Arginine-specific Regulation Mediated by the *Neurospora crassa* arg-2 Upstream Open Reading Frame in a Homologous, Cell-free in Vitro Translation System*

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Translational control mediated by an upstream open reading frame (uORF) in the 5′-leader of the *Neurospora crassa* arg-2 mRNA was reconstituted in a homologous, cell-free in vitro translation system. A cell-free *N. crassa* system was developed that required the presence of cap and poly(A) on RNA for maximal translation and that was amino acid-dependent. The 24-codon arg-2 uORF, when placed in the 5′-leader region of capped andadenylated synthetic luciferase RNAs, conferred Arg-specific negative regulation in this system. Improving the uORF translation initiation context decreased luciferase production and only slightly increased the magnitude of Arg-specific regulation. Mutation of uORF Asp codon 12 to Asn, which eliminates Arg-specific regulation, increased luciferase production and only slightly increased the magnitude of Arg-specific regulation. Mutation of uORF Asp codon 12 to Asn, which eliminates Arg-specific regulation. Arg-specific regulation

small subunit of arginine-specific carbamoyl phosphate synthetase, is subject to unique, arginine (Arg)-specific translational regulation (8). In Arg-containing medium, the translation of mRNA containing the wild-type arg-2 uORF decreases, and the decrease in translation is associated with a decrease in the number of ribosomes associated with the mRNA. The sequence of an uORF specifying a 24-residue peptide is critical for Arg-specific translational control: an Asp to Asn codon change at codon 12 of the arg-2 uORF (D12N) abolishes this control (9, 10). The corresponding *S. cerevisiae* gene, CPA1, contains a similar uORF whose sequence is also important for Arg-specific regulation, which presumably also occurs at the level of translation (11, 12).

The relatively common occurrence of uORFs in eukaryotic mRNAs (2,3,13) suggest that uORFs often have roles in modulating gene expression. As with the arg-2 and CPA1 uORFs, the sequences of the peptides specified by uORFs in the transcripts for mammalian β2-adrenergic receptor (14), human *S. cerevisiae* GCN4 (15), and maize *Lc* (17) RNAs appear to be important for uORF function. The sequences of uORF-encoded peptides in prokaryotes can also be important for translational control, and common mechanisms may be involved in uORF-mediated translational control in both kingdoms (5).

Cell-free translation systems have been invaluable for addressing many mechanisms of translational control (1). Here we describe an amino acid-dependent *N. crassa* cell-free translation system that reconstitutes cap, poly(A), and uORF effects on translation. Although there have been previous descriptions of programmable *N. crassa* cell-free translation systems (18–21), translational control has not been investigated using such a system. To our knowledge, these data represent the first instance in which translational control in response to the availability of a single amino acid has been reconstituted in a eukaryotic cell-free translation system.

**EXPERIMENTAL PROCEDURES**

Preparation of Templates Containing Wild-type and Mutant arg-2 Sequences—Megaprimer PCR (22) was used to obtain wild-type and mutant arg-2 DNA fragments to which 5′-BglII and 3′-XhoI sites were added. Templates for PCR reactions were plasmids pMF11-wt and pMF11-D12N (10), which contain the wild-type arg-2 uORF and the D12N (Asp to Asn) mutant uORF, respectively (see Fig. 7). The arg-2 region amplified by PCR and the nucleotide changes in mutant templates are indicated in Fig. 7. Primers for megaprimer PCR were: ZW1 (5′-CTGGAGATCTAAGCTTTTCGCG-3′), which includes the BglII site; ZW2 (5′-CCGCTCGAGCTTGAATATGT-3′), which includes the XhoI site; ZL19 (5′-TTGTCGCAATTCTGCCACAAGTCGTC-3′), which puts the uORF initiation codon in a better context (↑ uORF); and ZL17 (5′-ATCTGGCCCTTGGTAAAGCGG-3′), which moves the predicted uORF translation initiation codon (ΔAUG). Conditions for PCR were as described (10).

*BglII* and *XhoI*-digested megaprimer PCR products were gel-purified and ligated to *BglII* and *XhoI*-digested vectors pHLucS4 (see Fig.
Preparation of Synthetic RNA Transcripts—Synthetic RNA for N. crassa cox-5 (cytochrome oxidase subunit V) was obtained from plasmid pSRCOX5 (24), which had been linearized with HindIII. Synthetic RNA for N. crassa arg-2 was obtained from plasmid pARCG228 (25) linearized with EcoRI. Parallel were used in translation studies. These amounts were determined in Vogel’s minimal medium/1.5% sucrose at a concentration of 107 conidia/ml (8). To prepare cell-free extracts that were not incubated for 10 min at 21°C. EGTA was then added to a final concentration of 2.5 mM to inhibit the nuclease. Amino acid-dependent cell-free extracts were not treated with micrococcal nuclease. Extracts were aliquoted to Eppendorf tubes, frozen with liquid N2, and stored at −80°C.

Cell-free Translation and Analyses of Translation Products—Unless otherwise indicated, standard translation reactions (20 μl) contained 10 μl of N. crassa extract; 30 μM HERPS-ROH, pH 7.6; 3.75 mM MgOAc; 150 μM KOAc; 1 mM dithiothreitol; 1 mM ATP; 0.25 mM GTP; 25 mM creatine phosphate; 3.8 μM (0.9 units) of creatine phosphokinase; 25 μM of each amino acid; 4 units of ribonuclease inhibitor; and protease inhibitors (25 μg/ml p-aminophenylmethylsulfonyl fluoride, and 5 μg/ml each of pepstatin A, antipain, chymostatin, and leupeptin). Standard reactions were incubated at 25°C for 30 min and stopped by freezing in liquid N2.

Synthetic RNAs were prepared by run-off transcription of linearized DNA templates. Reactions (50 μl) to synthesize capped RNA contained 2 μg of linearized template, transcription buffer (40 mM Tris-HCl, pH 7.5, 6 mM MgCl2, 2 mM spermidine, 10 mM NaCl), 10 mM dithiothreitol, 0.5 mM each of ATP, CTP, and UTP, 2 μCi of [α-32P]UTP, 0.05 mM GTP, 0.5 mM GTP, 50 units of RNAsin, and 50 units of T7, T3, or SP6 RNA polymerase as appropriate. These synthesis conditions yield high levels of capped RNA (26) as borne out by analyses of the effects of different temperatures and different levels of K+ and Mg2+ on translation. Varying the concentrations of Mg2+ and K+ affected the translation of capped, polyadenylated luc RNA (capLUCpA RNA) and uncapped, unadenylated luc RNA (LUC RNA). Concentrations of these ions were chosen to yield the greatest relative translation of capLUCpA RNA compared with LUC RNA.

Translation of RNA in nucleus-treated reticulocyte lysates (Promega) was accomplished according to the supplier’s directions. Translation of RNA in S. cerevisiae cell-free extracts was as described (29).

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

RESULTS

Characterization of the N. crassa Cell-free Translation System—Analyses of [35S]methionine-labeled products from N. crassa and reticulocyte systems programmed with synthetic, capped cox-5, arg-2 or luc RNAs or containing no exogenous RNA indicated that these systems were comparable in activity (Fig. 1). The observed sizes of the COX5, ARG2, and LUC polypeptides were similar in these systems; measurements of luciferase enzyme activity produced were also comparable when similar amounts of capped, adenylylated luciferase RNA (capLUCpA RNA) were added to N. crassa and reticulocyte translation systems (see below).

A time-course experiment to measure production of luciferase (Fig. 2) from capLUCpA RNA revealed that within the first 10 min, there was little luciferase production; between 10 and 30 min, luciferase production increase linearly after 30 min. Luciferase production began to level off. [35S]methionine-labeling experiments and analyses of the synthesis of full-length, radiolabeled luciferase polypeptide yielded comparable results (data not shown). Synthesis of the full-length [35S]methionine-labeled COX5 polypeptide was observed earlier (6 min). Because COX5 polypeptide is smaller than LUC polypeptide, the lag in

ma)). The homogenized mycelia were centrifuged in a polycarbonate centrifuge tube for 10 min at 31,000 × g at 4°C in an SS34 rotor. The supernatant was carefully removed, avoiding both the pellet and the fatty upper layer; it was chromatographed on a 2.0 × 20-cm Sephadex G-25 Superfine column that was pre-equilibrated with buffer A. Fractions containing the peak of luciferase activity were pooled. Typical preparations yielded 6–7 ml of extract with an A260 nm = ~50. When extracts were treated with micrococcal nuclease, they were first adjusted to 1 mM CaCl2, then micrococcal nuclease was added to 50 units/ml, and the extracts were incubated for 10 min at 21°C. EGTA was then added to a final concentration of 2.5 mM to inhibit the nuclease. Amino acid-dependent cell-free extracts were not treated with micrococcal nuclease. Extracts were aliquoted to Eppendorf tubes, frozen with liquid N2, and stored at −80°C.
polypeptide synthesis was likely to be due in part to the time required for nascent polypeptide elongation (data not shown). The effects of cap and poly(A) on the efficiency of translation were examined in nuclease-treated \textit{N. crassa} extracts over a wide range of RNA concentrations (Fig. 3A). The level of luciferase production was linearly proportional to the level of RNA used to program translation in all cases over RNA concentrations varying by several orders of magnitude. Uncapped, unadenylated RNA (LUC RNA) was least efficiently translated. The addition of cap to RNA (capLUC RNA) increased translation. The addition of poly(A) alone to RNA (LUCpA RNA) stimulated translation more than addition of cap alone. The addition of both cap and poly(A) to mRNA (capLUCpA RNA) had synergistic stimulatory effects on translation, and capLUCpA RNA translated best. CapLUCpA typically translated more than 2 orders of magnitude more efficiently than LUC RNA.

Another observation consistent with the cap- and poly(A)-dependent translation that was seen in nuclease-treated extracts was that the addition of exogenous cap analog to \textit{N. crassa} translation reactions strongly inhibited the translation of capLUC RNA (Fig. 4). The addition of poly(A) to RNA relieved inhibition of translation by cap analog; translation of LUCpA RNA was least affected by the addition of analog, and translation of capLUCpA RNA was less affected than capLUC RNA (Fig. 4).

Removal of endogenous RNA by pretreatment of \textit{N. crassa} extracts with micrococcal nuclease was necessary for detecting \textsuperscript{35}S-methionine-labeled translation products (Fig. 1 and data not shown). However, this was not necessary for luciferase measurements. Thus, the addition of cap and poly(A) to RNA had similar, synergistic effects on translation of luciferase in \textit{N. crassa} extracts that were not treated with nuclease (Fig. 3B) as
extracts that were nuclease-treated (Fig. 3A). Commercial reticulocyte lysate did not show cap- and poly(A)-dependent effects on translation under the recommended translation conditions (Fig. 3B).

Direct measurement of the chemical stability of translated RNAs showed that RNA was degraded during incubation in N. crassa translation reactions (Fig. 5). Cap and poly(A) both stabilized the RNA; cap had a greater effect than poly(A).

**Effects of Upstream Open Reading Frames on Translation—**

The standard N. crassa translation system prepared from cells growing in minimal medium was not dependent on the addition of exogenous amino acids for translation (Fig. 6), and the addition of extra Arg did not affect translation of uORF-containing RNAs (data not shown). Therefore, as described under “Experimental Procedures,” we used a different set of growth conditions that enabled the preparation of amino acid-dependent N. crassa translation extracts to test the effects of adding Arg. The effects of adding different concentrations of 20 amino acids on production of luciferase in amino acid-dependent and -independent N. crassa translation reactions are shown in Fig. 6. The amino acid-dependent translation system enabled testing of Arg-specific translational regulation in vitro.

Both amino acid-independent and amino acid-dependent cell-free translation systems were used to examine the effects of the arg-2 uORF on translation of the luciferase coding regions in RNA. Wild-type and mutant arg-2 sequences (Fig. 7) were placed upstream of the luciferase coding regions in either of two vectors (Fig. 8, A and C). One vector contained the wild-type luciferase coding region, LUC; the other contained a luciferase coding region modified by site-specific mutagenesis to alter codon usage and eliminate several restriction enzyme cleavage sites, LUC+NF. Constructs were used to produce capped, polyadenylated RNA that contained the luciferase coding region and (i) no arg-2 sequence; (ii) the wild-type arg-2 5′ leader containing the uORF (wild-type uORF); (iii) an arg-2 5′ leader containing the wild-type arg-2 uORF in a better predicted initiation context ([Δ] uORF); (iv) the wild-type arg-2 5′ leader containing the arg-2 uORF with the Arg to Aas change that abrogates regulation in vitro (D12N); (v) a double mutant containing the D12N uORF in a better predicted initiation context (D12N ▼ uORF); and (vi) a mutated arg-2 5′ leader lacking the uORF AUG codon (∆AUG).

Equal amounts of the capped, adenylated RNAs obtained from the vectors in Fig. 8A were compared in the amino acid-independent, micrococcal nuclease-treated N. crassa in vitro translation system and nuclease-treated S. cerevisiae and reticulocyte systems. Transcripts containing uORFs were translated less well than transcripts lacking uORFs in the N. crassa system, as also observed in the amino acid-dependent system (discussed below); similar results were observed with the S. cerevisiae translation system but not with the reticulocyte system, which did not appear to respond to uORFs (data not shown).

In amino acid-dependent translation reactions, the addition of Arg to a final concentration of 10 μM and the addition of the other 19 amino acids to final concentrations of 10 μM were sufficient for near maximal translation of luciferase (Fig. 6), enabling testing of the effects of adding 10 or 500 μM Arg on the translation of uORF-containing and control RNAs (Fig. 8B). Excess Arg did not affect translation of RNA obtained from a construct that lacks all arg-2 sequences (T7LUC). Arg reduced translation of luciferase in RNA containing the wild-type uORF in its original translation initiation context or in an improved initiation context upstream of LUC (Fig. 8B). A reproducible, slight increase in Arg-specific regulation was seen with constructs containing the uORF in an improved initiation context.
Improving the translation initiation context of the uORF also decreased production of luciferase from the downstream initiation codon. In contrast to the wild-type uORF constructs, the D12N uORF construct did not show Arg-specific regulation. Eliminating the uORF initiation codon eliminated regulation and increased expression to the level observed in the construct lacking arg-2 sequences (Fig. 8B).

The data shown in Fig. 8B were highly reproducible. Considering the T7LUC, wild-type uORF, and D12N uORF constructs alone, five different batches of RNA translated in nine independently derived Arg-dependent N. crassa extracts gave similar results. Three independent batches of RNA synthesized from all five constructs have been translated in three independently derived Arg-dependent translation extracts with similar results.

The effects of uORFs on Arg-regulation were confirmed and extended using a second set of luciferase constructs in which LUC1NF (Promega) has been substituted for LUC (Fig. 8D). In N. crassa extracts, RNAs containing the LUC+NF polypeptide coding region instead of LUC produced similar yields of luciferase activity. The wild-type uORF construct, the $\uparrow$ uORF construct and the D12N uORF construct showed effects on translation similar to those observed in the original LUC constructs (Fig. 8, compare B and D). An additional construct, in which the D12N mutation was placed in an uORF that had an improved initiation context (D12N $\uparrow$ uORF), showed reduced translation of luciferase compared with the D12N mutation in the original uORF initiation context but still showed no Arg-specific regulation (Fig. 8D).

The primary mechanism of Arg-specific regulation mediated by the arg-2 uORF in the in vitro translation system does not appear to involve changes to the RNA. First, the chemical stability of each of the types of RNAs used in Fig. 8B was measured by determining the amount of trichloroacetic acid-soluble radioactivity released from RNA over the course of translation reactions. There were no discernible differences among RNAs; the addition of excess Arg did not appear to affect the stability of any of these RNAs (data not shown). Second, the negative effects of Arg on translation of the wild-type uORF RNA appeared to be reversible. Translation reactions containing excess Arg that were programmed with RNA containing the wild-type uORF translated RNA at a reduced rate compared...
with extracts without excess Arg (Fig. 9). 14-fold dilution of such translation mixtures with additional complete translation mixture lacking Arg after translation was initiated for 20 min resulted in an increased rate of RNA translation (Fig. 9). This ratio was comparable with that observed in translation reactions initiated without excess Arg that were monitored at a similar time and was substantially higher than the rates observed in reactions diluted with reaction mixture containing excess Arg (Fig. 9). Similar results were obtained when reactions were diluted at 15 or 30 min instead of 20 min after incubation (data not shown). These data indicate that (i) degradation or irreversible modification of uORF-containing RNA was unlikely to be responsible for its reduced translation in the presence of excess Arg and (ii) the effects of Arg on translation of uORF-containing RNA appeared reversible.

How specific is Arg-specific regulation? The effects of compounds that might be expected to act similarly to Arg were examined by comparing the addition of 500 mM of Arg or 500 mM of each of these compounds. The addition of 150 mM Arg was sufficient to observe maximal regulatory effects (data not shown). The addition of canavanine, an Arg analog that can be incorporated into polypeptides and, when incorporated, prevents their functional activity, resulted in a loss of luciferase activity in all cases, and its activity was not evaluated. Arginine methyl ester and arginine ethyl ester, which can be hydrolyzed to Arg, conferred regulation, but the Arg biosynthetic precursors citrulline and ornithine did not, nor did the basic amino acids His and Lys. Homoarginine, which has a side chain that is one methyl group longer than Arg, did not confer regulation. The Arg-related compounds agmatine, l-argininamide, phospho-l-arginine, l-argininic acid, and d-arginine did not confer regulation. These data strongly indicate that Arg-specific translational regulation has a high specificity for sensing the level of L-arginine.

**DISCUSSION**

In vivo, Arg-specific regulation has effects on the expression of the N. crassa arg-2 and S. cerevisiae CPA1 genes specifying the small subunit of Arg-specific carbamoyl phosphate synthetase at both transcriptional and translational levels (8, 25, 30, 31). Here we show that the N. crassa arg-2 uORF has a role in Arg-specific translational regulation using a homologous cell-free in vitro translation system programmed with synthetic RNA. The sequence of the uORF was critical for regulation in vitro, but the context of the uORF codon initiation codon was not. Furthermore, Arg-specific translational regulation appeared specific for l-arginine and was not elicited by related amino acids or by biosynthetic precursors to Arg. These data confirm that the arg-2 uORF modulates gene expression at the level of translation and to our knowledge represent the first demonstration of translational control in a eukaryotic in vitro system in response to the availability of a single amino acid.

Translation of RNA in the N. crassa cell-free translation system also was dependent on important features of eukaryotic RNA, cap, and poly(A). The addition of poly(A) to RNA stimulated translation in the N. crassa cell free system independently of the addition of cap to RNA, whereas the addition of both to RNA synergistically stimulated translation (Fig. 3). Although synergistic interactions between cap and poly(A) have been demonstrated in electroporated mammalian and fungal cells (32), only recently have such interactions been observed in vitro and only in S. cerevisiae cell-free translation systems (26, 29). The effects of adding cap and poly(A) to RNA used to program N. crassa and S. cerevisiae translation reactions are similar, and, in both systems, the addition of poly(A) to RNA relieves the inhibitory effects of adding exogenous cap analog to translation reactions. In the S. cerevisiae system, these results have been combined with additional studies to support the interpretation that cap and poly(A) stimulate translation initiation by recruiting different RNA-binding proteins (29), and it is likely that a similar situation holds in N. crassa.

In the N. crassa cell-free system, the presence of cap and poly(A) on mRNA appear to affect its chemical stability (Fig. 5 and data not shown). In S. cerevisiae, similar effects have been reported in some studies (33) but not others (26, 29). The reasons why cap and poly(A) have effects on RNA stability in some in vitro studies but not others remains unclear, but a variety of studies have implicated that these features of the RNA molecule are important in modulating RNA stability (34, 35).

The in vitro data indicate that the arg-2 uORF has sequence-independent and sequence-dependent effects on translation. Thus, either the wild-type uORF or the D12N uORFs in the wild-type translation initiation context reduce translation of the downstream luciferase coding region (Fig. 8, B and D). The uORF’s wild-type initiation context is not typical for N. crassa (8); changing the initiation context of either uORF to one resembling preferred N. crassa initiation contexts (36) further reduces translation of the downstream luciferase coding region. These data are consistent with the scanning model for translation initiation (37, 38).

Arg-specific and uORF-sequence-dependent regulation is observed in vitro in addition to sequence-independent uORF effects. Arg-specific regulation in wild-type cells mediated through the arg-2 uORF is approximately 2.5-fold in vivo, based on measurements of accumulated polypeptide products (8–10); a similar level of regulation is observed in vitro, based on measurements of accumulated luciferase product (Fig. 8). Measurements of relative rates of ARG2 polypeptide synthesis in vivo immediately after switching cells from minimal medium to fresh minimal medium or to Arg-containing medium indicate a 2.5-fold reduction in the rate of polypeptide synthesis upon exposure to Arg (8); a similar reduction in the rate of luciferase synthesis from uORF-containing RNA is observed in vitro.
when translation reactions contain excess Arg (Fig. 9), based on comparing the rates of synthesis of reactions containing 10 µM or 150 µM Arg.

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

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