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Site-Specific Release of Nascent Chains from Ribosomes at a Sense Codon▽

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"2A" oligopeptides are autonomous elements containing a D(V/I)EXNPGP motif at the C terminus. Protein synthesis from an open reading frame containing an internal 2A coding sequence yields two separate polypeptides, corresponding to sequences up to and including 2A and those downstream. We show that the 2A reaction occurs in the ribosomal peptidyltransferase center. Ribosomes pause at the end of the 2A coding sequence, over the glycine and proline codons, and the nascent chain up to and including this glycine is released. Translation-terminating release factors eRF1 and eRF3 play key roles in the reaction. On the depletion of eRF1, a greater proportion of ribosomes extend through the 2A coding sequence, yielding the full-length protein. In contrast, impaired eRF3 GTPase activity leads to many ribosomes failing to translate beyond 2A. Further, high-level expression of a 2A peptide-containing protein inhibits the growth of cells compromised for release factor activity and leads to errors in stop codon recognition. We propose that the nascent 2A peptide interacts with ribosomes to drive a highly unusual and specific "termination" reaction, despite the presence of a proline codon in the A site. After this, the majority of ribosomes continue translation, generating the separate downstream product.

Translation is generally linear from the initiating start to the terminating stop codon of an open reading frame (ORF). However, sequences within the mRNA or growing peptide chain can influence both the speed and the outcome of translation (29, 46). Such sequences bring about their effects by interacting with the ribosome and, in some cases, other factors. These interactions may be regulated or constitutive, and some allow specific sites in the mRNA to be "recoded," enriching the outcomes of translation beyond those predicted by simple mRNA sequence inspection. Recoding events include programmed ribosomal frameshift and stop codon readthrough, used extensively by viruses but also during translation of a number of cellular mRNAs to generate certain proteins or protein variants.

"2A" peptides are ~19-amino-acid sequences that, when encoded within longer ORFs, produce a discontinuity in the translated polypeptide (14, 16, 39). The separation point is at the C terminus of 2A, with the first amino acid of the downstream product being proline (13, 32, 34, 37, 50). This residue, termed proline 19 herein, is the final amino acid of a conserved, functionally important motif, D(V/I)EXNPGP. 2A peptides occur in many viral genomes and are key elements in the control of their protein biogenesis (4, 14, 15, 20, 27, 34, 40, 50). Functional 2A peptides are also found in repeated sequences within trypanosome genomes (16, 22). Remarkably, 2A peptides exert their effect in all of the eukaryotic systems in which they have been tested, indicating that they interact with highly conserved features of the eukaryotic cell. The unique features of 2A have led to its development as a tool for the coexpression of two or more separate proteins from a single ORF (11).

In vitro analysis supports the proposition that the reaction driven by 2A is a translational recoding event in which a peptide bond is "skipped" during elongation (16, 38, 39). In vitro translation of mRNAs that include sequences that encode 2A peptides within the ORF yielded some full-length protein, which did not undergo posttranslational processing at 2A. Further, a molar excess of the translation product up to 2A (the upstream product) relative to the downstream product is frequently generated, and thus, some ribosomes do not progress past 2A (16). Finally, the synthesis of a protein with an internal 2A in the presence of a low concentration of puromycin yields significant levels of puromycin-terminated polypeptide with a size corresponding to that of the upstream product, suggestive of a translational pause at 2A (16). We have further investigated the 2A reaction. We find that it takes place on substrates that extend only to the proline 19 codon and thus occurs within the ribosomal peptidyltransferase center. We also show that ribosomes pause over the codons that encode the final amino acids of 2A. Finally, we provide data consistent with the recruitment of release factors (RFs) to ribosomes at 2A to catalyze a "termination" reaction releasing the protein up to and

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TABLE 1. *S. cerevisiae* strains used in this study

Strain	Genotype[a]	Source or reference
JDY4	*his3Δ200 leu2Δ1 trp1Δ99 ade2-101 ura3Δ99 cir^o MAT*α	30
MTY557/2a	*leu2-3,112 ade2-1 ura3-1 MAT***a**	I. Stansfield
MTY557/1d	*leu2-3,112 ade2-1 ura3-1 sup45-2 MAT***a**	I. Stansfield
BSC483/1a	*his5-2 lys1-1 ade2-1 ura3-1 can1-100 SUQ5 PNM1 MAT*α	I. Stansfield
BSC483/1a-sal4-42	*his5-2 lys1-1 ade2-1 ura3-1 can1-100 SUQ5 PNM1 sup45-42 MAT*α	I. Stansfield
YDB498[b]	*his3-11,15 leu2-3,112 trp1-1 ade1-14 ura3-1 sup35::HIS3 pPW12.1 MAT***a**	42
74-D694	*his3Δ200 leu2-3,112 trp1-289 ade1-14 ura3-52 MAT***a**	M. Tuite
74-D694[PSI$^+$]s[c]	*his3Δ200 leu2-3,112 trp1-289 ade1-14 ura3-52 MAT***a** *[PSI$^+$]strong*	M. Tuite
74-D694[PSI$^+$]w[c]	*his3Δ200 leu2-3,112 trp1-289 ade1-14 ura3-52 MAT***a** *[PSI$^+$]weak*	M. Tuite
CSY550	*leu2Δ1 trp1Δ63 ura3-52 rat8-2*	C. Cole
HFY870	*his3-11,15 leu2-3,112 trp1-1 ade2-1 ura3-1 can1-100 upf1::HIS3*	A. Jacobson
CML476	*his3Δ200 leu2Δ1 ura3-52 CMVp(tetR′-SSN6)::LEU2 trp1::tTA*	N. Zhang
JDY808	*his3Δ200 leu2Δ1 ura3-52 CMVp(tetR′-SSN6)::LEU2 trp1::tTA sup45::kanMX4-tetO$_7$-SUP45*	This work

[a] All strains are [psi$^-$], unless otherwise noted.
[b] Strains expressing eRF3 variants were generated by transforming YDB498 with plasmids encoding Sup35p or the H348Q or R419G mutant protein and evicting pPW12.1 on 5-fluoroorotic acid plates.
[c] Weak and strong [PSI$^+$] 74-D694 variants were characterized in the laboratory of M. Tuite (Kent, United Kingdom).

including glycine 18 of 2A from tRNA^{Gly}, despite the presence of a proline codon in the A site.

MATERIALS AND METHODS

Strains, media, and constructs. The *Saccharomyces cerevisiae* strains used in this study (listed in Table 1) were grown in the media indicated. JDY808 (*sup45::kanMX4-tetO$_7$-SUP45*) was generated as recommended in reference 51. This strain was unable to grow on plates containing 10 μg/ml doxycycline, which was expected since Sup45p is an essential factor.

Constructs. TAP-DHFR-2A-pL was assembled in pMW20 (52) from fragments that encode the tandem affinity purification (TAP) tag, mouse dihydrofolate reductase (DHFR), and the foot and mouth disease virus (FMDV) 2A sequence including five upstream amino acids of 1D (from pSTA1/34) (15) and amino acids 51 to 128 of bovine prolactin appended by SHRGTGYERSPDAI MSM. Plasmids that encode ssΔαF-2A-GFP and ssΔαF-2A*-GFP (10) were modified for some experiments by the insertion of a *LEU2* fragment into the EcoRV site of *URA3*. Readthrough reporters were based on *lacZ-STOP-luc* (35) and constructed by replacing an NheI-BglII fragment that encodes the β-galactosidase–luciferase junction with fragments incorporating alternate stop signals. For in vitro translation and toeprinting, pDJ100 (21) was modified such that sequences coding for amino acids 10 to 66 of prepro-α-factor were replaced with a linker that encodes GM. The ORF was truncated at M101, and point mutations altered K96 and P100 to M. This was followed by sequences that encode the same 2A-prolactin fragment as in TAP-DHFR-2A-pL and a 28-nucleotide poly(A) tail (pJN141). Constructs incorporating the 2A* or *CPA1* upstream ORF (uORF) were generated by the exchange of an XbaI-ApaI fragment (see Fig. 3A). All constructs were sequenced. Truncated mRNAs ending at specific codons were transcribed from PCR products amplified from pJN141 with primers incorporating nucleotides up to the codon desired and the SP6 promoter primer.

Purification and mass spectrometric analysis of the 2A-containing protein. The TAP-DHFR-2A-pL fusion was expressed in yeast strain JDY4 grown in galactose-containing medium and purified from clarified cell lysate as previously described (36), except that NP-40 was omitted from buffers. One to two micrograms of purified protein was electrophoresed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie stained, and the protein band was excised. Following in-gel digestion with trypsin, peptides were extracted and analyzed on a Voyager DESTR (Applied Biosystems) matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometer or an LTQ FT (Thermo-Electron) electrospray tandem mass spectrometer equipped with a Nanomate (Adrian Biosciences) interface.

In vitro translation and toeprinting. Capped transcripts were generated from plasmids linearized with SalI or PCR products with SP6 RNA polymerase (Promega) or as described previously (47). In vitro translation in yeast lysates prepared from the strains indicated was done as previously described (30). Wheat germ extracts (Promega) were used as recommended, with 100 ng mRNA per 10-μl reaction mixture volume. For the experiments shown in Fig. 2, reactions were stopped by direct addition to sample buffer on ice. Half of each sample was then treated with RNase A (300 μg/ml) on ice for 10 to 20 min before being loaded directly onto 4 to 12% Bis-Tris NuPAGE gels (Invitrogen) without heating. In vitro translation and primer extension in *Neurospora crassa* lysates was done as previously described (41). Translation reaction mixtures contained 6 ng/μl transcript, and reactions were terminated by transfer to prechilled tubes on ice. Where indicated, cycloheximide was added to 0.5 mg/ml. Primer extensions were carried out with primer ppLanti (5′-GGACTTCATGGTGGGTCTG-3′), which is complementary to nucleotides 159 to 178 downstream of the proline 19 codon.

Readthrough assays. Cells ([psi$^-$] and strong [PSI$^+$] variants of yeast strain 74-D694) were grown overnight in medium containing 2% (wt/vol) raffinose, collected, and resuspended in 1% (wt/vol) each raffinose and galactose media. After 2 or 4 h, cells were harvested from 20 ml of culture and lysed in 1 ml TPO buffer (35) with zirconium beads (Biospec Products) in a Ribolyser (Hybaid). Luciferase and β-galactosidase activities in cleared lysates were measured with the Tropix kit (Applied Biosystems) as recommended by the manufacturer, with a Turner TD-20e luminometer, ensuring that readings were in the linear range. Luciferase activity was normalized to β-galactosidase activity and expressed as a percentage of that of the sense control.

Cell labeling, immunoprecipitation, and quantification were done as previously described (10). Briefly, cells of the indicated strains were grown and expression from the galactose promoter was induced as described above for readthrough assays. Cells were collected from a volume of culture with an optical density at 600 nm (OD_{600}) of 5, washed with medium lacking methionine, resuspended in 1 ml of the same, and transferred to a screw-top Eppendorf tube. After incubation at the appropriate temperature for 15 min, 50 μCi/ml Pro-mix (GE Healthcare) was added and incubation was continued for 7 min prior to cell harvesting and extract preparation. Extract derived from cells with an OD_{600} of 1 was used for each immunoprecipitation. Images of gels were captured and quantified with a Fuji BAS1500 PhosphorImager and TINA software (Raytest).

RESULTS

All codons of 2A are decoded once into translation products. An understanding of the 2A reaction mechanism requires definition of the C terminus of the upstream and N terminus of the downstream reaction products. In a number of cases, the first amino acid of the downstream product has been shown to be proline 19—or its equivalent in different 2A peptides (13, 32, 34, 37, 50). To complement these studies, the C terminus of the 2A upstream reaction product was determined. A 2A-containing fusion protein with an N-terminal TAP tag was expressed in *S. cerevisiae*, and the upstream product was purified and digested with trypsin (Fig. 1A and B and Materials and Methods). We considered the most likely position of the C terminus of the protein to be proline 17 (yielding a tryptic

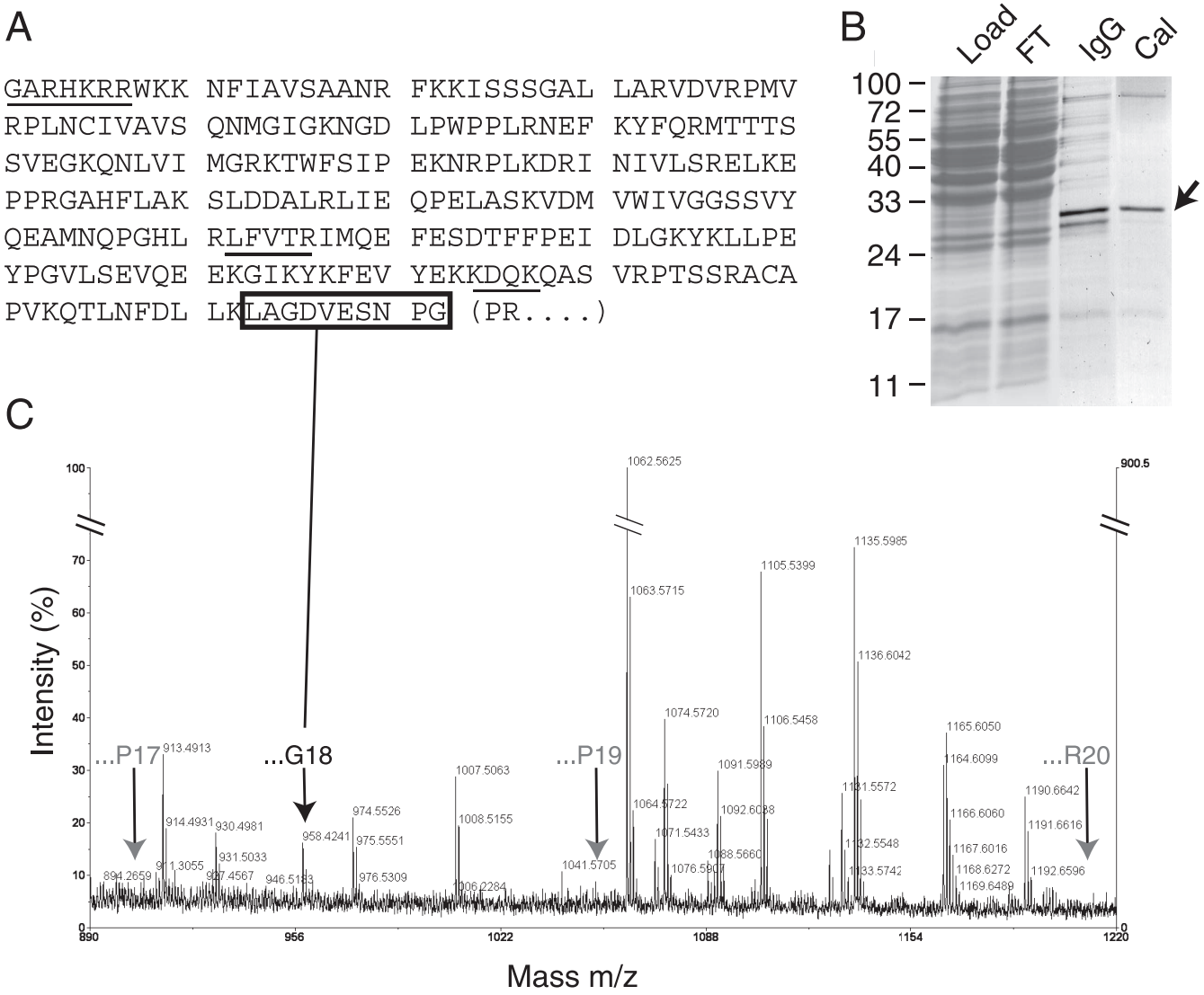

FIG. 1. Identification of the C-terminal amino acid of the upstream product as glycine 18. (A) Amino acid sequence of the expected upstream reaction product released from TAP-DHFR-2A-pL by the 2A reaction and tobacco etch virus protease up to the end of 2A plus, in parentheses, the first two downstream amino acids. The identified C-terminal peptide is boxed, and the corresponding peak in the mass spectrum is indicated (C). Underlined sequences were not represented in the mass spectrum. (B) Fractions from the purification (see Materials and Methods) were resolved by SDS-PAGE and stained with Coomassie blue. Positions of marker proteins are marked on the left (molecular sizes are in kilodaltons), as is the purified upstream product of the 2A reaction (arrow). Lanes are whole-cell extract (Load), material not bound to the IgG resin (FT), and eluates from the IgG resin (IgG) and calmodulin resin (Cal). No extension product (predicted molecular mass, 42 kDa) was seen, and Western blotting confirmed that the 2A reaction is very efficient in this construct (data not shown). (C) Partial MALDI-TOF mass spectrum of peptides derived by in-gel tryptic digestion of purified material cut from a gel similar to that shown in panel B. The C-terminal peptide peak is indicated (black arrow). Positions where peptides ending in proline 17, glycine 18, or the downstream arginine (R20) would have been seen are indicated with gray arrows.

peptide with a molecular mass of 901.43 Da), glycine 18 (958.45 Da), proline 19 (1,055.50 Da), or, if translation extended further and ribosomes shifted back to reinitiate on proline 19, a tryptic peptide ending at the next amino acid, arginine (1,211.60 Da). MALDI-TOF mass spectrometry (MS) of the trypsin-treated protein yielded nearly all of the predicted digestion products, and the mass spectrum revealed only one candidate for the C-terminal peptide, with a molecular mass of 958.42 Da (Fig. 1C). Mass calibration corrected this to 958.44 Da, within 0.01 Da of the mass of the peptide LAGDVESNPG. This peptide was also detected by electrospray MS, and fragmentation by electrospray tandem MS produced no masses corresponding to predicted y series ions, consistent with its representing the C-terminal peptide of the protein. Mass peaks were, however, seen that corresponded to a series of b ions predicted for the C-terminal sequence of the peptide, ESNPG (not shown). A report published during the course of this study demonstrated the inclusion of the terminal glycine in the upstream product of a 2A reaction that occurs during the replication of the *Perina nuda* virus (4). Our data confirm and extend these findings, suggesting that the reactions of all 2A peptides are similar. All codons of 2A are decoded with the

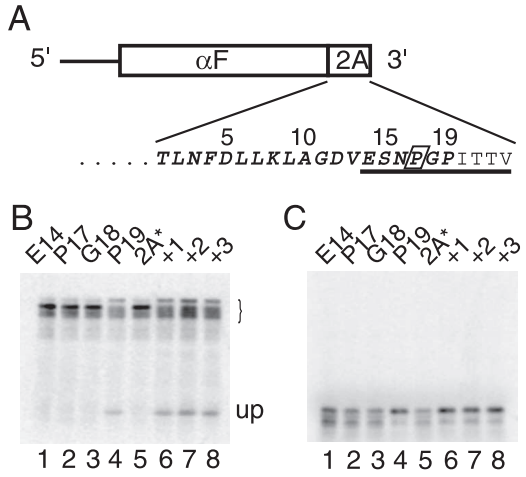

FIG. 2. The 2A reaction proceeds with the final codons that encode 2A in the peptidyltransferase center. (A) Outline of construct and 2A sequence. The construct comprises an abbreviated *S. cerevisiae* pro-α-factor, followed by sequences that encode the FMDV 2A peptide. PCR was used to generate transcription templates from this ending with nucleotides that encode different amino acids from glutamate at position 14 of 2A to proline 19, and also additional amino acids (one, two, three, or four) downstream of proline 19 (underlined). An additional template ended at proline 19 but included an inactivating proline 17 (boxed) mutation to alanine (2A*). (B) In vitro translation reaction mixtures prepared with wheat germ lysate and [^{35}S]methionine with equal amounts of the mRNAs from panel A. Reaction products were subjected to NuPAGE (Invitrogen) SDS-PAGE and visualized with a PhosphorImager. The position of the released upstream product is marked (up), as is that of tRNA adducts (brace). (C) Same as panel B, but samples were treated with RNase prior to resolution on the gel.

corresponding amino acids present once in the final translation products.

The 2A reaction takes place at the ribosomal decoding center. Next, the proposal that the 2A reaction is tightly linked to the peptidyltransferase center of the ribosome was tested. A series of RNAs ending at positions spanning the region from the glutamic acid at position 14 to the final proline at position 19 of the FMDV 2A sequence (40) was generated, along with additional mRNAs including one to four downstream amino acids (Materials and Methods and Fig. 2A). These were used to program translation reaction mixtures assembled with wheat germ extract. Ribosomes stall at the 3′ end of truncated transcripts, with nascent chains remaining covalently attached to ribosome-associated tRNA. The translation products were resolved at neutral pH by NuPAGE (Invitrogen) SDS-PAGE such that peptidyl-tRNA ester bonds were retained (28). If the 2A reaction was able to proceed during the translation of these RNAs, this would be revealed by the accumulation of the upstream products released from tRNA. Major products of reaction mixtures containing RNAs ending at positions ranging from glutamic acid 14 to glycine 18 of 2A were ~20 kDa larger than predicted for the encoded peptide, consistent with their being peptidyl-tRNAs (Fig. 2B, lanes 1 to 3; data not shown). Treatment of these reaction mixtures with RNase prior to electrophoresis increased the mobility of the products, which then migrated at the size expected for the translated polypeptides (Fig. 2C, lanes 1 to 3), confirming that the larger species were peptidyl-tRNAs. A similar result was obtained with a template extending to the proline 19 codon but including a proline 17 point mutation to alanine that inactivates 2A (15; termed 2A* herein) (Fig. 2B, lane 5). In contrast, in reaction mixtures programmed with RNA that encodes wild-type 2A extending to the proline 19 codon, a product of the size expected for the released polypeptide was generated (Fig. 2B, lane 4). This was also seen when the templates encoded further amino acids (lanes 6 to 8). The size of these products was not altered by RNase (Fig. 2C, lanes 4 and 6 to 8). These results indicate that the 2A reaction occurs within the ribosomal peptidyltransferase center. Further, there is no requirement for RNA sequence 3′ to the proline 19 codon.

Ribosomes pause at the end of the 2A coding sequence. Translation of a protein including 2A in the presence of a low concentration of puromycin yields significant puromycin-terminated product with a size similar to that of the upstream reaction product (16). This is consistent with a pause in translation at 2A. This was investigated further by the sensitive technique of primer extension inhibition (toeprint) analysis, in which reverse transcriptase is used to map the positions of ribosomes and other factors on RNA. Because of their size, toeprints of eukaryotic ribosomes map 15 to 17 nucleotides distal to the first base of the codon in the P site (1, 2, 26, 41, 48). *N. crassa* extracts were used for these experiments, as in this system toeprinting reveals ribosomes at various steps in translation without the addition of chemical inhibitors of translation. Capped, polyadenylated transcripts were generated from constructs (Fig. 3A) containing active 2A, the 2A* mutation, or, as a positive control, sequences that encode the *S. cerevisiae CPA1* arginine attenuator peptide (AAP). When implanted into a longer ORF, the AAP directs a strong pause in translation in response to a high arginine concentration (17).

Primer extension on translation reaction mixtures programmed with the AAP-containing mRNA yielded arginine-inducible signals corresponding to ribosomes at positions immediately distal to the AAP sequence (Fig. 3B, compare lanes 1 and 4). These toeprints were not significantly altered by the addition of cycloheximide after 10 min of translation (compare lanes 4 and 6) but were eliminated when translation events subsequent to initiation were blocked by the addition of cycloheximide at time zero (lane 5). In contrast, signals corresponding to ribosomes at the initiation codons were greatly strengthened when cycloheximide was added either prior to extract addition or during translation (upper panel, compare, e.g., lane 1 with lanes 2 and 3). These data are consistent with previous analyses (18, 47).

Primer extension on translation reaction mixtures programmed with mRNA that encodes 2A (Fig. 3B, lanes 9 to 12) yielded signals at +16/17 nucleotides to the first base of the glycine 18 codon (lane 9). This is consistent with ribosomes translating 2A pausing with this codon in the P site and that which encodes proline 19 in the A site. Again, the signals were translation specific and were not seen when the reaction was initiated in the presence of cycloheximide (lane 10) or in the absence of extract (lane 12). Signals were not seen at these positions with the 2A*-containing construct (lanes 13 to 16), and they were therefore specific to the functional 2A peptide. The 2A-specific signals were not enhanced when cycloheximide was added to the reaction mixtures (compare lanes 9 and

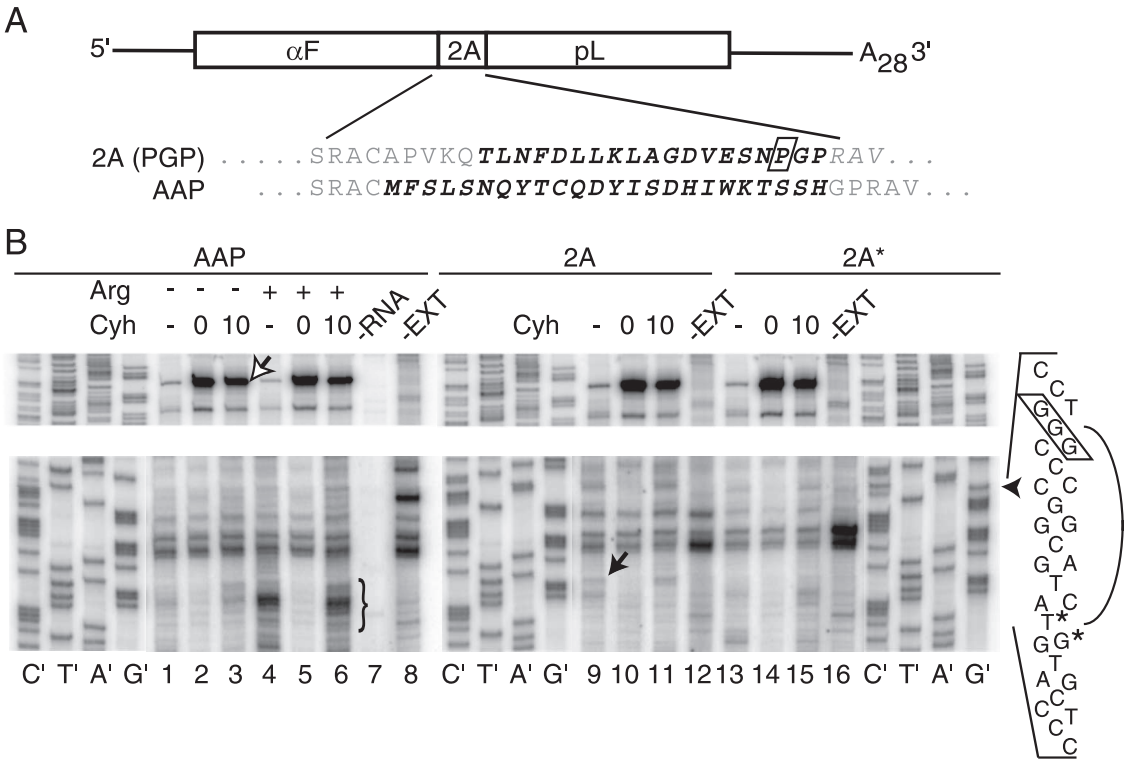

FIG. 3. Toeprinting identifies a ribosomal pause at 2A. (A) Outline of constructs. These contain abbreviated *S. cerevisiae* pro-α-factor and bovine prolactin sequences flanking amino acids corresponding to the 2A peptide and the *CPA1* uORF (AAP) (bold, italics) (see Materials and Methods for details). The proline changed to alanine in 2A* is boxed as in Fig. 1. (B) Toeprinting reactions were carried out on translation reactions initiated on the indicated transcripts with primer ppLanti (see Materials and Methods). Where indicated, arginine (Arg) and/or cycloheximide (Cyh) were added to the reaction mixtures. Cycloheximide was added at the beginning of the reaction (no-translation control) or after 10 min. Controls with no template (−RNA) or extract (−EXT) are shown. Positions of specific pauses on mRNA are marked as follows: initiator codon, open arrow; AAP, brace; 2A, filled arrow. Dideoxynucleotide sequencing reactions with primer ppLanti were run alongside the toeprinting reactions. The mutation that yields the proline-alanine change is marked (arrowhead). The nucleotide complementary to the dideoxynucleotide added to each sequencing reaction mixture is indicated below the corresponding lane so that the sequence of the template can be directly deduced; the 5′to-3′ sequence reads from top to bottom. The relevant portion of the 2A sequence is shown to the right as a series of triplets corresponding to codons in the mRNA. The glycine 18 codon is boxed, and asterisks mark the positions of the 2A-specific toeprints.

11), suggesting that ribosomes paused at 2A are not in the pretranslocation state.

Rapid release of the upstream product from peptidyl(2A)-tRNA. Next, time course translations were used to determine if the pause seen in toeprinting experiments was prolonged and if a species corresponding to peptidyl(2A)-tRNA persisted before its resolution from ribosomes. These were carried out with *N. crassa*, *S. cerevisiae*, and wheat germ cell-free translation systems and focused on early times, up to those at which ribosomes first reached the end of the ORF. Similar results were obtained with each system, and those from *N. crassa* and wheat germ are presented (Fig. 4A and B, respectively). Duplicate gels were run with samples left untreated or treated with RNase (removing tRNA) to reveal polypeptides that accumulated as paused translation intermediates.

In translation reaction mixtures assembled on mRNA that encodes the AAP, a high arginine concentration led to the accumulation of a translation product (asterisks in the lower parts of Fig. 4; compare lanes 11 to 13 with lanes 14 to 16 in panel A and lanes 11 to 15 with lanes 16 to 20 in panel B) of a size consistent with its being the portion of the ORF up to and including the AAP. This was not seen when tRNA adducts were retained, confirming its identification as the AAP-paused intermediate (upper panels); evidence for this intermediate was also obtained previously (17).

In translation reaction mixtures assembled with mRNA that encodes the 2A peptide, fragments of the expected sizes of the upstream, downstream, and extended full-length ORF products were generated (Fig. 4A and B, lanes 1 to 5). The upstream product accumulated first in the time courses, consistent with the translation of all of the products being driven from the same initiation point and the known dependence of synthesis of the downstream fragment on translation of the 2A peptide (16). This species was seen at equal intensity, regardless of whether or not the sample was treated with RNase (compare the upper and lower panels). Thus, unlike ribosomes stalled at the AAP, those at 2A efficiently release the polypeptide up to this point. Further, no significant difference was detected in the kinetics of accumulation of the downstream and extension products translated from mRNA including 2A and the full-length ORF product generated in reaction mixtures programmed with the 2A*-containing mRNA (Fig. 4A and B, compare lanes 1 to 5 with lanes 6 to 10). The resolution of these experiments is thus not sufficient to detect the pause in translation seen by toeprinting.

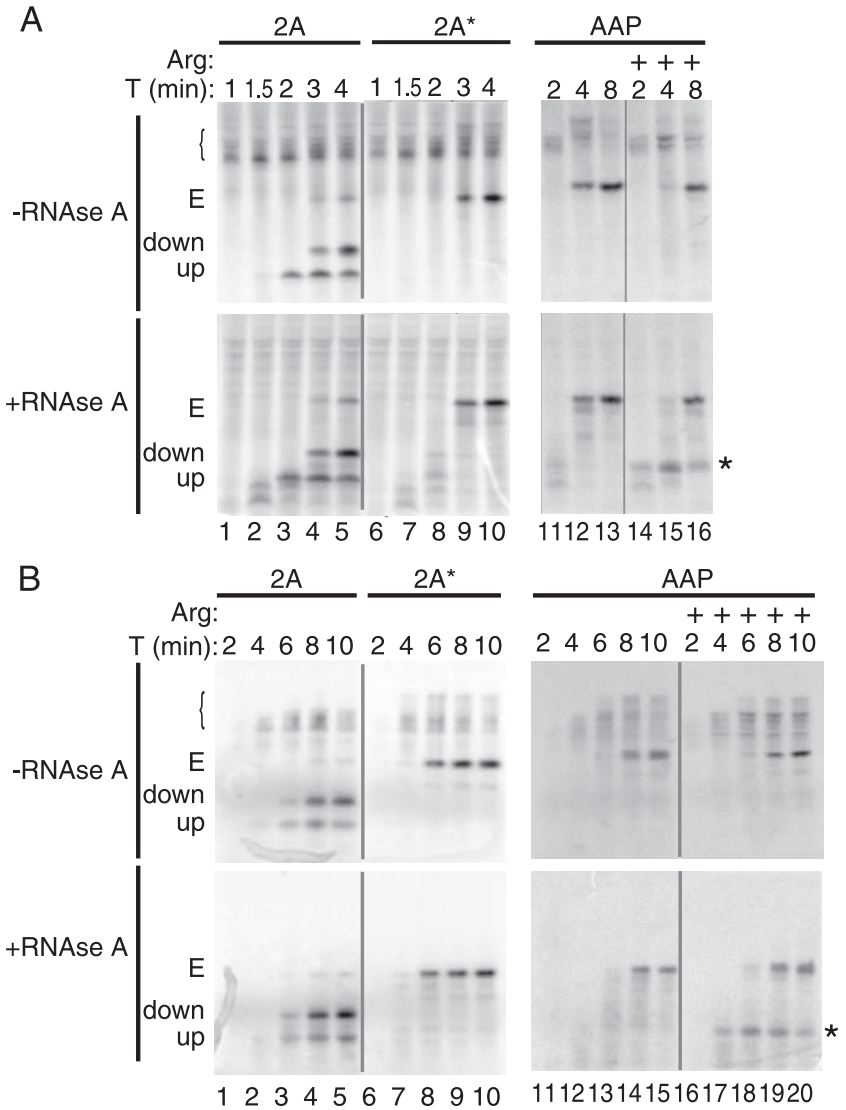

FIG. 4. Rapid release of the upstream 2A reaction product from tRNA. (A) In vitro translation reactions were carried out with *N. crassa* translation extract and [^{35}S]methionine, and equal amounts of synthetic capped, polyadenylated transcripts that encode the constructs indicated (as in Fig. 3A) were run on NuPAGE gels (Invitrogen) and visualized by autoradiography. The extension (E) and separated upstream (up) and downstream (down) 2A reaction products are indicated, as are tRNA adducts (brace) and the AAP-paused species (*). Reactions including the AAP template were carried out at either a low (10 μM) or a high (500 μM) arginine concentration. Samples were taken at the times indicated. Edeine was added to 1 mM at 2 min to inhibit further initiation. Note that the upstream product is degraded over time in the *N. crassa* extracts. (B) Same as panel A, except that translations were carried out with wheat germ extract. Samples were taken at the times indicated, and edeine was added at 1 min.

Cells limited in RF activity are sensitive to the overexpression of 2A-containing proteins. To this point, data indicate that the 2A reaction occurs within the peptidyltransferase center of the ribosome and ribosomes pause briefly on the mRNA at the C-terminal end of the 2A coding sequence, over its final glycine and the subsequent proline codons. The translation product predicted by the ORF is separated into two polypeptides at this point. What could mediate this event? In an attempt to uncover factors that play a role in or influence the 2A reaction, a 2A-containing reporter (Fig. 5A) was expressed in a number of *S. cerevisiae* strains in each of which the activity of a specific translation factor was altered. The reporter comprised yeast pro-α-factor, 2A, and green fluorescent protein (GFP) in a single ORF (ssΔαF-2A-GFP) (10). Transcription of mRNA that encodes ssΔαF-2A-GFP is driven from the strong, inducible *GAL1* promoter. Among the initial yeast strains tested was one with constitutively reduced translation-terminating RF activity (BSC483/1a-sal4-42), due to a mutation in the gene that encodes yeast eRF1, *sup45-42* (43). Expression of ssΔαF-2A-GFP in this strain, but not an isogenic wild-type strain, strongly inhibited growth (Fig. 5B). Expression of a control construct that encodes ssΔαF-2A*-GFP had no effect on the growth of the *sup45-42* mutant strain.

The growth-inhibiting effect of ssΔαF-2A-GFP was not *SUP45* allele specific. The eRF1 protein encoded by a second mutant allele, *sup45-2*, is thermosensitive (44), and the growth

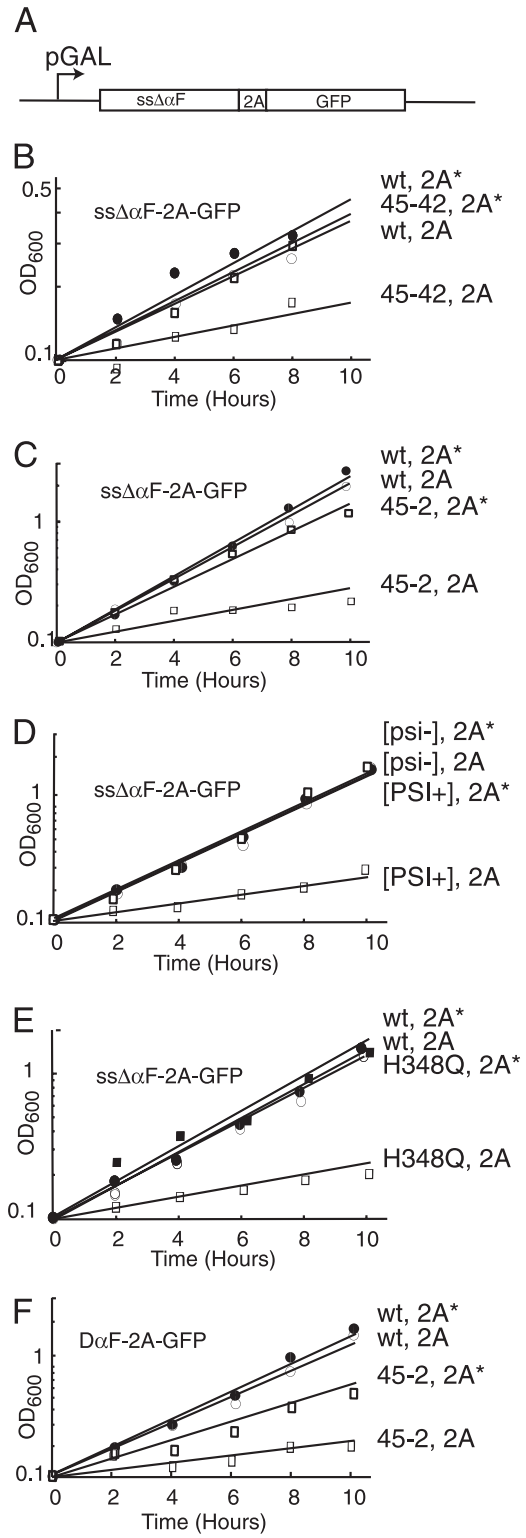

FIG. 5. Cells limited in eRF1 activity are sensitive to the overexpression of a 2A-containing protein. (A) ssΔαF-2A-GFP reporter. Transcription is driven by the strong *GAL1* promoter. (B) Isogenic wild-type (wt; circles) and *sup45-42* mutant (45-42; squares) cells were transformed with plasmids that encode ssΔαF-2A-GFP (2A; open symbols) or ssΔαF-2A*-GFP (2A*; closed symbols), grown to mid-log phase at 30°C in 2% raffinose medium, and transferred into medium containing 1% (wt/vol) each galactose and raffinose to induce transcription from *pGAL1*. The growth of the cultures, which were diluted to maintain conditions suitable for logarithmic growth, was monitored at 30°C as the relative OD_{600} over time (in hours). (C) Same as panel B but with isogenic wild-type (wt; circles) or *sup45-2* (45-2; squares) cells, which were grown at 34°C, the semipermissive temperature for the *sup45-2* mutant cells. (D) Same as panel B but with isogenic [psi$^-$] and strong [PSI$^+$] strains. (E) Same as panel B, except that isogenic wild-type eRF3 and H348Q mutant eRF3 were used. (F) Same as panel B, but cells were transformed with modified reporters containing an N-terminal *DAP2* signal sequence which directs cotranslational translocation into the endoplasmic reticulum.

of *sup45-2* mutant cells at the semipermissive temperature of 34°C, but not the permissive temperature of 25°C, was inhibited by the expression of ssΔαF-2A-GFP (Fig. 5C and not shown). As with *sup45-2* mutant cells, growth inhibition was due to the presence of the functional 2A, as expression of ssΔαF-2A*-GFP had no effect on *sup45-2* mutant cells. Supporting this conclusion, the expression of ssΔαF-2A-GFP variants containing functional 2A sequences from other viruses (*Thosea asigna* virus and Theiler's murine encephalitis virus) (15) also inhibited the growth of *sup45-2* mutant cells (data not shown).

Eukaryotic translation termination is driven by a complex of two proteins—eRF1, which binds to the ribosomal A site, decodes all three stop codons, and stimulates the hydrolysis of the peptidyl-tRNA ester linkage, and the GTP binding protein eRF3 (Sup35p in yeast). GTP hydrolysis by eRF3 is proposed to facilitate stop codon decoding by eRF1 and coupling of this to efficient release of the nascent peptide (1, 42).

The growth of strains with limited, or altered, eRF3 activity was also inhibited by expression of ssΔαF-2A-GFP but not ssΔαF-2A*-GFP. [PSI$^+$] is an aggregating, prion-like conformation of yeast eRF3 (Sup35p). Distinct [PSI$^+$] strains can be isolated with more (strong [PSI$^+$]) or less (weak) Sup35p aggregated and unavailable to function in termination (12). ssΔαF-2A-GFP expression slightly impaired the growth of a weak [PSI$^+$] variant (data not shown) and strongly inhibited the growth of a strong [PSI$^+$] variant of the same strain (Fig. 5D). The H348Q and R419G mutations in eRF3 reduce the key GTPase activity of the protein (42). Of these, eRF3-H348Q is more severely compromised and the growth rate of a strain expressing this mutant protein was significantly decreased when ssΔαF-2A-GFP was expressed in it (Fig. 5E), further supporting a link between RF function and 2A. As with *sup45* mutants, the effect on the [PSI$^+$] strains and eRF3 GTPase mutants was specific to the expression of the functional 2A sequence. Finally, the expression of a 2A-containing protein from a moderate-strength promoter (*PHO5* lacking regulatory sequences [9]) did not affect the growth of cells limited in RF activity (data not shown). In conclusion, high-level expression of 2A severely inhibited the growth of strains with limited eRF1 or eRF3 activity.

Next, we tested whether the growth-inhibiting effect of the expression of proteins containing 2A extended to strains compromised for the activities of Dpb5p and Upf1p, further factors required for efficient translation termination (19, 25). Dbp5p is implicated in loading eRF3 into termination complexes, while Upf1p is a key factor in the degradation of mRNAs containing

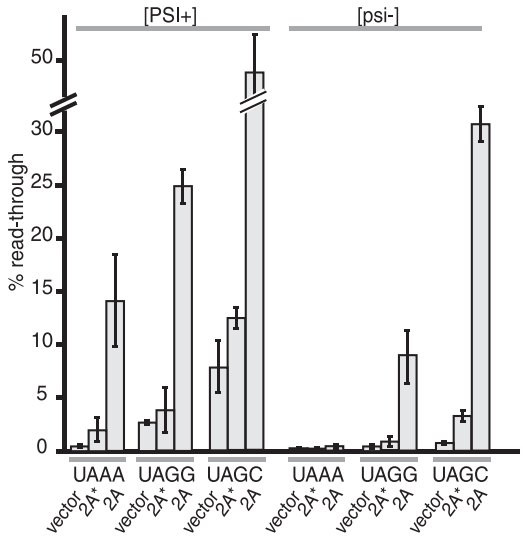

FIG. 6. Expression of 2A leads to increased stop codon suppression in *trans*. [psi$^-$] and strong [PSI$^+$] strains were transformed with plasmids that encode *lacZ-STOP-luc* reporters containing the indicated stop codon (plus the downstream nucleotide) along with an empty vector (vector) or a plasmid expressing ssΔαF-2A-GFP (2A) or ssΔαF-2A*-GFP (2A*). These were grown, expression of 2A/2A* was induced, and readthrough assays were carried out (see Materials and Methods). Readthrough is presented as a percentage of the reading obtained with a sense control with a GCA (alanine) codon in place of the stop codon. Each bar is the average measurement from three or more independent transformants with standard deviations indicated.

premature stop codons. The growth of cells compromised for either Upf1p (*upf1*Δ) or Dbp5p (*rat8-2*) activity was not affected by the expression of 2A (data not shown). The effect of 2A on eRF1- and eRF3-compromised cells appears to extend, therefore, no further than the core components of the termination machinery.

RF activity is reduced by 2A. The specific inhibition of the growth of RF-limited cells by the expression of 2A suggested a titration effect, such that there was insufficient RF to function in translation termination. Dual-reporter constructs comprising β-galactosidase followed by a stop codon and luciferase allow the assessment of translation termination efficiency by comparing the activities of the two enzymes. Thus, increased luciferase activity relative to β-galactosidase activity indicates increased readthrough of the stop codon (7, 35). A *lacZ-STOP-luc* fusion containing an efficient stop codon and context, GAUUAAACG (stop codon underlined; *lacZ-UAAA-luc*), was expressed in [psi$^-$] and isogenic, eRF3-limited strong [PSI$^+$] strains that also contained the *GAL1* promoter-driven ssΔαF-2A-GFP or ssΔαF-2A*-GFP construct or an empty-vector control (Fig. 6). Comparison of β-galactosidase and luciferase activities showed that stop codon readthrough for *lacZ-UAAA-luc* in the presence of the empty vector was fivefold higher in [PSI$^+$] than in [psi$^-$] cells (0.5% and 0.1%, respectively), consistent with a previous analysis of the effects of reduced eRF3 activity on readthrough. Expression of ssΔαF-2A-GFP in [PSI$^+$] cells yielded a 30-fold increase in readthrough in *lacZ-UAAA-luc*, over and above the elevated basal level, to nearly 15%. In contrast, 2A barely affected the readthrough of *lacZ-UAAA-luc* in [psi$^-$] cells (0.2% compared to 0.1% in the control).

This analysis was extended with two additional *lacZ-STOP-luc* constructs. These contained the stop signals CAAUAG-CAA, which promotes high basal readthrough (7, 35) and is an extremely sensitive indicator of defects in termination, and CAAUAGGAA, which is expected to be a relatively poor stop signal (8). *lacZ-UAGG-luc* and *lacZ-UAGC-luc* yielded 3% and 9% readthrough, respectively, in [PSI$^+$] cells with the empty vector and greatly elevated readthrough when ssΔαF-2A-GFP was expressed (25% and 49%, respectively). Increased readthrough was also detected in [psi$^-$] cells with these reporters when ssΔαF-2A-GFP was expressed. *lacZ-UAGG-luc* and *lacZ-UAGC-luc* yielded 0.8% and 1.4% readthrough, respectively, in the presence of the empty vector and 9.1% and 31% with ssΔαF-2A-GFP expression.

Finally, β-galactosidase and luciferase activities were monitored from a *lacZ-luc* fusion containing an inactivating arginine-serine mutation within the active site of luciferase (35). Luciferase activity is only obtained from this construct through mistranslation of the AGC (serine) codon to AGA or AGG (arginine) by near-cognate tRNA. Expression of ssΔαF-2A-GFP had no effect on luciferase activity from this missense reporter (data not shown). This suggests that 2A does not impose a general defect on translational accuracy.

The above results indicate that RF activity is titrated by the expression of the 2A-containing protein—and that this extends to wild-type ([psi$^-$]) cells. This could be explained by the 2A peptide, as part of a cytosolic protein, binding and inhibiting or inactivating the RF. Another possibility is that 2A, directly or indirectly, affects cellular RF levels. These possibilities were discounted by several observations. The addition of a synthetic peptide corresponding to the FMDV 2A sequence to in vitro translation reaction mixtures at up to 2.5 μM had no effect on the 2A reaction (data not shown). Western blotting indicated that high-level expression of the TAP-tagged 2A construct used to identify the C terminus of the upstream reaction product did not alter eRF1 or -3 levels, and material isolated on immunoglobulin G (IgG)-Sepharose from these extracts contained neither Sup35p nor Sup45p (data not shown). Finally, the expression of 2A-containing proteins cotranslationally targeted to, and quantitatively translocated into, the endoplasmic reticulum (ssΔαF-2A-GFP modified to contain the N-terminal signal sequence of Dap2p or a Pho8-2A-GFP fusion) (10) had effects on RF-limited cells similar to those of ssΔαF-2A-GFP (Fig. 5F and data not shown). In this situation, the 2A peptide is not present as a free cytosolic species and, combined, these observations suggest an alternative model for the inhibitory effect of 2A on RF-limited cells: recruitment of RF to ribosomes paused at 2A. A twofold decrease in Sup45p levels is sufficient to drive significant increases in readthrough in reporters similar to those used here (42). Since the expression of the 2A constructs is driven by the *GAL1* promoter, induced by >4 orders of magnitude by galactose (45), a considerable pool of mRNA that encodes 2A accumulates in cells grown in galactose medium. This might titrate out the limited amount of RF in such strains to such an extent that growth is inhibited.

In all of the experiments with the dual reporter with poor stop codon contexts and with the efficient context in [PSI$^+$] cells, readthrough measured when the control ssΔαF-2A*-

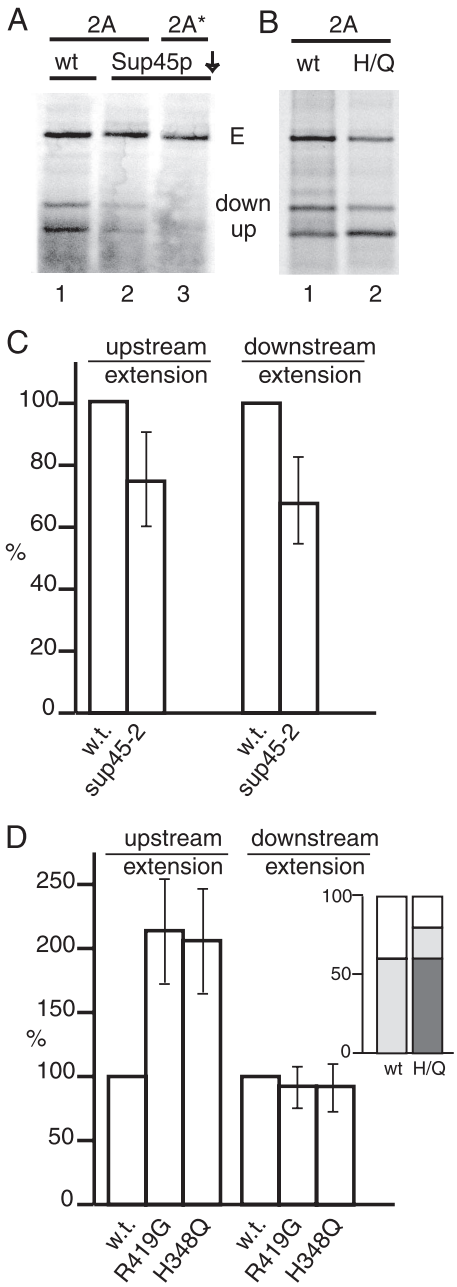

FIG. 7. The outcome of ribosomal transit through the 2A coding sequences is altered by changes in RF activity. (A, B) In vitro translation reaction mixtures assembled with mRNA templates including 2A and 2A* as in Fig. 2 but with *S. cerevisiae* translation extract. Reaction mixtures were assembled with extract from wild-type (wt) or Sup45p-depleted (Sup45p ↓) cells (A) and cells expressing wild-type Sup35p or the H438Q (H/Q) variant of Sup35p (B) (42). Reaction products are labeled as in Fig. 4. (C) Anti-2A and anti-GFP antibodies were used to isolate extension and either upstream or downstream 2A reaction products from lysates of isogenic wild-type and *sup45-2* mutant cells expressing ssΔαF-2A-GFP and pulse-labeled with [^{35}S]methionine following growth at 37°C for 1 h (see Materials and Methods and Results). Immunoprecipitated products were resolved by SDS-PAGE and quantified, and the ratio of the upstream or downstream product to the extension product was determined. Results of three independent experiments were averaged, in each case setting the value obtained for wild-type cells to 100% and expressing that for *sup45-2* mutant cells relative to this, with 1 standard deviation indicated. To test the significance of these data, an unpaired, two-tailed *t* test was carried out on the initial data sets, yielding *P* values of 0.03 and 0.04, respectively, for the means of the data for upstream/elongation and downstream/elongation ratios from the two strains being the same. (D) Same as panel C, except that strains were the isogenic wild type and the R419G and H348Q mutants. In the inset, data for a single experiment are shown as the proportion of each of the different translational outcomes—extension (clear box), complete 2A reaction (light gray), and ribosomal drop off at 2A (dark gray).

GFP construct was expressed was higher than with the empty vector. Thus, while this 2A mutant is not active for the 2A reaction per se, it may nevertheless "recruit" RF to some extent.

RF plays a key role in the 2A reaction. If RF associates with ribosomes at 2A, then could it be responsible for generating the upstream 2A reaction product by hydrolysis of the peptidyl-tRNA^{Gly} ester bond? To determine if this is the case, translation products generated from mRNA including 2A were examined both in vitro and in vivo in situations where RF activity was altered. Sup45p-depleted translation extract was prepared from a strain in which the transcription of *SUP45* could be repressed by the addition of doxycycline to the growth medium (see Materials and Methods). Extract was prepared from these cells grown in the presence of doxycycline to the point at which eRF1 was reduced to approximately 20% of wild-type levels. Further depletion of eRF1 yielded cells from which we were unable to generate extracts with significant translation activity. Translation of a 2A-containing protein in the eRF1-depleted extract yielded a lower proportion of both upstream and downstream 2A reaction products compared to the extension product than in control extract prepared from isogenic wild-type cells grown in the presence of doxycycline (Fig. 7A, compare lanes 1 and 2). Quantification of experiments such as that shown in Fig. 7A revealed an approximately 40% reduction in the 2A reaction in the eRF1-depleted extract.

For in vivo analysis, *sup45-2* mutant and isogenic wild-type cells transformed with the plasmid that encodes ssΔαF-2A-GFP were grown in galactose-containing medium to induce its expression, transferred to 37°C to inactivate eRF1 in the mutant cells, and pulse-labeled with [^{35}S]methionine. 2A- and GFP-containing species were then collected from cell lysates by immunoprecipitation with specific antibodies, resolved by SDS-PAGE, and quantified (see Materials and Methods). The ssΔαF-2A-GFP construct was particularly useful for these analyses, as in this case the context upstream of 2A results in a relatively inefficient 2A reaction (10), facilitating the determination of changes in product ratios. Consistent with the results obtained in vitro, the ratio of both up- and downstream 2A reaction products to the full-length extension product decreased by approximately 30% in *sup45-2* mutant cells compared to the ratio in wild-type cells (Fig. 7C). Thus, a reduction of eRF1 activity is accompanied by a reduction in the efficiency of the 2A reaction.

As a second test, the products of translation through sequences that encode 2A in the presence of eRF3 mutants with reduced GTPase activity (H348Q and R419G) were examined. Translation reaction mixtures assembled with extract prepared from cells expressing eRF3-H348Q as the sole source of the protein yielded a result very different from that obtained when

eRF1 activity was decreased: compared to wild-type extract, the proportion of upstream product in the reaction products was increased while those of both the downstream and full-length extension products were reduced (Fig. 7B). This result was recapitulated in vivo: in cells expressing either eRF3-H348Q or -R419G, the amount of the upstream translation product relative to that of the extension product was increased approximately twofold, while the ratio of the downstream translation product to the extension product was maintained (Fig. 7D). Thus, while in wild-type cells ribosomes either extend through the 2A sequence or complete the 2A reaction, in cells expressing these eRF3 mutant proteins, an increased proportion of ribosomes generate the upstream product but then fail to continue translation (Fig. 7D, inset). Thus, a reduced GTP hydrolysis rate on eRF3 leads to a significantly reduced number of ribosomes translating beyond 2A and the rate at which eRF3 hydrolyzes GTP strongly influences the outcome of the 2A reaction.

DISCUSSION

The 2A reaction drives a critical primary "cleavage" event in the processing of many picornaviral polyproteins (14, 20, 39, 40). Active "2A-like" sequences are also found in a number of insect viruses, in type C rotaviruses, and in *Trypanosoma* species repeated sequences (4, 15, 22, 27, 34, 50; http://www.st-andrews.ac.uk/ryanlab/2A_2Alike.pdf). We set out to gain deeper insight into the 2A reaction, specifically, to determine if it occurs at the ribosomal peptidyltransferase center and to identify cellular factors that influence the reaction. Our observations suggest that 2A represents a new class of recoding element that dictates a "stop-carry on" or "StopGo" (4) form of translation in which a peptide bond is skipped. Site-specific termination on a sense codon (as opposed to reassigning it to stop) has not been previously documented. All codons of 2A are decoded with the corresponding amino acids present in the final translation products. 2A does not, therefore, promote ribosomal movement on the mRNA as in frameshifting and shunting events.

The 2A reaction takes place at the peptidyltransferase center of the ribosome, generating upstream and downstream products separated between glycine 18 and proline 19. Release of the upstream reaction product from ribosomes translating RNA that only extends to the proline 19 codon is key evidence for this. In this context, the reaction is mechanistically restricted to either hydrolytic release of the nascent chain from $tRNA^{Gly}$ or possibly formation of the glycine 18-proline 19 peptide bond, followed directly by its scission. This second possibility is strongly disfavored by data implicating RF in the 2A reaction. Ribosomal pausing at 2A is consistent with the hypothesis that the 2A peptide interacts with the ribosomal exit tunnel. The toeprint signal at +16/17 nucleotides to the first base of the glycine 18 codon (Fig. 3B) indicates that ribosomes pause with this codon in the P site and the proline 19 codon in the A site. Replacement of proline 19 as the final codon in truncated RNAs with alanine or glycine codons results in loss of activity (V.A.D. and J.D.B., unpublished data), confirming previous results indicating that proline is critical at position 19 for 2A activity (16, 20). Previously, the necessity for a proline codon at position 19 was rationalized by suggesting that the conformation of the 2A-paused ribosome was such that it was not possible for the sterically constrained secondary amine of proline to act as a nucleophile to generate a peptide bond to glycine 18 (16). The data presented here are entirely consistent with this.

The strength of the toeprint signal at 2A is relatively weak compared to those seen under some circumstances, including that due to stalling at the AAP in the presence of arginine. However, this correlates well with the facts that peptidyl(2A)-tRNA does not accumulate significantly during translation and a delay in translation through 2A was not detected within the resolution of the time course experiments carried out (Fig. 4). In contrast, the stall at the AAP is sufficient to delay the accumulation of full-length protein by minutes in time course experiments (Fig. 4) (17). It should be noted that the toeprint at 2A may represent ribosomes poised to undergo the 2A reaction, after the release of the upstream product prior to synthesizing the downstream product, or a mixture of both.

RFs plays a key role in the 2A reaction. To date, these factors are only known to function at stop codons. The A site must be available for RF to gain entry into the ribosome; i.e., the ribosome must be in the posttranslocation state. This possibility is supported by the observation that the 2A toeprint was unaffected by the presence of cycloheximide, which prevents ribosomal translocation. The previous observation that ribosomes at 2A are susceptible to puromycin-mediated chain termination also supports this (16). Cognate prolyl-$tRNA^{Pro}$ is presumably not stably bound to 2A-paused ribosomes, possibly due to an inability to form a peptide bond. Alternatively, the ribosomal conformation imposed by 2A disfavors the entry of the tRNA. We considered that prolyl-$tRNA^{Pro}$ and RF might compete on ribosomes paused by 2A. However, overexpression of cognate $tRNA^{Pro}$ neither reversed the toxic effects of overexpressing 2A in RF-limited cells nor altered the ratio of products of translation through 2A (V.A.D. and J.D.B., unpublished). Thus, the peptidyl(2A)-ribosome interaction and the conformation of the complex apparently disfavor further extension unless RF acts to release the nascent chain or the 2A-ribosome interaction is lost.

Termination at sense codons is extremely rare, with premature, nonspecific termination in *S. cerevisiae* and *Escherichia coli* estimated to occur at frequencies of 10^{-4} or lower and 10^{-5}, respectively (3, 24). How could 2A promote productive RF-ribosome binding in the absence of a stop codon in the A site? Ribosomes undergo large-scale conformational changes as they cycle through each round of peptide bond synthesis, with different conformations binding different factors. A number of antibiotics, small molecules compared to the nascent chain, alter the progress or fidelity of translation and, in some cases, drive conformational changes within the ribosome (6, 31). By analogy with these situations, 2A may bypass the necessity for stop codon decoding by RF by directing the ribosome into a conformation similar to that which it takes once RF has bound productively to the A site. Candidate movements might be at the peptidyl transferase centre toward the conformation in which the hydrolytic termination reaction takes place and in the decoding region to mimic productive proofreading of incoming RF.

Overexpression of the ssΔαF-2A-GFP polyprotein led to (i) increased readthrough of stop codons in *trans* in both RF-

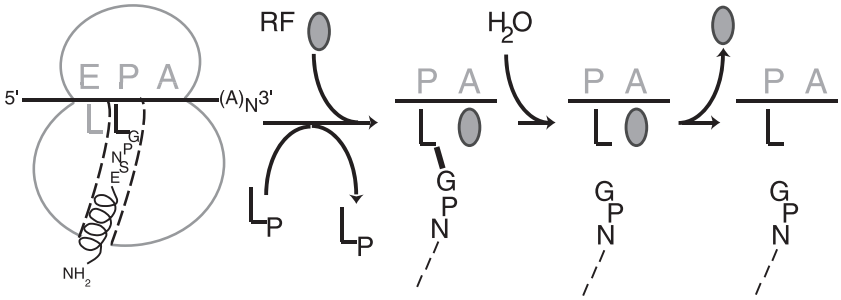

FIG. 8. Model for translation termination at 2A. See text for details.

limited [PSI$^+$] and [psi$^-$] cells (Fig. 6) and (ii) inhibition of the growth of a variety of RF-limited strains (Fig. 5). Several lines of evidence indicate that these effects are not due to free cytosolic 2A interacting with and inhibiting RF: RF was not coisolated with a 2A-containing protein; a 2A-containing protein cotranslationally targeted to the endoplasmic reticulum inhibited the growth of RF-limited cells in the same way as one that was cytoplasmically localized; exogenously added 2A peptide did not inhibit the 2A reaction in in vitro translation reaction mixtures. An explanation may then be that RF remains bound to the peptidyl(2A)-ribosome complex for an extended time compared to when it engages in termination on stop codons, resulting in its concentration effectively being lowered. However, as we could not detect a lengthy pause of ribosomes at 2A (Fig. 3), we considered other alternatives to such a "simple" titration model. Once RF has catalyzed termination, it must be recycled. Could the abnormal termination complex at 2A result in release of RF in a form that is less readily recycled (and thus effectively functionally impaired) than after termination at a stop codon?

Alteration of RF activity affected the outcome of the 2A reaction both in vitro and in vivo (Fig. 7). Reduced eRF1 levels were accompanied by the reduced synthesis of separated upstream and downstream products, consistent with RF catalyzing the hydrolytic termination event. Impaired GTP hydrolysis on eEF3 led to the increased production of the upstream product and a reduction in both the extension and downstream products. During termination at stop codons, GTP hydrolysis on eRF3 couples stop codon recognition by eRF1 to efficient peptide release and is proposed to stimulate correct positioning of eRF1 in the peptidyltransferase center (1, 42). In the peptidyl(2A)-ribosome complex, there is no stop codon recognition. Perhaps the conformation of the complex is such that the termination reaction does not require GTP hydrolysis, and in this context the energy of GTP hydrolysis on eRF3 drives the complex out of this conformation. The increased efficiency of release of the upstream product seen in the eRF3 mutants would then reflect an increased opportunity for termination at 2A. An extended time window before GTP hydrolysis would also increase the opportunity for dissociation of the upstream product from the ribosome before initiation of synthesis of the downstream product. Since 2A-ribosome interaction is likely to facilitate this, dissociation of the upstream product would decrease the synthesis of the downstream product—as seen with these mutants. The two eRF3 GTPase mutants tested had similar effects on the 2A reaction, whereas only the H348Q strain was seriously compromised for growth when the 2A-containing protein was overexpressed. These mutants have similarly reduced GTPase activities (the k_{cat}/K_m ratio is reduced by about twofold for each). However, the H348Q mutant protein is much less functional on stop codons than is the R419G mutant protein, and cells containing the H348Q mutant protein as the only source of eRF3 grow slower than those supported by the R419G mutant protein (42). The growth of cells containing the H348Q mutation is thus likely to be more sensitive to further perturbation of RF levels/function than those carrying the R419G mutation.

2A is one of a small number of characterized nascent peptides that pause or stall translation, and even fewer are known to direct recoding (5, 46). uORF2 of the human cytomegalovirus UL4 gene is intriguing in that ribosomes stall with the uORF2 stop codon in the A site stably bound to eRF1 but not to eRF3 (23). Further, mutations to conserved glycines in a catalytically important motif in eRF1 (GGQ) decrease the activity of uORF2, supporting a key role for these amino acids in generating the stalled complex. In this case, interaction between eRF1 and the peptidyl transferase center containing the C terminus of the uORF2-encoded peptide stabilizes, rather than resolves, the paused complex—which is the "opposite" of the effect of 2A. 2A is not, though, unique in inhibiting the formation of glycine-proline bonds. The arrest peptide of *E. coli secM* also pauses ribosomes with peptidyl-tRNA^{Gly} in the P site and a proline codon in the A site (28). However, in this case, prolyl-tRNA^{Pro} is retained in the A site, despite its not forming a bond to the peptidyl-tRNA^{Gly}, and RF is excluded from the complex (28).

A model for the initial steps in the 2A reaction based on the data presented here is shown in Fig. 8. In this model, ribosomes translate the 2A coding sequence until glycine 18 has been incorporated into the nascent chain and translocate on the mRNA to position the glycine 18 and proline 19 codons in the P and A sites, respectively. At this point, the conformation of the peptidyl(2A)-ribosome complex, driven by the 2A peptide within the peptidyltransferase center and exit tunnel, militates against the incorporation of proline into the nascent chain, instead promoting the binding of RF to the ribosome. Hydrolytic termination catalyzed by RF then resolves the nascent chain from tRNA^{Gly}. Further effort is required to fully define the role of RF in the reaction; the transient existence of the peptidyl(2A)-ribosome complex makes it a significant challenge to isolate it and study the interaction of RF with it. Following termination, the 2A peptide is envisaged to remain

associated with the ribosome, facilitating the restart to translation that generates the downstream product. This continued translation is perhaps most likely to be precipitated by the entry of prolyl-tRNA^{Pro} into the A site and its subsequent translocation to the P site. Such an event is not without precedent. Translation initiation directed by the internal ribosome entry site of the cricket paralysis virus intergenic region requires the translocation of a charged A site tRNA^{Ala} to the P site, with alanine becoming the first amino acid of the translated protein (33, 49). Defining the requirements for the synthesis of the downstream 2A reaction product is another challenge for the future.

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