes, translational attenuation of *cat-86* and *cmlA* occurs by a mechanism of ribosome stalling caused by leader peptide inhibition of the activity of peptidyl transferase (15). The sequences of the prokaryotic leader peptides that inhibit peptidyl transferase are important for this effect and bear resemblance to the *arg-2* and *CPA1* uORF sequences, suggesting the possibility that these phenomena are related (12).

How Arg is involved in Arg-specific regulation remains unknown. Presumably levels of charged tRNA or an arginine metabolite affect the activity of a translational component to cause increased uORF-mediated inhibition of translation during growth in Arg. The *N. crassa* and *M. grisae arg-2* uORF peptides each contain two Arg residues in identical positions, but the *CPA1* uORF peptide lacks Arg residues. The lack of Arg codons in the *CPA1* uORF indicates that uORF Arg codons are not necessary for Arg-specific regulation. Possibly, Arg codons in the *N. crassa* uORF may still affect gene expression. The second Arg codon in this uORF is the rarest Arg codon (11, 26), and there is evidence that rare codons in a eukaryotic uORF can reduce downstream gene expression (5). Another aspect of the role of Arg must be considered: direct translational fusions of *lacZ* to the *arg-2* uORF show reproducible increases in expression in Arg (pZL6a and pZL2a) (Fig. 1). Should this effect prove to be translational (and the Northern analysis data indicate a posttranscriptional component), then the reason why Arg stimulates translation from the uORF AUG must also be considered in understanding Arg-specific control through the *arg-2* uORF.

Sequences in other eukaryotic genes may have functions analogous to those of the *arg-2* uORF. Thus, a transcript might contain a uORF that has sequence-independent effects on translation and additional sequence-dependent effects in response to specific elicitors. Finally, because translation termination may not be an integral part of sequence-dependent translational regulation through uORF sequences, the possibility might be considered that other peptide coding sequences, which do not form a separate reading frame in the mRNA, might affect translation through similar mechanisms.

ACKNOWLEDGMENTS

We thank Michael Freitag, Alan Sachs, Margaret Alic, and Julie Carroll for helpful suggestions. This work was supported by the National Institutes of Health (grant GM-47498) and the Medical Research Foundation of Oregon.

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