MOLECULAR AND CELLULAR BIOLOGY, Sept. 1997, p. 4904–4913 Vol. 17, No. 9
0270-7306/97/$04.00+0

Ribosome Stalling Is Responsible for Arginine-Specific Translational Attenuation in *Neurospora crassa*

ZHONG WANG AND MATTHEW S. SACHS*

Department of Biochemistry and Molecular Biology, Oregon Graduate Institute of Science & Technology, Portland, Oregon 97291-1000

Received 16 April 1997/Returned for modification 15 May 1997/Accepted 3 June 1997

The *Neurospora crassa arg-2* upstream open reading frame (uORF) plays a role in negative arginine-specific translational regulation. Primer extension inhibition analyses of *arg-2* uORF-containing RNA translated in a cell-free system in which arginine-specific regulation was retained revealed "toeprints" corresponding to ribosomes positioned at the uORF initiation and termination codons and at the downstream initiation codon. At high arginine concentrations, the toeprint signal corresponding to ribosomes at the uORF termination codon rapidly increased; a new, broad toeprint that represents additional ribosomes stalled on the uORF appeared 21 to 30 nucleotides upstream of this site; and the toeprint signal corresponding to ribosomes at the downstream initiation codon decreased. These data suggest that arginine increases ribosomal stalling and thereby decreases translation from the downstream initiation codon.

Upstream open reading frames (uORFs) are present in the 5′ leaders of a number of eukaryotic mRNAs, particularly those involved in growth and development (12, 14, 18, 22, 30). Some of these are known to reduce translation from downstream initiation codons. In the best-understood example of eukaryotic uORF control, *Saccharomyces cerevisiae GCN4*, the predicted primary sequences of the uORFs appear relatively unimportant for their function. In other cases, the primary sequences of the uORFs are critical. The mechanistic basis for the action of these uORFs has been hypothesized to involve the sequence-dependent arrest of the ribosomes translating them. This creates a blockade to ribosomal scanning that reduces ribosome loading at the downstream initiation codon (13). Only for uORF2 of the cytomegalovirus *gp48* transcript has arrest of ribosomes at a eukaryotic uORF been directly demonstrated (3, 4). In this case, ribosomes appear to arrest by an unregulated mechanism at the uORF translation termination site. However, the mechanisms by which other sequence-specific eukaryotic uORFs act and how they might play regulatory roles remain unknown.

Ribosome stalling can be detected by a primer extension inhibition ("toeprint") assay (15, 16). Toeprinting showed ribosome arrest at the *gp48* uORF2 termination codon (3). The toeprinting technique has also been applied to eukaryotic systems to detect ribosomes and translation factors at initiation codons (1, 20, 28, 29). In these cases, ribosomes bound at an AUG initiator were found to cause reverse transcriptase to terminate primer extension at a site 15 to 17 nucleotides (nt) downstream from the A of this codon. A related technique has been used in other eukaryotic cell-free systems treated with cycloheximide to detect stalled, elongating ribosomes (39) and ribosomes at initiation codons and termination codons (8, 9).

The level of *Neurospora crassa* arginine-specific carbamoyl phosphate synthetase, which generally determines flux through the Arg biosynthetic pathway (6), is determined by the level of the *arg-2*-encoded polypeptide subunit (5). *arg-2* is the only gene encoding an *N. crassa* Arg biosynthetic enzyme that is negatively regulated by Arg (5). The *arg-2* mRNA contains an uORF specifying a 24-residue peptide (Fig. 1A) (26) that appears to be translated in vivo as determined by using fusions of the uORF to *Escherichia coli lacZ* (24). The *arg-2* uORF sequence is important for negative translational regulation by Arg in vivo. For example, changing uORF Asp codon 12 to Asn (D12N) eliminates regulation by Arg (11). The number of ribosomes associated with RNA containing the wild-type uORF, but not the D12N uORF, is reduced in cells exposed to excess Arg (11, 23, 24).

The sequence of the *arg-2* uORF is evolutionarily conserved, which is consistent with its having functional significance. The homologous *arg-2* gene from the rice blast fungus *Magnaporthe grisea* contains an uORF specifying a nearly identical peptide (34); the corresponding *CPA1* gene from *S. cerevisiae* contains an uORF specifying a closely related peptide (Fig. 1B). Arg-specific regulation of *CPA1* also requires the uORF; a mutation corresponding to the *arg-2* uORF D12N mutation eliminates Arg-specific regulation (7, 38).

To gain a better understanding of the mechanism of Arg-specific translational control, we developed a cap-, poly(A)-, and amino acid-dependent cell-free *N. crassa* protein-synthesizing system (37). In this system, Arg-specific translational control by the *arg-2* uORF is reconstituted, as judged by translation of dicistronic *arg-2-LUC* RNA containing uORF and luciferase (*LUC*) coding regions (37). Arg-specific regulation and the effects of uORF mutations on regulation appear similar in vitro and in vivo.

Here we present toeprint data indicating the presence of ribosomes on *arg-2–LUC* RNA during its translation in vitro, including ribosomes at the uORF initiation codon, the uORF termination codon, and the *LUC* initiation codon. We show that in the presence of excess Arg, ribosome stalling at the uORF is increased. Stalling of ribosomes translating the uORF was accompanied by a decrease in the number of ribosomes associated with the downstream *LUC* initiation codon. The sequence of the uORF, and its capacity to be translated, were necessary for these effects. These data suggest that ribosome stalling accounts for Arg-specific negative regulation mediated by the *arg-2* uORF.

* Corresponding author. Mailing address: Department of Biochemistry and Molecular Biology, Oregon Graduate Institute of Science & Technology, P.O. Box 91000, Portland, OR 97291-1000. Phone: (503) 690-1487. Fax: (503) 690-1464. E-mail: msachs@admin.ogi.edu.

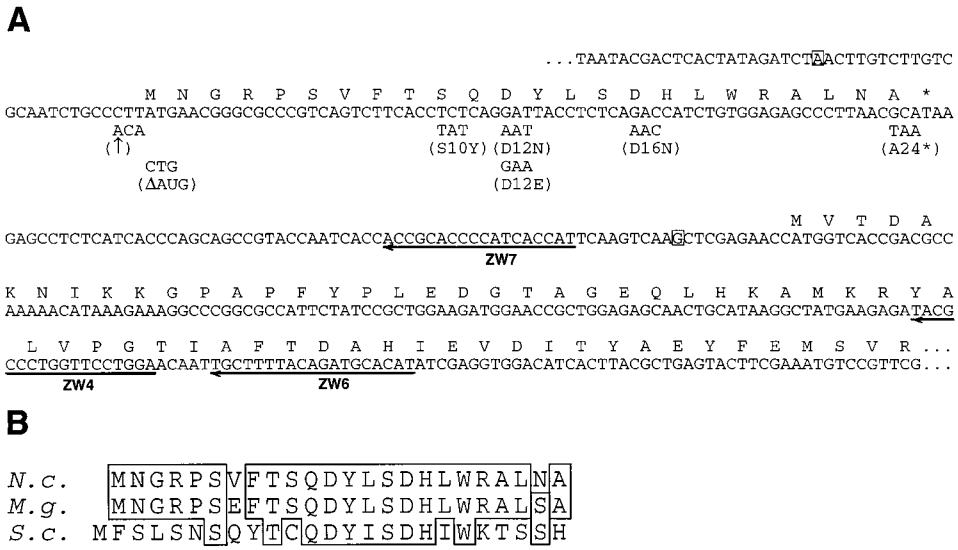

FIG. 1. The 5′ region of the *arg-2–LUC* gene and comparison of *arg-2* uORF-related peptides. (A) Sequences of wild-type and mutant *arg-2–LUC* templates. The sequence shown begins with the T7 RNA polymerase-binding site and ends within the luciferase coding region. The 5′ and 3′ boundaries of the *arg-2* region are boxed. The amino acid sequences of the *arg-2* uORF and the amino terminus of luciferase are indicated. Specific mutations and their predicted consequences for uORF translation are shown below the wild-type sequence. The (↑) mutation improves the initiation context for uORF translation. The sequences for which the reverse complements were synthesized as primers ZW4, ZW6, and ZW7 are indicated by horizontal arrows. (B) Alignment of the peptide sequences encoded by the uORFs in the transcripts of the homologous genes *N. crassa arg-2* (*N.c.*) (26), *M. grisea arg-2* (*M.g.*) (34), and *S. cerevisiae CPA1* (*S.c.*) (38).

MATERIALS AND METHODS

Preparation of templates containing wild-type and mutant *arg-2* sequences. Megaprimer PCR (33) was used to construct mutated *arg-2* sequences (Fig. 1A) flanked by 5′ *Bgl*II and 3′ *Xho*I sites. Mutagenic primers for megaprimer PCR included ZL20 (5′-ATCTGCCCTTCTGAACGGGC-3′), which eliminates the uORF AUG codon; OJC102 (5′-GTCAGTCTTCACCTATCAGGA-3′), which introduces the S10Y mutation; OJC103 (5′-ACCTCTCAGGAATACCTCTCA-3′), which introduces the D12E mutation; OJC104 (5′-TACCTCTCAAACCA TCTGTGG-3′), which introduces the D16N mutation; and OJC108 (5′-GCCC TTAACTAATAAGAGCCTC-3′), which introduces the A24* mutation. *Bgl*II- and *Xho*I-digested PCR products were placed into the corresponding sites of pHLUC+NFS4, and the sequences of these constructs were confirmed as described previously (37).

Preparation of synthetic RNA transcripts. Plasmid DNA templates were purified by equilibrium centrifugation and linearized with *Ppu*10I. Capped, polyadenylated RNA was synthesized with T7 RNA polymerase, and the yield of RNA was quantitated (37).

Cell-free translation and analyses of translation products. Amino acid-dependent *N. crassa* cell-free translation extracts were prepared, used, and assayed for luciferase enzyme activity as described previously (37). For reaction mixtures subjected to toeprint analyses, the concentration of *arg-2–LUC* transcript was increased to 6 ng/μl (which was near the maximal concentration of transcript for which luciferase production remained linearly proportional to the level of transcript added to translation reaction mixtures) and the concentration of RNasin RNase inhibitor was increased from 0.2 to 0.8 U/μl.

Preparation of 5′-^{32}P-labeled primers for toeprinting and sequencing reactions. Oligodeoxynucleotides ZW4, ZW6, and ZW7 (Fig. 1A) were labeled at their 5′ termini with T4 polynucleotide kinase (New England Biolabs) and [γ-^{32}P]ATP (>6,000 Ci/mmol; Andotek Life Sciences, Irvine, Calif.). The reaction mixtures (100 μl) contained 50 pmol of oligodeoxynucleotide, 50 mM Tris-HCl (pH 8.0), 20 mM $MgCl_2$, 4 mM spermidine, 4 mM dithiothreitol, 400 μCi [γ-^{32}P]ATP, and 10 U of T4 kinase. The primer was first mixed with Tris-HCl, and the volume was adjusted to 34 μl; the primer was then heated at 90°C for 3 min and chilled on ice. Water, $MgCl_2$, spermidine, dithiothreitol, [γ-^{32}P]ATP, and kinase were added, and the mixture was incubated for 45 min at 37°C. EDTA was added to a final concentration of 50 mM, and the reaction mixture was extracted with 120 μl of buffered phenol-chloroform. The aqueous phase was transferred to a new tube; the phenol-chloroform phase was back-extracted with 80 μl of Tris-EDTA (TE), and this wash was combined with the original aqueous phase. This aqueous solution was extracted once more with an equal volume of chloroform and then chromatographed on a Sephadex G-25 superfine column (5-ml bed volume in a 5-ml disposable pipette) that was preequilibrated with 10 mM NH_4HCO_3 and developed in the same buffer. Fractions were collected, and portions (1 μl) were analyzed by polyethyleneimine thin-layer chromatography to check for the presence of inorganic phosphate and unreacted ATP. Fractions containing radiolabeled oligonucleotide were pooled and lyophilized. Oligonucleotides were dissolved in 250 μl of water (yielding a primer concentration of 0.1 μM if a recovery of 50% is assumed).

Primer extension inhibition (toeprint) assays. The primer extension inhibition (toeprint) assay was modified from previously described procedures (3, 16). Toeprint assays were performed by adding 3 μl of translation reaction mixtures (or pure RNA) to 5.5 μl of reverse transcription buffer, which was already in a tube precooled on ice. This buffer contained components to bring the final 10-μl reverse transcription reaction mixture to 50 mM Tris-HCl (pH 8.3); 75 mM KCl; 10 mM $MgCl_2$; 0.01 M dithiothreitol; 0.25 mM each dATP, dCTP, dGTP, dTTP; and 1,000 U of RNasin RNase inhibitor per ml (ignoring the contributions of components of the translation reaction mixture). The tubes were heated at 50°C for 2 min and then immediately placed on ice again. This heating step was essential to observe toeprinting. ^{32}P-labeled primer (1 μl; approximately 2×10^6 cpm of Cerenkov) was added to each tube and annealed to the template by placing tubes in a 37°C water bath for 5 min. Then 0.5 μl (100 U) of Superscript II RNase H^- reverse transcriptase (Gibco BRL) was added, and the reverse transcription reaction mixtures were incubated for 30 min at 37°C. The reactions were terminated by extraction with 10 μl of phenol-chloroform. The aqueous phase was removed and mixed with an equal volume of DNA sequencing stop solution (91% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF). Samples were heated at 85°C for 5 min, cooled on ice, and loaded on 6% DNA denaturation sequencing polyacrylamide gels (4 μl of sample per lane). The gels were dried and exposed for at least 3 days to Kodak XAR-5 film without intensifying screens (screens decreased the sharpness of bands); photographs of these autoradiograms are presented here (see Fig. 4 to 7). Gels were also analyzed with a Molecular Dynamics PhosphorImager for quantitative data. All toeprint data presented are representative of multiple experiments.

Typically, translation reaction volumes for toeprint experiments were 20 μl. For time course experiments (see, e.g., Fig. 7), larger volumes were used. In time course studies, coordinated teamwork was required for precise, accurate handling of serial samples.

To study the effects of chemical inhibitors on translation, compounds were added (0.5 μl of 40X stock solutions) to the indicated final concentrations: puromycin (640 μM), cycloheximide (320 μM), hygromycin (8 mM), and EDTA (5 mM). Additional $MgCl_2$ (5 mM) was added back to reaction mixtures incubated with EDTA prior to primer extension analyses.

DNA sequencing markers were obtained by using the same ^{32}P-labeled oligonucleotide primers used for toeprinting to sequence the plasmid template containing the wild-type uORF. Non-cycle sequencing was performed with the ΔTaq cycle-sequencing kit (United States Biochemicals) by modifying the supplier's procedure; labeling reactions were omitted, and the termination reactions were incubated for 10 min at 67°C (primers ZW4 and ZW7) or 20 min at 57°C (primer ZW6).

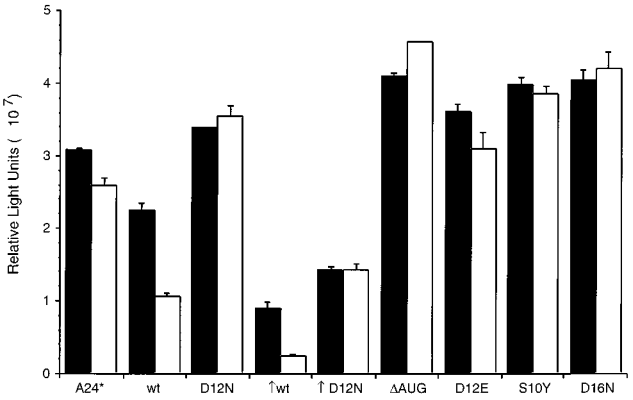

FIG. 2. Effects of uORF mutations on Arg-specific translational control in vitro. Equal amounts (1.2 ng) of wild-type (wt) and mutant (Fig. 1A) RNA were translated in *Neurospora* extracts containing 10 μM (solid bars) or 500 μM (open bars) Arg and 10 μM each of the other 19 amino acids (37). Mean values and standard deviations obtained from measuring the luciferase enzyme activity in three independent translation reactions are given. The ↑wt RNA has the wild-type uORF in an improved initiation context; the ↑D12N RNA has the D12N uORF in this context.

RESULTS

Translational arrest mediated by the *arg-2* uORF and Arg. Arg-specific translational control mediated by the *arg-2* uORF can be observed in a homologous cell-free translation system (37). When the concentration of Arg is increased from 10 μM (low Arg) to 500 μM (high Arg) in reaction mixtures programmed with dicistronic *arg-2–LUC* RNA containing the wild-type uORF in the 5′-leader sequence (Fig. 1A), the synthesis of luciferase is reduced (Fig. 2). Mutations (Fig. 1A) which alter the *arg-2* uORF initiation codon to eliminate uORF translation (ΔAUG) or which change a critical Asp residue at codon 12 of the uORF to Asn (D12N) eliminate this negative regulatory effect of Arg (Fig. 2) (37).

Arg may exert its negative effect on translation of *arg-2* through stalling of ribosomes translating the uORF, thereby hindering access to the downstream luciferase translation initiation codon (22). We tested this possibility by primer extension inhibition (toeprinting) (16) assays, in which reverse transcriptase is used to extend a radiolabeled primer on an RNA template in the presence or absence of cellular factors (Fig. 3A). Toeprinting should reveal the positions of translational components, such as ribosomes, at sites where they accumulate on RNA (e.g., at sites of rate-limiting steps in translation). Below, we will use the term "ribosome" inclusively to refer to 80S ribosomes, translation initiation complexes, and termination complexes.

In the absence of extract, reverse transcription of synthetic *arg-2–LUC* RNAs with any of three radiolabeled primers (ZW4, ZW6, and ZW7 in Fig. 1A) yielded cDNA extension products predominantly corresponding to full-length transcripts (see, e.g., Fig. 4, lane 12, and Fig. 6, lane 19 [data not shown]). This was determined by comparing the positions of the primer-extended cDNA products to dideoxynucleotide-sequencing products obtained by using the same radiolabeled primers to sequence corresponding DNA templates. Several minor, shorter termination products were also reproducibly observed.

When *arg-2–LUC* RNA present in the cell-free translation reaction mixture was used for toeprint analyses, additional premature, site-specific termination products were observed (Fig. 4, lanes 1 and 2). Several modifications of previously described procedures for toeprinting RNA in eukaryotic translation-competent extracts (1, 3) were important to obtain these results. Both heat treatment of the translation reaction mixture prior to adding primer and maintenance of high magnesium concentrations during reverse transcription appeared necessary.

Among the additional species observed in translation reaction mixtures containing low concentrations of Arg and wild-type *arg-2–LUC* RNA were toeprint sites ≈16 nt downstream of the A of the uORF AUG initiation codon, ≈13 nt downstream of the U of the uORF UAA termination codon and ≈16 nt downstream of the A of the LUC AUG initiation codon (Fig. 4, lane 1; indicated by arrows as AUG_{uORF}, UAA_{uORF}, and AUG_{LUC}, respectively). The location of these products was determined by comparing series of alternating lanes of toeprinted translation reactions and sequencing reactions (data not shown). While the migration of dideoxy-sequencing products and cDNA products differed slightly, the precision of these measurements was within a nucleotide.

The toeprint signals observed in the *Neurospora* extracts arose from interactions between the extract and the input

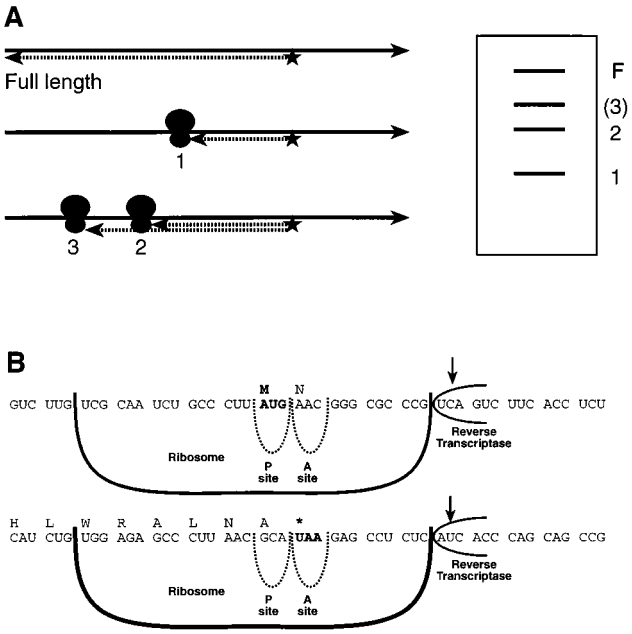

FIG. 3. The primer extension inhibition (toeprint) assay. (A) Principle of the assay. Radiolabeled oligonucleotide primer (star) anneals to the RNA template (solid arrow) and is extended by reverse transcriptase (dotted arrow). On pure RNA, extension proceeds to the 5′ end of the template. Ribosomes accumulated at different discrete positions on the RNA will inhibit primer extension (e.g., ribosome 1). Analyses of primer extension (toeprint) products on denaturing sequencing gels (right) enable the determination of the sizes of primer extension products; the full-length product (F) migrates most slowly. Ribosomes could also accumulate on RNA at multiple sites (e.g., ribosomes 2 and 3). When multiple complexes are associated with RNA, a toeprint analysis would be expected to yield only signals corresponding to the complex that is first encountered on an RNA template by the elongating reverse transcriptase, if this first complex were infinitely stable; however, dissociation of this complex would allow primer extension beyond this site. (B) The relationship between toeprint sites and the P and A sites of the ribosome. Eukaryotic ribosomes or 40S subunits bound to RNA with an initiation codon at their P site cause toeprints 15 to 17 nt downstream (1, 28). (Top) A ribosome with the *arg-2* uORF initiation codon in its P site blocks the movement of reverse transcriptase on the RNA template, causing premature termination (arrow) 16 nt downstream of the A of the AUG initiator. (Bottom) A ribosome with the uORF termination codon, UAA, at its A site causes premature termination 13 nt downstream of the U of the termination codon).

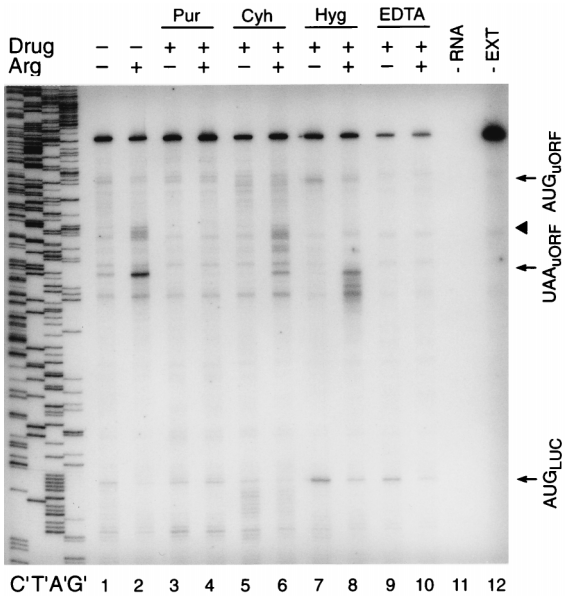

FIG. 4. Effects of translational inhibitors on toeprinting. The RNA transcript containing the wild-type uORF (120 ng) was translated for 20 min in 20-μl reaction mixtures at 25°C. Reaction mixtures contained 10 μM (−) or 500 μM (+) Arg as indicated and 10 μM each of the other 19 amino acids. Translation inhibitors were added as described in the text: puromycin (Pur), cycloheximide (Cyh), hygromycin (Hyg), and EDTA. Radiolabeled primer ZW6 was used for primer extension analyses (lanes 1 to 12) and for sequencing of the wild-type template (the four left-hand lanes). For the dideoxynucleotide sequencing of the wild-type template, the nucleotide complementary to the dideoxynucleotide added in each reaction mixture is indicated above the corresponding lane so that the sequence of the wild-type template (Fig. 1) can be directly deduced: the 5′-to-3′ sequence reads from top to bottom. The products obtained from primer extension of translation extract in the absence of input RNA (−RNA [lane 11]) and from a corresponding amount of pure RNA (18 ng) in the absence of translation extract (−EXT [lane 12]) are shown for comparison. Arrows indicate the positions of premature transcription termination products corresponding to ribosomes bound at AUG_{uORF}, UAA_{uORF}, and AUG_{LUC}; the arrowhead indicates the position of an additional toeprint site induced in 500 μM Arg.

RNA template; parallel primer extension analyses of extracts to which no *arg-2–LUC* RNA was added did not yield these products (see, e.g., Fig. 4, lane 11, and Fig. 6, lane 20 [data not shown]). Because eukaryotic translation initiation complexes and 80S ribosomes, when bound at initiation codons, cause toeprints at sites approximately 15 to 17 nt distal from the A of AUG initiation codons (1, 28) and for additional reasons given below, these toeprint sites appear to correspond to ribosomes positioned on RNA with AUG_{uORF} and AUG_{LUC} initiation codons at their P sites and UAA_{uORF} termination codons at their A sites (Fig. 3B).

Addition of high instead of low Arg levels to translation reaction mixtures containing RNA with the wild-type uORF caused a substantial increase in the toeprint signal corresponding to the uORF termination codon (Fig. 4, compare lanes 2 and 1). In addition, high Arg levels caused the appearance of a cluster of strong toeprint signals 21 to 30 nt upstream of the termination codon toeprint. These effects of Arg on uORF toeprinting were observed with all three primers (Fig. 1A) used for primer extension. Addition of Arg also reduced the toeprint signal at the *LUC* initiation codon. Therefore, the translational response of this RNA to Arg, as measured by the twofold reduction in luciferase synthesis (Fig. 2), was correlated, at the 20-min time point of the translation reaction, with increased toeprint signals at the uORF and a decreased toeprint signal at the *LUC* initiation codon. In some experiments (see, e.g., Fig. 6), high Arg levels also caused a slightly increased signal at a site downstream of the uORF termination codon; a corresponding signal was present at a lower level in primer extension analyses of RNA in the absence of extract. This toeprint signal also increased when hygromycin was added to reaction mixtures as described below. The significance of this species is unknown. Finally, an additional strong toeprint was observed downstream of the LUC initiation codon at both low and high Arg concentrations. This toeprint might correspond to ribosomes with the rare *N. crassa* Lys codon, AAA (10), in their A sites or might arise from the binding of nonribosomal factors to the RNA.

Effects of limiting protein synthesis. Several alternative explanations could be given for the additional toeprint signals observed on *arg-2–LUC* RNA when it is translated in cell-free extracts. These toeprints could arise by nucleolytic cleavage of the RNA at specific sites when the RNA is incubated in the cell-free translation extracts. Alternatively, they could arise from factors in the extract that complexed to RNA in a manner independent of translation or from secondary structures in the RNA that formed in the presence of extract independent of translation and that impede primer extension. To discriminate among these possibilities, we examined the effects of inhibiting translation by using chemical inhibitors and by limiting the amino acids. Inhibiting translation in these ways should preferentially affect toeprint signals arising from the association of ribosomes with RNA.

Puromycin is an antibiotic analog of aminoacyl-tRNA that binds to the ribosomal A site and undergoes a transpeptidation reaction. The peptide-puromycin product that is formed cannot be extended further; translation terminates prematurely and ribosomes dissociate from the RNA. A second inhibitor, cycloheximide, specifically blocks the translocation of peptidyl-tRNA from A site to the P site in eukaryotic ribosomes and stabilizes polysomes. A third inhibitor, hygromycin B, affects ribosomes at the translocation step and is implicated in increased codon misreading; in prokaryotes, it also affects translation termination. EDTA dissociates Mg^{2+}-dependent complexes and thus would be expected to release ribosomes from RNA. Pretreatment of translation reaction mixtures containing low or high Arg concentrations with these compounds prior to adding RNA resulted in the loss of luciferase translation, as measured by enzyme assay (data not shown). In all cases, primer extension inhibition analyses of the transcripts in pretreated reaction mixtures showed the loss of the toeprint signals corresponding to ribosomes at the uORF stop codon (data not shown) and at the site 21 to 30 nt upstream. Therefore, these toeprint signals required translation.

To further examine the effects of chemical inhibitors of translation on toeprint signals, translation reaction mixtures containing low or high Arg concentrations and wild-type *arg-2–LUC* RNA were incubated for 20 min; puromycin, cycloheximide, hygromycin, or EDTA was added; and the mixtures were incubated for a further 5 min (Fig. 4). Compared to the control, to which no translation inhibitors were added, puromycin or EDTA addition caused the loss of toeprints that corresponded to the uORF stop codon and to the site 21 to 30 nt upstream that occurred at high Arg concentrations (Fig. 4, lanes 1 to 4, 9, and 10). Puromycin did not reduce the toeprint signals corresponding to the uORF and *LUC* initiation codons but abrogated the effect of high Arg levels to reduce the toeprint signals corresponding to AUG_{LUC}. Thus, it appeared that under these assay conditions, puromycin and EDTA preferentially released nascent peptides and their associated ribosomes from RNA but did not fully release initiation complexes from RNA. These data indicate that the Arg-induced toeprint

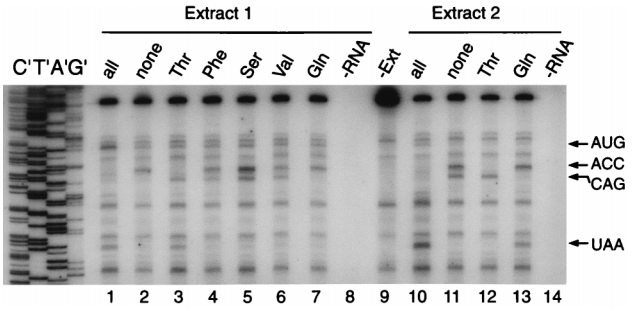

FIG. 5. Effects of amino acid limitation on toeprinting. RNA containing the wild-type uORF in the improved initiation context (120 ng) was incubated in 20-μl translation reaction mixtures for 20 min. The reaction mixtures contained either of two independently derived *N. crassa* cell extracts (extract 1 [lanes 1 to 8] and extract 2 [lanes 10 to 14]) and were toeprinted and analyzed with controls as described in the legend to Fig. 4, except that primer ZW4 was used. The reaction mixtures contained different pools of amino acids. These extracts showed slight differences in their responses to added amino acids that presumably reflect differences in the growth of the cultures or in the preparation of the extracts. Reactions marked "all" contained 10 μM (lane 1) or 50 μM (lane 10) each of all 20 amino acids. Reactions marked "none" (lanes 2 and 11) contained no exogenously supplied amino acids. For reactions marked Thr (lanes 3 and 12), Phe (lane 4), Ser (lane 5), Val (lane 6), and Gln (lanes 7 and 13), only the indicated amino acid was exogenously supplied, to 500 μM. Arrows indicate the toeprint products corresponding to ribosomes at AUG_{uORF}, ACC (uORF codon Thr-9), CAG (uORF codon Gln-11), and UAA_{uORF}.

signals arise from the continued association of ribosomes with RNA and not from cleavage of the RNA.

In contrast to the results obtained with puromycin, the effects of Arg on toeprint signals were still apparent in ongoing translation reaction mixtures that were treated with cycloheximide or hygromycin (Fig. 4, lanes 3 to 8). With cycloheximide, the toeprint signal corresponding to the uORF termination codon was substantially diminished, but the Arg-induced toeprint 21 to 30 nt upstream was not. Cycloheximide also caused a series of additional toeprints immediately downstream of the uORF and luciferase initiation codons. These additional toeprints may represent elongating ribosomes arrested by the drug. Thus, the decrease in the toeprint at the uORF termination site observed in these experiments might have occurred because cycloheximide did not inhibit termination to the same extent as it inhibited elongation, and in the presence of the drug, ribosomes did not translocate to the termination site to replace those that had dissociated from this site.

Hygromycin reduced toeprint signals at sites corresponding to elongating ribosomes and increased toeprint signals at sites corresponding to translation initiation. Studies on the effects of hygromycin on bacteria indicate that hygromycin does not block elongating ribosomes except when the initiation codon is in the P site (17), results which are consistent with our observations. The toeprint signal corresponding to ribosomes at the uORF termination codon was reduced, but an increase in the toeprint signal immediately downstream of this site was observed. This may reflect hygromycin-related effects on termination (2).

Experiments with chemical inhibitors indicated that the Arg-mediated differences in toeprint signals arose from the presence of translating ribosomes. Synthesis of luciferase in the *Neurospora* cell-free system requires exogenously supplied amino acids (37). Therefore, we also tested the effects of limiting the supply of amino acids in the toeprint assay. Toeprints corresponding to translating ribosomes would be expected to be influenced by charged-tRNA limitation. Representative results obtained with two independently derived extracts are shown in Fig. 5. Leaving out the amino acids caused a loss of the toeprint signal corresponding to the UAA_{uORF} (Fig. 5, lanes 1, 2, 10, and 11), as well as eliminating luciferase synthesis as determined by measurement of enzyme activity (data not shown). The toeprint signals corresponding to AUG_{uORF} and AUG_{LUC} (data not shown) remained; in addition, several new toeprint sites were observed within the uORF coding region (lanes 1, 2, 10, and 11).

Limitation for an aminoacyl-tRNA would be expected to cause a ribosome to stall with the corresponding codon in its A site; therefore, a toeprint would appear about 13 nt downstream. Mapping toeprints in this way revealed that two prominent new toeprint sites observed in the absence of added amino acids corresponded to ribosomes stalled with the A of Thr-9 codon ACC and the C of Gln-11 codon CAG at their A sites, respectively (Fig. 5). Addition of the amino acid Thr alone (Fig. 5, compare lanes 2 and 3 and lanes 11 and 12) eliminated the toeprint corresponding to this site. In contrast, adding single amino acids corresponding to nearby codons (Phe, Ser, and Val) did not eliminate this toeprint (lanes 4, 5, and 6). While adding Ser alone increased the toeprint at the Thr-9 codon, adding both Ser and Thr together eliminated this toeprint (data not shown). Addition of Gln alone eliminated the toeprint signal corresponding to this site (compare lanes 11 and 13). Thus, the toeprint assay detected elongating ribosomes stalled at codons for which the corresponding amino acid was limiting.

The toeprint signals appeared in a manner consistent with their arising from translocating ribosomes (Fig. 5). In extract 1, Thr appears to be most limiting: when Thr was added, the toeprint at the uORF Thr codon disappeared and new toeprints appeared at the downstream Gln codon and at the termination codon. In extract 2, Gln is also highly limiting: when Gln was added, the toeprint at the upstream Thr codon was not affected but the toeprint at the Gln codon disappeared and a new toeprint appeared at the termination codon. Thus, the signal corresponding to the UAA_{uORF} termination codon appears to require ribosomes to translocate to that site.

Effects of uORF mutations on the distribution of ribosomes. Analyses of toeprint data obtained with a construct lacking the uORF initiation codon (ΔAUG) showed that the signals corresponding to AUG_{uORF} and UAA_{uORF} were missing (Fig. 6; compare lanes 3 and 4 and lanes 11 and 12). Thus, consistent with these toeprint sites arising as a consequence of uORF translation, they were absent when RNA without a translatable uORF was toeprinted. Furthermore, the RNA lacking an uORF initiation codon showed unregulated luciferase synthesis (Fig. 2). However, in contrast to results obtained with constructs containing the wild-type uORF, in which the intensity of the toeprint at the luciferase initiation codon was always reduced at high Arg compared to low Arg concentrations, in the ΔAUG construct the intensity of the toeprint at the luciferase initiation codon sometimes appeared unaffected by high Arg levels (data not shown) or was sometimes reduced by high Arg levels (lanes 11 and 12). The reason for this discrepancy is not known but might reflect effects of Arg not requiring uORF translation (e.g., effects on RNA structure).

The uORF D12N mutation eliminates Arg-specific translational regulation in vivo and in vitro (11, 37). The results of toeprinting RNA containing the D12N uORF revealed that while toeprints corresponding to ribosomes at the uORF initiation and termination codons were present, Arg-specific differences in toeprint signals were lost (Fig. 6, lanes 5 and 6 compared to lanes 3 and 4). This corresponded to the loss of regulation of luciferase synthesis for this RNA (Fig. 2). In contrast, a conservative mutation at this codon, from Asp to

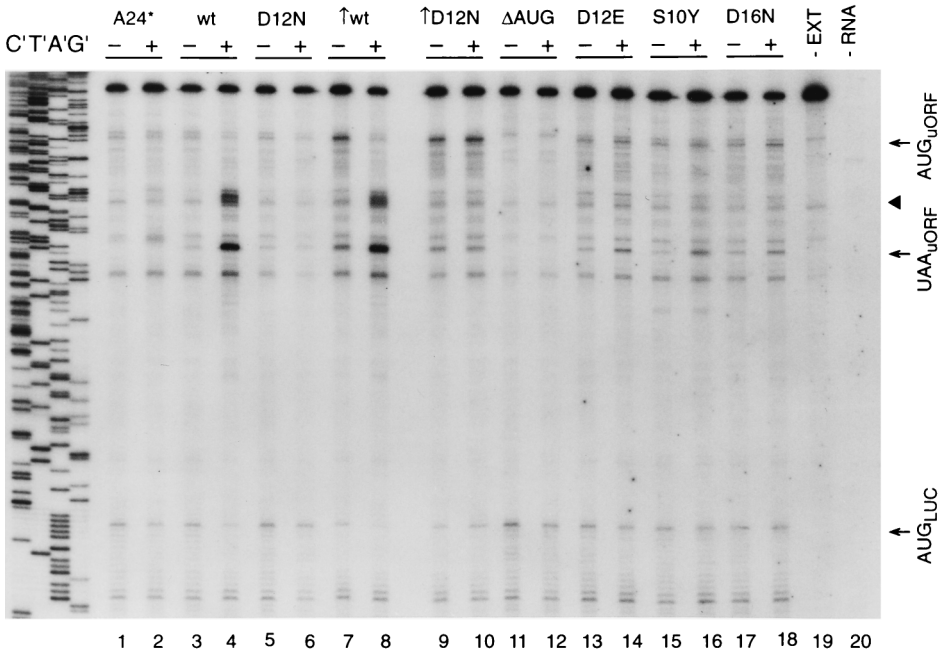

FIG. 6. Toeprinting reveals Arg-specific, sequence-specific effects. Equal amounts of RNA transcripts (120 ng) were translated in 20-μl reaction mixtures containing 10 μM (−) or 500 μM Arg (+) and 10 μM each of the other 19 amino acids. The transcripts examined are indicated at the top and correspond to those assayed in Fig. 2. After 20 min of translation, the translation mixtures were toeprinted with primer ZW4. Slightly less sample is present in lane 6.

Glu (D12E), reduced regulation but did not eliminate it, as judged by either assay (Fig. 2; Fig. 6, lanes 13 and 14).

Both *arg-2* and *CPA1* uORFs contain a second, conserved Asp codon (Fig. 1). Mutation of this Asp codon to Asn (D16N) also strongly reduced Arg-specific effects on toeprints (Fig. 6, lanes 17 and 18) and eliminated Arg-specific regulation (Fig. 2).

Placing the wild-type uORF in a predicted improved initiation context decreased the synthesis of luciferase and slightly increased the magnitude of Arg-specific regulation. In contrast, placing the D12N uORF in this improved initiation context decreased the synthesis of luciferase but did not confer Arg-specific regulation (Fig. 2) (37). At low Arg levels, improving the translation initiation context for either the wild-type or D12N uORF increased the toeprint at the uORF initiation codon and decreased the toeprint at the *LUC* initiation codon (Fig. 6, lanes 7 and 9). At high Arg levels, the wild-type uORF in an improved initiation context showed increased toeprint signals corresponding to ribosomes at the uORF termination site and at the site 21 to 30 nt upstream of this site and a decreased toeprint signal corresponding to ribosomes at the *LUC* initiation codon (lanes 8 and 7 compared to lanes 4 and 3). These Arg-specific changes were not observed for the D12N uORF in an improved initiation context (lanes 10 and 9). Finally, at high Arg levels, the signal from the uORF initiation codon was reduced for the wild-type uORF in an improved initiation context but not for the D12N uORF in this context (lanes 8 and 10). This may reflect reduced primer extension to this site arising from increased occupancy by ribosomes of downstream sites in the wild-type uORF at high Arg levels (Fig. 3A) and not diminished occupancy of this site by ribosomes.

The effect of shortening the uORF by one codon, by replacement of the GCA (Ala) codon with a UAA (stop) codon (A24*, Fig. 1A), was examined. When compared to the wild-type RNA, the UAA$_{uORF}$ ribosome arrest site in the A24* mutant RNA was shifted exactly 3 nt in the 5′ direction, consistent with the one-codon shortening of the uORF (Fig. 6, lanes 1 and 2 compared to 3 and 4). Translation of the A24* RNA at high Arg levels caused only a slight increase in the toeprint signal at the uORF termination codon, in contrast to the large increase observed for the wild-type uORF. Also, the strong signal 21 to 30 nt upstream of the uORF termination codon observed with wild-type RNA translated at high Arg levels appeared correspondingly diminished, and possibly shifted, in the A24* mutant. Consistent with the reduction in Arg-mediated effects on uORF translation observed in toeprint assays, Arg-specific translational regulation was also reduced by the A24* mutation, as determined by the luciferase assay (Fig. 2).

The *N. crassa* and *M. grisea arg-2* uORFs encode Ser at codon 10; the corresponding codon of the *S. cerevisiae CPA1* uORF is conservatively substituted with Cys (Fig. 1B). Mutation of the *CPA1* Cys codon to Tyr eliminates Arg-specific regulation (38); therefore, we examined the effect of the corresponding mutation in the *arg-2* uORF, S10Y (Fig. 1A). This mutation eliminated Arg-specific regulation (Fig. 2) and largely reduced Arg-specific effects on toeprint signals (Fig. 6, lanes 15 and 16). An additional primer extension product obtained with this RNA downstream of the uORF (lanes 15 and 16) was also observed in primer extension reaction mixtures with pure S10Y RNA (data not shown) and does not represent novel interactions between this RNA and extract. Thus, a variety of different mutations that changed the predicted and evolutionarily conserved primary amino acid sequence of the *arg-2* uORF affected Arg-specific regulation.

The effect of Arg is rapid. Translation reaction mixtures containing wild-type *arg-2–LUC* RNA were initiated with low or high Arg concentrations, and samples were examined by toeprint analyses at intervals thereafter (Fig. 7A). At either low or high Arg levels, a toeprint corresponding to the uORF termination codon became visible after 1 min of translation

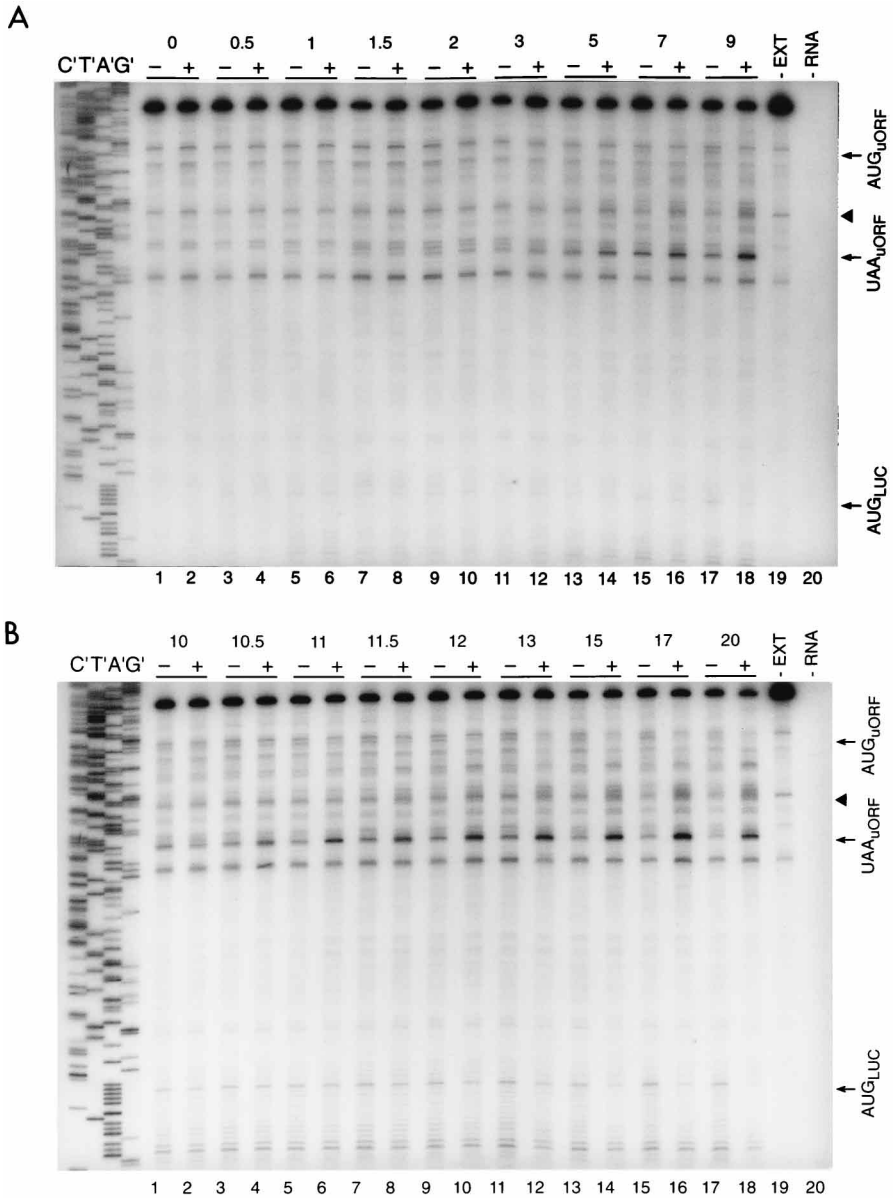

FIG. 7. Time course analysis of the effect of Arg by toeprinting. (A) Time course of toeprinting in translation reactions initiated at low or high Arg concentrations. RNA transcripts containing the wild-type uORF were added to translation reaction mixtures containing 10 μM (−) or 500 μM (+) Arg to final concentrations of 6 ng of transcript/μl. At the time points (0 to 9 min) indicated aliquots (3 μl) of the reaction mixtures were removed, added to precooled tubes containing reverse transcription buffer, and toeprinted, as described in Materials and Methods and the legend to Fig. 4, with ZW4 primer. (B) Time course analysis of toeprinting on RNA switched from translation at low Arg levels to translation at high Arg levels. RNA transcript containing the wild-type uORF was added to a translation reaction mixture containing 10 μM Arg (−) and incubated for 10 min as described for panel A. Then the reaction mixture was split into two tubes, one of which was supplemented with Arg to a final concentration of 500 μM (+). At the time points (10 to 20 min) indicated aliquots (3 μl) of the reaction mixtures were removed, added to precooled tubes containing reverse transcription buffer, and toeprinted as described with ZW4 primer. The 10-min time point represents the time at which the reaction mixture was divided and extra Arg was added to one portion.

and continued to increase in intensity for the next several minutes. The effect of high Arg levels on ribosome stalling at the uORF termination codon was detectable after 3 min and readily discernible after 5 min; it increased with time. The appearance at high Arg levels of the broad toeprint that corresponded to the additional stalled ribosomes translating the uORF paralleled the appearance of the toeprint at the uORF termination codon. The toeprint corresponding to the LUC initiation codon was faint in the autoradiogram shown in Fig. 7A but was readily detectable after 30 s of translation through PhosphorImager analysis of this and other gels (data not shown). The effect of Arg on reducing the toeprint at this site was observed after 5 min of translation, later than when its initial effect on the uORF toeprints was observed. The toeprint corresponding to the uORF initiation codon became detectable after 7 min of translation at low Arg levels and was not detectable at high Arg levels.

The effect of preincubating extracts with high Arg concentrations in the absence of *arg-2–LUC* RNA was also examined (data not shown). Complete reaction mixtures lacking exogenous RNA were incubated for 10 min at 25°C with low or high Arg concentrations; then, at time zero, high Arg concentra-

tions and RNA were added to the reaction mixture preincubated at low Arg concentrations, and RNA was added to the reaction mixture preincubated with high Arg concentrations. The progress of the reactions was monitored as in the experiment in Fig. 7A. Prewarming the translation reactions shortened the time required to observe ribosomes at the uORF termination codon to 30 s. However, preincubation with high Arg concentrations but without RNA increased neither the rapidity nor the magnitude of the translational response to Arg, relative to preincubation with low Arg concentrations, as measured both by toeprint and luciferase enzyme assays (data not shown). This suggests but does not prove that the *arg-2* RNA must be present for Arg to exert its effect on the translational machinery.

Finally, the effect of high Arg levels on translation reaction mixtures containing low Arg levels that were already translating *arg-2–LUC* RNA for 10 min was examined (Fig. 7B). Under these conditions, the effect of adding high Arg levels on ribosome stalling at the uORF termination codon was detectable after 30 s, the earliest time analyzed. Within 1.5 min of adding high Arg, toeprints at both the uORF termination codon and the site 21 to 30 nt upstream were apparent, but the toeprint signal corresponding to ribosomes at the downstream *LUC* initiation codon was not discernibly reduced until 3 min after addition of high Arg levels. The magnitude of this effect then increased with time. Thus, the effect of Arg on causing ribosomes to stall while translating the *arg-2* uORF appeared to precede its effect on luciferase synthesis.

DISCUSSION

We used primer extension inhibition (toeprinting) to examine the role of the *N. crassa arg-2* uORF in negative, Arg-specific translational regulation of *arg-2* RNA. Data obtained with an *N. crassa* cell-free system can be interpreted as revealing the presence of ribosomes at a variety of positions on an actively translated RNA. These positions include translation initiation and termination codons and codons for which the cognate amino acid is limiting for translation. This assay presumably resolves ribosomes that have specific codons in their P sites and ribosomes with codons in their A sites (illustrated in Fig. 3B). Ribosomes at initiation codons cause toeprints to appear 16 nt downstream from the start codons, whereas ribosomes at a termination codon or stalled at a codon due to limitation for an amino acid caused a toeprint to appear 13 nt downstream from that codon. Such differences in the extent of shielding of the RNA may be a consequence of physical differences between the P and A sites in the ribosome (35). The toeprint assay revealed that Arg had substantial effects on translation of the *arg-2* uORF and that the primary sequence of the uORF was critical for this Arg-specific translational control.

Possible mechanisms for Arg-specific translational control. The translation of leader peptides in amino acid biosynthetic operons of bacteria is essential for regulation by amino acid availability through coupled processes of translation and transcription, now classically known as transcription attenuation (21). In eukaryotes, transcription and translation are not intimately coupled and eukaryotic ribosomes generally do not bind directly at translation initiation sites but reach these sites by scanning from the 5′ end of the RNA. These differences have ramifications for how the eukaryotic *arg-2* uORF, which in a formal genetic sense is similar to short bacterial leader peptides, must act to attenuate translation from a downstream initiation codon in the presence of high Arg. Toeprinting data obtained with the *Neurospora* in vitro system, in which a high Arg level changes the distribution of ribosomes on *arg-2–LUC* RNA, provides clues into this mechanism.

Do ribosomes reach the initiation codon downstream of the *arg-2* uORF primarily by leaky scanning of the 40S subunit past the uORF or by translation reinitiation following uORF translation? The scanning model posits that 40S ribosomal subunits scan from the 5′ cap of the RNA and initiate translation at the first AUG codon in an mRNA. Leaky scanning, in which the second as well as the first initiation codon in an RNA serves to initiate translation, occurs more frequently when the initiation context for the first AUG is poor (20). These conditions hold for *N. crassa arg-2*.

The in vitro data presented here indicate that ribosomes do not efficiently reinitiate at the downstream initiation codon following termination of *arg-2* uORF translation. At low Arg concentrations, improving the uORF initiation context increased the association of ribosomes with AUG_{uORF}, decreased their association with AUG_{LUC}, and reduced luciferase translation (Fig. 2 and 6). Were reinitiation (or internal initiation) important, increased translation of the uORF gained by improving its initiation context should not have been accompanied by decreased translation from the downstream initiation codon. Thus, it appears that most ribosomes reach the downstream initiation codon by leaky scanning. Consistent with this interpretation, analyses of the in vivo expression of an *arg-2–lacZ* gene in which the uORF and *lacZ* coding regions were overlapping led to the conclusion that reinitiation of ribosomes at a downstream start codon was not essential for Arg-specific translational control (24). In yeast, reinitiation following uORF translation also appears to be the exception.

High concentrations of Arg caused an increase in the association of ribosomes with the uORF and a reduction in their association with AUG_{LUC}. High Arg concentrations might directly or indirectly increase initiation at AUG_{uORF}, which would correspondingly decrease the number of ribosomes reaching AUG_{LUC} by leaky scanning. Improving the initiation context of the uORF start codon caused increased loading of ribosomes at that site and decreased luciferase translation, indicating that such a mechanism would be functional. However, the increased association of ribosomes with the uORF at high Arg concentrations did not appear to arise from increased initiation of uORF translation as judged from toeprinting. Rather, Arg caused ribosomes translating the uORF to stall.

Ribosome stalling at the uORF termination codon is measurable within 30 s of adding high Arg concentrations to translation reaction mixtures in which the RNA is actively translating (Fig. 7B). This is soon accompanied by the appearance of additional stalled ribosomes in the uORF, and followed within minutes by a reduction in ribosomes at the downstream initiation codon. The relative rate of in vivo synthesis of ARG2 is also reduced within minutes of switching cells from minimal to Arg-containing medium (23).

Our data suggest the following model for Arg-specific translational control mediated by the *arg-2* uORF. At low Arg concentrations, the movement of scanning preinitiation complexes or translating ribosomes through the uORF is not hindered and translation initiation at the downstream start codon is relatively high (Fig. 8A). At high Arg concentrations, ribosomes stall while translating the uORF. This hinders the movement of scanning preinitiation complexes or other ribosomes translating the uORF, reducing initiation at the downstream start codon (Fig. 8B).

An important remaining question concerns whether the ribosomal stall site 21 to 30 nt upstream of the ribosomal stall site at the uORF termination codon represents an independent, Arg-mediated stalling event or occurs as a consequence

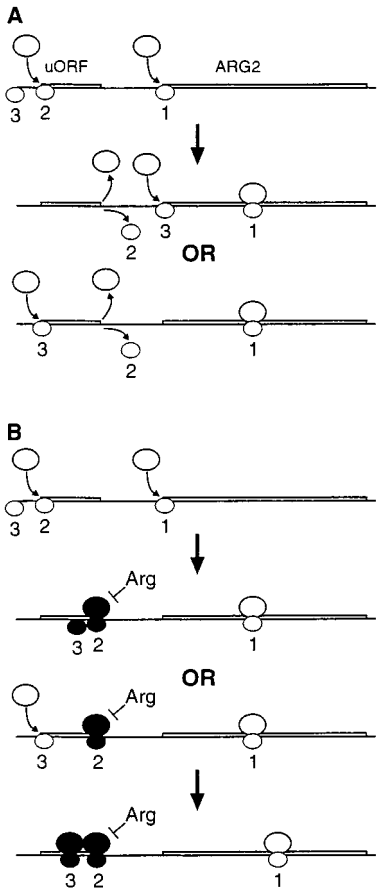

FIG. 8. A method in which ribosome stalling could mediate Arg-specific attenuation of translation from a downstream start codon. In this model, all ribosomes scan from the 5′ end of the RNA. (A) At low Arg concentrations, ribosomes do not stall at the uORF termination site. 40S ribosomal subunits (ribosomes 1 to 3) loaded from the 5′ end scan for initiation codons. Ribosome 1 joins a large subunit and initiates translation at the ARG2 start codon; ribosome 2 similarly initiates translation at the uORF start codon; ribosome 3 begins scanning from the 5′ end. As time elapses (thick arrow), ribosome 1 elongates ARG2, ribosome 2 terminates uORF translation and dissociates, and ribosome 3 either scans past the uORF and initiates translation at the downstream start codon or initiates translation at the uORF (shown) and then terminates translation and dissociates (not shown). (B) At high Arg levels, ribosomes stall at the uORF termination site. 40S ribosomal subunits (ribosomes 1 to 3) loaded from the 5′ end scan for initiation codons as in panel A. As time elapses, ribosome 1 elongates ARG2, ribosome 2 reaches the uORF termination codon but Arg blocks termination and/or dissociation, and ribosome 3 either scans past the uORF initiation codon but its further progress is arrested by stalled ribosome 2 or it initiates translation at the uORF start codon and stalls behind ribosome 2. Hindered movement is indicated by the use of solid symbols. This model predicts that the reduced ability of ribosomes to complete uORF translation at high Arg concentrations prevents ribosomes from loading at the ARG2 initiation codon.

of Arg-mediated increased stalling at the termination codon. Stacked ribosomes have their centers 27 to 29 nt apart (39). The position and the broadness of the upstream toeprint cluster (21 to 30 nt upstream of the termination codon toeprint) is consistent with its arising from ribosomes or scanning 40S ribosomal subunits that stack behind ribosomes stalled at the termination codon. The upstream toeprint might be broad because these complexes would begin to translocate when ribosomes stalled at the termination codon release (as they must for toeprints of upstream ribosomes to be observed by primer extension inhibition [Fig. 3A]).

The A24* data (Fig. 2 and 6) also appear inconsistent with the upstream toeprint representing an independent stalling site. The upstream toeprint corresponds to ribosomes positioned 7 to 10 codons upstream of the termination codon. Shortening the uORF by one codon at the carboxyl terminus of the predicted peptide should not affect ribosomes that have not reached this last codon. The effect of the A24* mutation (and all other mutations tested) to alter the toeprints at the upstream and termination sites concomitantly thus suggests that they are not independent. The obvious caveat to this interpretation is that effects on RNA secondary structure might occur in mutant RNAs that fortuitously eliminate the independent upstream stall site. That the stalled upstream ribosome "reins in" the downstream ribosome is also possible although unprecedented. Direct positive evidence to support the linkage of the stall sites, for example by moving the uORF termination codon to a new position so that regulation is retained and determining whether both stall sites move correspondingly, is not available at present.

Toeprints corresponding to hypothetical ribosomes stalled at the two Arg codons in the *arg-2* uORF were not observed. This argues against a regulatory mechanism in which ribosome stalling at these codons at low Arg levels is important, although such codon-specific stalling is critical in transcription attenuation of bacterial amino acid biosynthetic operons. The absence of Arg codons in the homologous yeast *CPA1* uORF (Fig. 1B), whose predicted peptide sequence but not nucleotide sequence appears important for Arg-specific negative regulation (7, 38), also implies that uORF Arg codons are not required for Arg-specific regulation.

Relation of *arg-2* uORF regulation to other regulatory phenomena. uORFs that arrest ribosomes at the stage of translation termination are found in prokaryotes and eukaryotes. A prokaryotic uORF peptide involved in regulating chloramphenicol acetyltransferase inhibits translation termination, possibly by blocking peptidyltransferase (22, 31). Synthesis of the bacterial TnaC leader peptide also regulates gene expression, and events at its termination codon appear important for regulation (19). The cytomegalovirus mRNA encoding gp48 contains an uORF whose sequence is critical for translational control (12, 13); biochemical studies indicate that ribosomes stall at the uORF termination codon (3, 4). Control at the termination step of uORF translation also is critical for regulation of *S. cerevisiae GCN4* (18).

How Arg controls the translation of *arg-2* is an important, unanswered question. Control may be exerted directly by the level of the free amino acid (or a closely related metabolite). Arg can interact with the ribosomal peptidyltransferase center and inhibit transpeptidation in a puromycin-based assay (27). Conceivably, the interaction of Arg with this center might affect *arg-2* uORF translation. Arg might bind to polypeptides to mediate regulation. The *E. coli* arginine repressor is a DNA-binding protein whose conformation changes when it is bound to Arg corepressor (25). The crystal structure of the Arg-binding domain complexed to Arg reveals that Arg binds to the protein through Asp residues in the repressor (36). This is interesting because two Asp residues that are conserved in the *arg-2* and *CPA1* uORF peptides are critical for Arg-specific attenuation (Fig. 1B, 2, and 6).

Alternatively, levels of Arg-tRNA^{Arg} charging might effect Arg regulation. tRNA charging affects uORF control in bacteria (21) and yeast (18). If so, effects of tRNA charging must be rapid or require the mRNA, because preincubation of extracts with high Arg concentrations but without *arg-2* uORF-containing RNA did not increase the rapidity or magnitude of its effect. Initial efforts to examine the regulatory effects of depleting extracts of tRNA and/or of adding exogenous tRNA

in the *Neurospora* system have been inconclusive (data not shown).

The negative, Arg-specific regulation conferred by the *arg-2* uORF represents one of the few demonstrated examples in which a eukaryotic uORF modulates translation in response to a specific signal (18, 22, 32). Translation of the *N. crassa arg-2* uORF, whose sequence and function are evolutionarily conserved, appears to be a choke point to control the synthesis of the ARG2 polypeptide and therefore to control flux through the Arg biosynthetic pathway. The common occurrence of uORFs in mRNAs specifying polypeptides important in growth control and development (12, 14) and the recent demonstration that uORFs are critical in tissue-specific regulation of retinoic acid receptor expression (30) indicate that eukaryotic uORF function will prove to be of general significance.

ACKNOWLEDGMENTS

We thank Julie Carroll for help in preparing mutant templates; Randolph Addison and Jianhong Cao for valuable discussions; Michael Freitag, Adam Geballe, Alan Hinnebusch, Alan Sachs, and Charles Yanofsky for critical reading of the manuscript; and Carolyn Tapia and Guiqin Zhang for encouragement.

This work was supported by the National Institutes of Health (grant GM47498).

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