Ribosomes in a Stacked Array

ELUCIDATION OF THE STEP IN TRANSLATION ELONGATION AT WHICH THEY ARE STALLED DURING S-ADENOSYL-L-METHIONINE-INDUCED TRANSLATION ARREST OF CGS1 mRNA*

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Background: S-Adenosyl-l-methionine arrests ribosomes on CGS1 mRNA at the pre-translocation step, and ribosomes stack behind it.

Results: Puromycin reactivity of stacked ribosomes was intermediate between ribosomes at the pre- and post-translocation steps.

Conclusion: Ribosomes are stacked at nine-codon intervals, and the majority are stalled at an early step of translocation.

Significance: This study is the first report of ribosomal states in a stacked array.

Expression of CGS1, which codes for an enzyme of methionine biosynthesis, is feedback-regulated by mRNA degradation in response to S-adenosyl-l-methionine (AdoMet). In vitro studies revealed that AdoMet induces translation arrest at Ser-94, upon which several ribosomes stack behind the arrested one, and mRNA degradation occurs at multiple sites that presumably correspond to individual ribosomes in a stacked array. Despite the significant contribution of stacked ribosomes to inducing mRNA degradation, little is known about the ribosomes in the stacked array. Here, we assigned the peptidyl-tRNA species of the stacked second and third ribosomes to their respective codons and showed that they are arranged at nine-codon intervals behind the Ser-94 codon, indicating tight stacking. Puromycin reacts with peptidyl-tRNA in the P-site, releasing the nascent peptide as peptidyl-puromycin. This reaction is used to monitor the activity of the peptidyltransferase center (PTC) in arrested ribosomes. Puromycin reaction of peptidyl-tRNA on the AdoMet-arrested ribosome, which is stalled at the pre-translocation step, was slow. This limited reactivity can be attributed to the peptidyl-tRNA occupying the A-site at this step rather than to suppression of PTC activity. In contrast, puromycin reactivities of peptidyl-tRNA with the stacked second and third ribosomes were slow but were not as slow as pre-translocation step ribosomes. We propose that the anticodon end of peptidyl-tRNA resides in the A-site of the stacked ribosomes and that the stacked ribosomes are stalled at an early step of translocation, possibly at the P/E hybrid state.

An actively translating mRNA bears many ribosomes as a polysome complex. When one ribosome stalls, the following ones will form a stacked array behind it. Polysomes have been extensively studied because of their contribution to overall translation activity (1–7). In contrast, reports on stacked ribosomes are limited (8). However, they are not static but sometimes play a role in the control of gene expression. One such example occurs in the Arabidopsis CGS1 gene, which encodes the first committed enzyme in methionine biosynthesis. Expression of CGS1 is negatively feedback-regulated by its mRNA degradation in response to S-adenosyl-l-methionine (AdoMet), a direct metabolite of methionine (9, 10). The amino acid sequence of the MTO1 region in CGS1 acts as the cis element in this response. Studies using an in vitro translation system of wheat germ extract (WGE) revealed that, in the presence of AdoMet, the nascent MTO1 peptide induces translation arrest at the Ser-94 codon located immediately downstream of the MTO1 region, causing peptidyl-tRNASer (PrRSer) to accumulate (11). Subsequently, several ribosomes stack behind the AdoMet-arrested one, and multiple mRNA degradation events occur to produce a ladder of 5′-truncated RNA species as degradation intermediates, most likely through the action of an endoribonuclease, suggesting that the stacked ribosomes function in determining the sites of CGS1 mRNA degradation (12).

In the AdoMet-arrested ribosome, PrRSer resides in the A-site of the small subunit (11), implying that the ribosome

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3 The abbreviations used are: AdoMet, S-adenosyl-l-methionine; CHX, cycloheximide; nt, nucleotide(s); PTC, peptidyltransferase center; PrR, peptidyl-tRNA; WGE, wheat germ extract.
TABLE 1

Plasmids used for in vitro transcription and PCR primers used for their construction

| Mutagenesis methods used are: 0, overlap extension PCR method; i, inverse PCR. |
|-------------------------|-----------------------------------|----------------------|
| Mutagenesis            | M8:Ex1(WT)-based synonymous codon substitutions | M8:ND5(C80A)-based cysteine substitutions |
| Plasmid                | Forward primer (5'-3')         | Reverse primer (5'-3')         | Mutagenesis |
| pYY23                  | Ille-74(AUA)                    | TGACATATAGAAGGCCCTAGAA | GCCTTTTATGCCTAGCTGCA 0 |
| pYY22                  | Lys-75(AAG)                     | GCATTAAGGGCCATGAAATCT    | ACAGCCCTATATGCTAGCTG 0 |
| pYY24                  | Ala-76(GCG)                     | TAAGCCTTAGAAAGACATAGC    | TGCTACGTGCTTATAAGCTCAG 0 |
| pYY25                  | Ala-76(GCA)                     | TAAGCCTAGGAAAGACATAGC    | TCTACTACGTGCTTATAAGCTCAG 0 |
| pYY26                  | Arg-77(CGG)                     | TAAGCCTCGGAAAGACATAGC    | GTCTCTCGGCTTATAAGCTG 0 |
| pYY28                  | Arg-77(GGG)                     | TAAGCCTCGGAAAGACATAGC    | GCTTCTCGGCTTATAAGCTCAG 0 |
| pYY27                  | Arg-77(AGA)                     | TAAGCCTCGGAAAGACATAGC    | GCTTCTCUGGCTTATAAGCTCAG 0 |
| pYY21                  | Ille-83(AUA)                    | AGAATGTTTCTTAGATAGC      | GCAAACGTCTATGCTAGCTG 0 |
| pYY20                  | Gly-84(GGC)                     | ATCGCCGTTTCCAGTGGT      | GCACTCAGCTTGCTATCTGAC 0 |
| pYY19                  | Gly-84(GGA)                     | ATCGCCGTTTCCAGTGGT      | GCACTCAGCTTGCTATCTGAC 0 |
| pYY17                  | Val-85(GUG)                     | CGGTGTTGCAAGCTGAGCT    | TGCCGACACCAGATGGTGC 0 |
| pYY18                  | Val-85(GUA)                     | CGGTGTTGCAAGCTGAGCT    | TGCCGACACCAGATGGTGC 0 |
| pYY43                  | Ser-94(UCG)                     | CTAATGTTGCCGCAAAACATCC  | GCTGTTGCGACCCATGAC 0 |
| pYY44                  | Ser-94(AGC)                     | CTAATGTTGCCGCAAAACATCC  | GCTGTTGCGACCCATGAC 0 |
| pYY37                  | K75C, C80A                      | TTTAATACGTACGCTAGGAC    | GCTCTAGAAGACGGATACACT 0 |
| pYY38                  | K76C, C80A                      | TTTAATACGTACGCTAGGAC    | GCTCTAGAAGACGGATACACT 0 |
| pYY39                  | R77C, C80A                      | TTTAATACGTACGCTAGGAC    | GCTCTAGAAGACGGATACACT 0 |
| pYY40                  | R78C, C80A                      | TTTAATACGTACGCTAGGAC    | GCTCTAGAAGACGGATACACT 0 |
| pYY41                  | N79C, C80A                      | TTTAATACGTACGCTAGGAC    | GCTCTAGAAGACGGATACACT 0 |
| pYY42                  | K92C, C80A                      | TTTAATACGTACGCTAGGAC    | GCTCTAGAAGACGGATACACT 0 |

Stalls during the translocation step. AdoMet-induced arrest of CGS1 mRNA translation is temporary, and resumption of translation is blocked by cycloheximide (CHX), which inhibits ribosomal translocation (11). A recent report revealed that CHX blocks movement of the deacylated tRNA toward the E-site within the large subunit at an early step of translocation (13). This enables reinterpretation of our previous data as demonstrating that AdoMet induces translation arrest at the pre-translocation step in CGS1.

Stacking of ribosomes also occurs during normal translation termination (8); however, no report has linked this stacking to mRNA degradation. A ladder of possible mRNA degradation intermediates similar to those of CGS1 was recently reported in the no-go mRNA decay system in yeast (14), possibly also suggesting a role of stacked ribosomes in mRNA degradation. Under what conditions do the stacked ribosomes take part in mRNA degradation? To answer this question, we must better understand the stacked array of ribosomes. RNase protection experiments have indicated that mRNA protected by a stacked ribosome is 27–29 nucleotides (nt) in length (8), suggesting that ribosomes are arranged at either nine- or ten-codon intervals in a stacked array. However, the step in the translation elongation cycle at which the stacked ribosomes are stalled has not been studied. Also, the exact intervals of the ribosomes in a stacked array have not been reported in terms of codons.

In the present study, we addressed two questions: (i) where are the stacked ribosomes located relative to the arrested ribosome, and (ii) at which step in the translation elongation cycle are the stacked ribosomes stalled? Primer extension inhibition (toeprint) experiments are used to determine the location of an arrested ribosome on a mRNA, based on the established distance between the P-site of the small subunit and toeprint signal (15, 16), but the location of the stacked ribosomes must be identified by other methods. Here, we determined the tRNA species of the PtRs in the stacked ribosomes and analyzed the puromycin reactivity of AdoMet-arrested and stacked ribosomes. Puromycin, an analog of aminoaconit-tRNA, binds to the A-site of the peptidyltransferase center (PTC) and reacts with PtR in the P-site, releasing the nascent peptide as peptidyl-puromycin. To deduce the ribosomal location on the mRNA by identifying PtR species, occupation of PtR in the A- or P-site of the small subunit must be determined. Because puromycin reactivity of PtR changes depending on the step in the translation elongation cycle, integrating the results of PtR location on mRNA and the arrested step is required. Therefore, our two questions are mutually interrelated.

EXPERIMENTAL PROCEDURES

**Chemicals**—AdoMet was purchased from Sigma-Aldrich (iodide salt, A4377; St Louis, MO, USA) and puromycin from Wako Pure Chemicals (Osaka, Japan). Edeine was a gift from the National Cancer Institute (Bethesda, MD, USA). Sources of other chemicals were previously reported (17).

**Plasmids**—pYK00 and pYK01 carry M8:Ex1(WT) and M8:Ex1(mto1–1) DNA, respectively, in the pSP64 poly(A) vector (Promega, Madison, WI, USA) (17). pNO57 carries M8:ND5(C80A) DNA in pSP64 poly(A) (17).

Plasmids pYY17 through pYY28, pYY43, and pYY44 carry synonymous codon substitutions of M8:Ex1(WT) (Table 1). In *vivo* mutagenesis was accomplished by the overlap extension PCR method (18, 19). The Xbal-BamHI fragment containing the wild-type CGS1 exon 1 coding region of pYK00 was replaced with the Xbal-BamHI fragment generated by overlap extension PCR of pYK00 DNA using PrimeSTAR HS DNA polymerase (Takara Bio, Ohtsu, Japan). The franking primers used for overlap extension PCR were described (17). Plasmids pYY37 through pYY42 carry cysteine substitutions of M8:ND5(C80A) (Table 1). Except for pYY37 and pYY38, *in vitro* mutagenesis was accomplished by the overlap extension PCR method as above using pNO57 as a template. *In vitro* mutagenesis for pYY37 and pYY38 plasmids was accomplished by inverse PCR using KOD Plus Neo DNA polymerase.

**TABLE 1**

| Plasmid | Mutation | Forward primer (5'-3') | Reverse primer (5'-3') | Mutagenesis |
|---------|----------|------------------------|------------------------|-------------|
| pYY23   | Ille-74(AUA) | TGACATATAGAAGGCCCTAGAA | GCCTTTTATGCCTAGCTGCA 0 |
| pYY22   | Lys-75(AAG) | GCATTAAGGGCCATGAAATCT | ACAGCCCTATATGCTAGCTG 0 |
| pYY24   | Ala-76(GCG) | TAAGCCTTAGAAAGACATAGC | TGCTACGTGCTTATAAGCTCAG 0 |
| pYY25   | Ala-76(GCA) | TAAGCCTAGGAAAGACATAGC | TCTACTACGTGCTTATAAGCTCAG 0 |
| pYY26   | Arg-77(CGG) | TAAGCCTCGGAAAGACATAGC | GTCTCTCGGCTTATAAGCTG 0 |
| pYY28   | Arg-77(GGG) | TAAGCCTCGGAAAGACATAGC | GCTTCTCGGCTTATAAGCTCAG 0 |
| pYY27   | Arg-77(AGA) | TAAGCCTCGGAAAGACATAGC | GCTTCTCUGGCTTATAAGCTCAG 0 |
| pYY21   | Ille-83(AUA) | AGAATGTTTCTTAGATAGC | GCAAACGTCTATGCTAGCTG 0 |
| pYY20   | Gly-84(GGC) | ATCGCCGTTTCCAGTGGT | GCACTCAGCTTGCTATCTGAC 0 |
| pYY19   | Gly-84(GGA) | ATCGCCGTTTCCAGTGGT | GCACTCAGCTTGCTATCTGAC 0 |
| pYY17   | Val-85(GUG) | CGGTGTTGCAAGCTGAGCT | TGCCGACACCAGATGGTGC 0 |
| pYY18   | Val-85(GUA) | CGGTGTTGCAAGCTGAGCT | TGCCGACACCAGATGGTGC 0 |
| pYY43   | Ser-94(UCG) | CTAATGTTGCCGCAAAACATCC | GCTGTTGCGACCCATGAC 0 |
| pYY44   | Ser-94(AGC) | CTAATGTTGCCGCAAAACATCC | GCTGTTGCGACCCATGAC 0 |

M8:Ex1(WT)-based synonymous codon substitutions

M8:ND5(C80A)-based cysteine substitutions
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(Toyobo, Tokyo, Japan) followed by digestion of the plasmid DNA by DpnI (Toyobo) using pNO57 as a template. Then, PCR products were self-ligated using T4 DNA ligase (Wako Pure Chemicals) and T4 polynucleotide kinase (Toyobo). Primers used for in vitro mutagenesis are shown in Table 1. In all cases, the mutations were confirmed by DNA sequencing.

In Vitro Transcription—In vitro transcription of M8:Ex1(WT), M8:NDS(C80A), and their derivatives by SP6 RNA polymerase (CellScript, Madison, WI) in the presence of a cap analog was carried out as described previously (17). DNA templates for nonstop RNAs, M8:Ex1(S94ns) and M8:Ex1(V85ns), were prepared by PCR amplification of corresponding regions from pYK00 using PrimeSTAR HS DNA polymerase. The forward primer used was SP65’F (17). The reverse primers used for M8:Ex1(S94ns) and M8:Ex1(V85ns) were S94r (17) and 5’-AACACCGATGTTGCTACAGT-3’ (V85r), respectively. DNA template for M8:Ex1(mto1–1, S94ns) was prepared as for M8:Ex1(S94ns), except that pYK01 was used instead of pYK00. The amplified fragments were used for in vitro transcription as described previously (17).

In Vitro Translation—In vitro translation reactions using WGE (Promega) were carried out as described (10), except for adding [35S]methionine or [35S]cysteine (37 TBq mmol⁻¹, 370 MBq ml⁻¹, American Radiolabeled Chemicals, St. Louis, MO) in the translation reaction mixture. M8:Ex1 RNA (1 pmol) or nonstop RNA (2 pmol) was translated in a 20-μl WGE reaction mixture in the presence or absence of 1 mM AdoMet. The reaction mixture for the [35S]methionine labeling experiment consisted of 10 μl of WGE, 1.6 μl of a 1 mM amino acid mixture lacking methionine (Promega), 0.3 μl 5 μM 1-methionine, and 0.06 μl of [35S]methionine. For [35S]cysteine labeling, the reaction mixture consisted of 10 μl of WGE, 1.6 μl of a 1 mM amino acid mixture lacking methionine and cysteine (Promega), 0.4 μl of 5 μM 1-methionine, and 0.5 μl of [35S]cysteine. RNase A treatment was performed as described (11). Translation samples were boiled for 3 min, separated on a NuPAGE 4–12% BisTris gel in MOPS running buffer (Invitrogen, Carlsbad, CA), dried, and visualized using a FLA-7000 image analyzer (Fuji Photo Film, Tokyo, Japan). Because migration of PtRs is affected by temperature, SDS-PAGE was carried out in a 28 °C chamber. Measurements of band intensities and digital data acquisition were carried out using MultiGauge software (Fuji Photo Film).

Puromycin Reaction—For puromycin reaction of PtR, in vitro translation was carried out in a 40-μl translation reaction mixture. After 30 min of translation at 25 °C, puromycin was added to a final concentration of 2 mM and further incubated at 25 °C until an aliquot was sampled for SDS-PAGE. The unreacted PtR band was quantified, and the puromycin reactivity was assessed by dividing the band intensity at a given time point by the intensity at time 0.

To test the effect of exogenously added MTO1 peptide on puromycin reactivity, in vitro translation and puromycin reaction were carried out in the presence of 500 nM synthetic peptide.

Primer Extension Experiment—Primer extension of RNA after translation in WGE was carried out as described (12).

Pseudo-First-Order Kinetic Analysis of Puromycin Reaction and Statistical Analysis—Puromycin reaction will follow pseudo-first order kinetics provided that puromycin is present in large excess relative to the stalled ribosome (20, 21), as in the present study. In simple first-order kinetics, the amount of unreacted PtR will follow (Equation 1),

\[ y = \exp(-k_{app} t) \]  

where \( y \) is amount of the unreacted PtR, \( k_{app} \) is the apparent rate constant of puromycin reaction, and \( t \) is reaction time.

If PtR consists of two or three PtR species with different \( k_{app} \) the amount of the unreacted PtR will follow a two-component (Equation 2) or three-component (Equation 3) model, and will give a curved line on a semi-log plot.

\[ y = f_1 \exp(-k_{app,1} t) + (1 - f_1) \exp(-k_{app,2} t) \]  

where \( f_i \) is the fraction of \( i \)-th component of PtR, and \( k_{app,i} \) is the apparent rate constants of puromycin reaction of \( i \)-th component of PtR. Fitting to these models by non-linear least square method was carried out by using Mathematica ver. 9 software (Wolfram Research, Champaign, IL, USA). The Akaike information criterion (22) was used to choose the best fitted model among (Equation 1) - (Equation 3). The rate of spontaneous hydrolysis of PtR was analyzed by (Equation 1).

Statistical analyses were carried out using Welch’s t test. For multiple comparisons, the false discovery rate correction of Benjamini and Hochberg (23) was applied.

RESULTS

Identification of the Peptidyl-tRNA Band That Was Formed on the AdoMet-arrested Ribosome—In the in vitro translation condition used in this study (50 fmol RNA μl⁻¹ translation mixture), three major translation arrest products were detectable (Fig. 1), and we previously determined that at least two additional ribosomes were stacked behind the arrested ribosome (12). To identify the stalled sites of the stacked ribosomes, we first identified the PtR of the arrested ribosome. M8:Ex1(WT) RNA (Fig. 1A) was translated in WGE in the presence of [35S]methionine, and the translation products were analyzed by SDS-PAGE. M8 is a small tag that encodes eight consecutive methionine residues (17, 24). When M8:Ex1(WT) RNA was translated in the presence of AdoMet, three PtR bands were detected at ~30 kDa, whereas no such band was observed when RNA was translated in the absence of AdoMet (Fig. 1B, lanes 2 and 1, respectively). We designated these bands PtR(a), PtR(b), and PtR(c) according to their gel mobilities. RNase A treatment of the translation products caused these bands to disappear, and new bands of ~10 kDa were detected that corresponded to the molecular weights of the arrested peptides (Fig. 1B, lane 5).

When the amount of mRNA increases, fewer ribosomes are expected to load on one mRNA, and fewer ribosomes will stack upon translation arrest. When the concentration of M8:Ex1(WT) RNA in the translation reaction mixture was increased, the
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FIGURE 1. Detection of the three translation arrest products. A, schematic representation of M8:Ex1(WT) RNA. B, detection of three PtRs by SDS-PAGE. M8:Ex1(WT) RNA (50–250 fmol µl⁻¹) as indicated) was translated in the absence (lane 1) or presence (lanes 2–7) of 1 mM AdoMet. After 30 min of translation, samples were treated with RNase A (lanes 5–7). The AdoMet-dependent PtR (≈30-kDa), the 21-kDa full-length translation product (FL), and the 10-kDa arrested peptide bands (AP) produced by the RNase A treatment are marked. PtR(a), blue circle; PtR(b), magenta circle; PtR(c), green circle. A representative result of triplicated experiments is shown.

Intensities of the PtR(a) and PtR(c) bands decreased, whereas that of PtR(b) barely changed (Fig. 1B, lanes 2–4), suggesting that PtR(b) is PtR\textsuperscript{Ser94} formed on the AdoMet-arrested ribosome that stopped at the Ser-94 codon, whereas PtR(a) and PtR(c) were formed on the stacked second or third ribosomes.

To verify these band assignments and to determine the stalled sites of the stacked ribosomes, we performed a gel mobility shift assay. Mobility of PtRs in SDS-PAGE depends on the tRNA species of the PtR, even though the peptide part is the same. Therefore, by changing the codon at the stalled site of the stacked ribosome, we expect a mobility shift of the arrest products, allowing us to determine the PtR species. Because the stalled site of the second stacked ribosome is expected to reside within the MTO1 region, where only limited amino acid changes are allowed (25), we introduced synonymous codon substitutions.

We substituted the wild-type Ser-94(UCG) codon with UCG or AGC codons (25). When these RNAs were translated in the presence of AdoMet, the PtR(b) band disappeared (Fig. 2B, lanes 2–4, magenta circle). In the case of Ser-94(UCG), a new band, PtR(b1), appeared that was larger than PtR(a) (Fig. 2B, lane 3, magenta triangle), whereas with Ser-94(AGC), a new band, PtR(b2), appeared slightly above the PtR(a) band (Fig. 2B, lane 4, magenta square). The results unequivocally determined that PtR(b) was formed on the ribosome that arrested at the Ser-94 codon. Hereafter, we refer to PtR(b) as PtR(S94).

The Stacked Second Ribosome Stalls at Val-85—An RNase protection assay with stacked ribosomes in WGE (8) suggested that ribosomes are stacked at nine- or ten-codon intervals. Therefore, we introduced synonymous codon substitutions at the Ile-83, Gly-84, and Val-85 codons (Fig. 2A). Synonymous substitution of Val-85(GLU) to GLIG or GLIA codons shifted the mobility of PtR(a). When M8:Ex1(Val-85 GLIG) RNA was translated, the resulting PtR(a1) band migrated slightly faster than PtR(a) (Fig. 2C, lane 3, blue triangle). Likewise, when M8:Ex1(Val-85 GLIA) RNA was translated, PtR(a) disappeared, whereas the new PtR(a2) overlapped with PtR(b) (Fig. 2C, lane 4, blue square). Introduction of synonymous codon substitutions at the Ile-83 and Gly-84 codons did not result in PtR mobility shifts (Fig. 2D). These results indicate that PtR(a) was formed on the stacked second ribosome and that this ribosome stalled at the Val-85 codon, nine codons upstream of Ser-94. Hereafter, we refer to PtR(a) as PtR(V85).

The Majority of Stacked Third Ribosomes Stalls at Ala-76—We tested synonymous codon substitutions for the Ile-74, Lys-75, Ala-76, and Arg-77 codons to identify where the third ribosome stalled (Fig. 2A). The only difference that we detected was a new band, PtR(c1), that migrated faster than PtR(c), when Ala-76(GCC) was substituted to GCA (Fig. 2E, lane 6, green triangle). Because PtR(c) did not disappear with this substitution, we verified the stalled site of the third ribosome by another approach.

Cysteine substitutions were introduced to M8:ND5(C80A) RNA, which carries no cysteine residue within CGS1 exon 1 (Fig. 2F) (17), and the translation products were labeled with \[^{35}S\]cysteine. When M8:ND5(C80A) RNAs carrying a single cysteine residue at Lys-75 or Ala-76 were translated in the presence of AdoMet, three PtR bands were detected (Fig. 2G, lanes 1 and 2), indicating that Lys-75 and Ala-76 codons were translated by the third ribosome. In contrast, M8:ND5 RNAs carrying a single cysteine at Arg-77, Arg-78, Asn-79, and Cys-80 produced two PtR bands (Fig. 2G, lanes 3–6), showing that these codons were not translated by the third ribosome. These results corroborate the findings of our synonymous codon substitution experiments (Fig. 2E) that the third ribosome translates up to Ala-76 codon.

The PtR bands were weak in M8:ND5(R78C, C80A). The Arg-78 to cysteine substitution probably affected AdoMet-dependent translation arrest, because Arg-78 is located within the MTO1 region. A similar case was also observed in M8:ND5(K75C, C80A), in which three PtRs were observed, but the PtR(c) band was weak. Possibly, although Lys-75 was outside the MTO1 region and an alanine substitution was tolerated at this position (25), substitution to cysteine may have affected the translation arrest at Ser-94.

Because PtR(c) did not disappear with the Ala-76(GCC) to GCA substitution, there may be ambiguity in the stall site of the third ribosome, and/or PtR of the stacked fourth ribosome may overlap with PtR(c); however, these factors would be minor, because the PtR(c) band produced by M8:ND5(A76C, C80A) RNA (Fig. 2G, lane 2) was as intense as that detected by translation of M8:Ex1(WT) RNA in the presence of AdoMet (Fig. 2H). Alternatively, the Ala-76 codon may be recognized by more than one tRNA species; however, this would not affect the band assignment of PtR(c). Taken together, the results indicated that the majority of stacked third ribosomes translated up to the Ala-76 codon. Hereafter, PtR(c) will be referred to as PtR(A76).

Puromycin Reactivity of the AdoMet-arrested Ribosome—To gain insight into the state(s) of the ribosomes in a stacked array, the puromycin reactivity was analyzed (Fig. 3). The rate of spontaneous hydrolysis of PtR under our experimental conditions was slow enough not to hinder the puromycin reaction assay (Fig. 3, B and F). M8:Ex1(WT) RNA was translated in WGE...
in the presence of AdoMet for 30 min, and then 2 mM puromycin was added and the time course of puromycin reaction was analyzed. Because excess puromycin was added relative to the stalled ribosome, the reaction will follow pseudo-first order kinetics (20, 21) (Equation 1, “Experimental Procedures”). Simple pseudo-first order kinetics will give a straight line on a semi-log plot, but the line was curved (Fig. 3F). This fact can be interpreted, regarding the puromycin reactivity, as indicating that the PtRs are composed of a mixture of fast-reacting and slow-reacting ribosomes (Equation 2, “Experimental Procedures”). Fitting to the two-component model by the least-square method showed that PtR(S94) is curved (Fig. 3E) and PtR(S94) was similar to PtR(S94fast) and PtR(S94slow) (Table 2). To evaluate this puromycin reactivity, we used M8:Ex1RNA (V85ns) RNA, a nonstop RNA that is truncated at Val-85 codon (Fig. 3A), as a control of post-translocation step ribosome. A ribosome will translate a nonstop RNA up to the last codon on the mRNA and will remain in the post-translocation state until it is released from the mRNA (26, 27). Puromycin has been shown to react most efficiently with PtR at the post-translocation state, in which PtR resides in the P-site whereas the A-site is vacant (20, 21). Puromycin reaction of PtR(V85ns), which is produced with M8:Ex1RNA (V85ns) RNA, was generally fast both in the presence and absence of AdoMet (Fig. 3D, F). In this case, the time course of puromycin reaction followed a three-component model (Equation 3, “Experimental Procedures”), rather than a two-component model (22). Fitting to the three-component model showed that ~70% of PtR exhibited $k_{app}$ of 0.59 ± 0.10 s⁻¹ in the absence of AdoMet ($k_{app}(V85ns−AdoMet)$) (Table 2). We consider this value to be puromycin reactivity of the post-translocation state ribosome and will refer to it as $k_{app}(Post)$. This value was not significantly different from the corresponding $k_{app}(V85ns+AdoMet)$ obtained in the presence of AdoMet (Table 2). The remaining 30% of PtR showed much lower puromycin reactivity. Although we do not know in what state these PtR exist, accumulation of similar slow-reacting PtR on nonstop RNA was also reported in another system (27). Approximately 10% of PtR(S94) were in the fast-reacting state. Although the observed S.E. was large, and statistical analysis would not be valid, the $k_{app}(S94fast)$ was similar to
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$\kappa_{app}(\text{Post})$ (Table 2), suggesting that this 10% fraction was stalling at the post-translocation state.

Puromycin Reactivity of the Stacked Second and Third Ribosomes—Puromycin reactivities of PtR(V85) and PtR(A76) followed a similar time course (Fig. 3, C and F). In the case of PtR(V85), fitting to the two-component model showed 36% fast-reacting and 64% slow-reacting PtR with $\kappa_{app}$ of $0.54 \pm 0.11 \text{ s}^{-1}$ ($\kappa_{app}[V85^{\text{fast}}]$) and $1.7 \times 10^{-3} \pm 0.1 \times 10^{-4} \text{ s}^{-1}$ ($\kappa_{app}[V85^{\text{slow}}]$) (Table 2), respectively. Similar values were obtained with PtR(A76) (Table 2). The $\kappa_{app}(V85^{\text{fast}})$ and $\kappa_{app}(A76^{\text{fast}})$ were not significantly different from $\kappa_{app}(\text{Post})$ (Table 2), whereas $\kappa_{app}(V85^{\text{slow}})$ and $\kappa_{app}(A76^{\text{slow}})$ were significantly larger than $\kappa_{app}(S94^{\text{slow}})$ ($p < 0.05$, Welch’s $t$ test).

FIGURE 3. Puromycin reactivities of stalled ribosomes. A, schematic representation of M8:Ex1(WT) RNA and the $3^{'}$-truncated nonstop RNAs M8:Ex1(S94ns) and M8:Ex1(V85ns). B, stability of PtR(S94ns) in the translation reaction conditions used in the present study was analyzed. M8:Ex1(S94ns) RNA (100 fmol $\mu$L$^{-1}$) was translated in the absence of AdoMet. Translation reaction started at $-10$ min, and edeine, an inhibitor of translation initiation, was added at a final concentration of 2.5 mM at $-5$ min. Samples were removed at the indicated times for SDS-PAGE. Positions of PtR(S94ns) and the hydrolyzed peptide are marked. C, M8:Ex1(WT) RNA (50 fmol $\mu$L$^{-1}$) was translated in the presence of 1 mM AdoMet. After 30 min of translation, puromycin was added to a final concentration of 2 mM. At the indicated times, samples were removed for SDS-PAGE. The AdoMet-dependent PtR(S94) (magenta circle), PtR(V85) (blue circle) and PtR(A76) (green circle), the 21-kDa full-length translation product (FL), and peptidyl-puromycin (PP) bands are marked. D and E, M8:Ex1(V85ns) RNA (D) or M8:Ex1(S94ns) RNA (E) (100 fmol $\mu$L$^{-1}$) was translated in the presence or absence of 1 mM AdoMet and subjected to SDS-PAGE as in C. A representative result of triplicated experiments is shown in B–E. Positions of PtR(V85ns) and PtR(S94ns) are marked in D and E, respectively. F and G, radioactive signals in B–D were quantified. For each time point, the intensity of unreacted PtR was normalized to that at time 0. Average $\pm$ S.D. ($n = 3$) are shown. Filled circles, fraction of unreacted PtRs in C; filled and open triangles, fraction of unreacted PtR(V85ns) in the presence and absence of AdoMet, respectively, in D; gray dashed line, spontaneous hydrolysis in B. G, radioactive signals of unreacted PtR in E were quantified and presented as in F. Filled and open triangles, fraction of unreacted PtR(S94ns) in the presence and absence of AdoMet, respectively, in E. Puromycin reaction of PtR(S94) in F is reproduced for comparison (gray line).
Puromycin Reactivity of the PtR(S94ns) Formed on M8:Ex1(S94ns) RNA—When M8:Ex1(S94ns), a nonstop RNA that is truncated at the Ser-94 codon (Fig. 3A), was translated in the presence of AdoMet (Fig. 3E, G), puromycin reaction followed a time course similar to that of AdoMet-arrested PtR(S94), with >80% of PtR showing \( k_{\text{app}} \) of 4.2 \( \times 10^{-3} \pm 0.5 \times 10^{-3} \) s\(^{-1}\) (\( k_{\text{app}}(\text{S94ns-AdoMet}) \)), suggesting that the ribosomes that translated Ser-94 nonstop RNA in the presence of AdoMet were in a state similar to that of the AdoMet-arrested ribosome. In contrast, when the same RNA was translated in the absence of AdoMet, the overall puromycin reaction was much faster than that in its presence (Fig. 3E, G). Fitting to the three-component model showed that >80% of the PtR exhibited \( k_{\text{app}} \) of 3.1 \( \times 10^{-2} \pm 0.3 \times 10^{-2} \) s\(^{-1}\) (\( k_{\text{app}}(\text{S94ns-AdoMet}) \)), which is intermediate between \( k_{\text{app}}(\text{S94ns}) \) and \( k_{\text{app}}(\text{Post}) \) (Table 2). To test whether the low puromycin reactivity of PtR(S94ns) produced by translation of M8:Ex1(S94ns) RNA in the presence of AdoMet was dependent on MTO1 function, M8:Ex1(mto1–1, S94ns) RNA was translated in the presence and absence of AdoMet, and puromycin reactivity was analyzed (Fig. 4, C and D). The results showed that puromycin reactivity of PtR(S94ns) formed on M8:Ex1(mto1–1, S94ns) RNA was not appreciably different between the presence and absence of AdoMet (\( p > 0.05 \), Welch’s t test). These findings indicated that the slow puromycin reactivity of M8:Ex1(S94ns) RNA in the presence of AdoMet was dependent on MTO1 function.

Table 2

| PtR           | AdoMet | Fraction ± S.E. | \( k_{\text{app}} \) ± S.E. | Note* | \( k_{\text{app}}(\text{S94ns-AdoMet}) \) | \( k_{\text{app}}(\text{V85ns-AdoMet}) \) |
|---------------|--------|----------------|-----------------------------|-------|----------------------------------------|----------------------------------------|
| M8:Ex1(WT) RNA |        |                |                             |       |                                        |                                        |
| PtR(S94)      | +      | 10.3 ± 1.3     | 0.6 ± 0.7                   |       | \( k_{\text{app}}(\text{S94ns}) \)   | \( k_{\text{app}}(\text{S94ns}) \)   |
| PtR(V85)      | +      | 89.7 ± 1.3     | 8.4 \( \times 10^{-3} \) ± 0.6 \( \times 10^{-3} \) | *     | *                                     | *                                     |
| PtR(A76)      | +      | 35.8 ± 1.0     | 0.54 ± 0.11                 |       | \( k_{\text{app}}(\text{V85ns}) \)   | \( k_{\text{app}}(\text{V85ns}) \)   |
|               |        | 64.2 ± 1.0     | 1.7 \( \times 10^{-3} \) ± 0.1 \( \times 10^{-3} \) | *     | *                                     | *                                     |
|               |        | 39.3 ± 1.6     | 0.54 ± 0.16                 |       | \( k_{\text{app}}(\text{A76ns}) \)   | \( k_{\text{app}}(\text{A76ns}) \)   |
|               |        | 60.7 ± 1.6     | 1.8 \( \times 10^{-3} \) ± 0.2 \( \times 10^{-3} \) | *     | *                                     | *                                     |
| M8:Ex1(V85ns) RNA |        |                |                             |       |                                        |                                        |
| PtR(V85ns)    | +      | 70.4 ± 5.1     | 0.51 ± 0.09                 |       | \( k_{\text{app}}(\text{V85ns}) \)   | \( k_{\text{app}}(\text{V85ns}) \)   |
|               |        | 19.3 ± 4.8     | 3.8 \( \times 10^{-2} \) ± 1.9 \( \times 10^{-2} \) | *     | *                                     | *                                     |
|               |        | 10.3 ± 7.0     | 1.9 \( \times 10^{-2} \) ± 1.1 \( \times 10^{-2} \) | *     | *                                     | *                                     |
| PtR(V85ns)    | −      | 71.0 ± 5.0     | 0.59 ± 0.10                 |       | \( k_{\text{app}}(\text{V85ns}) \)   | \( k_{\text{app}}(\text{V85ns}) \)   |
|               |        | 15.6 ± 4.7     | 5.2 \( \times 10^{-2} \) ± 2.7 \( \times 10^{-2} \) | *     | *                                     | *                                     |
|               |        | 13.4 ± 6.9     | 2.6 \( \times 10^{-3} \) ± 0.8 \( \times 10^{-3} \) | *     | *                                     | *                                     |
| M8:Ex1(S94ns) RNA |        |                |                             |       |                                        |                                        |
| PtR(S94ns)    | +      | 17.6 ± 1.4     | 6.0 \( \times 10^{-2} \) ± 3.1 \( \times 10^{-2} \) |       | \( k_{\text{app}}(\text{S94ns-AdoMet}) \) | \( k_{\text{app}}(\text{Pre}) \)         |
|               | −      | 82.4 ± 1.4     | 4.2 \( \times 10^{-4} \) ± 0.5 \( \times 10^{-4} \) |       | \( k_{\text{app}}(\text{S94ns-AdoMet}) \) | \( k_{\text{app}}(\text{Pre}) \)         |
| PtR(S94ns)    | −      | 10.2 ± 3.3     | 0.9 ± 1.5                   |       | \( k_{\text{app}}(\text{S94ns-AdoMet}) \) | \( k_{\text{app}}(\text{Pre}) \)         |
|               | −      | 81.0 ± 3.4     | 3.1 \( \times 10^{-2} \) ± 0.3 \( \times 10^{-2} \) |       | \( k_{\text{app}}(\text{S94ns-AdoMet}) \) | \( k_{\text{app}}(\text{Post}) \)         |
|               | −      | 8.7 ± 4.7      | 1.3 \( \times 10^{-3} \) ± 0.9 \( \times 10^{-3} \) |       | \( k_{\text{app}}(\text{S94ns-AdoMet}) \) | \( k_{\text{app}}(\text{Post}) \)         |

* Asterisks indicate significant difference (\( p < 0.05 \), Welch’s t test for multiple test correction). − denotes that the S.E. was large, and the data were excluded from statistical analysis; their \( k_{\text{app}} \) values are shown here for reference.
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X-ray crystallographic studies of prokaryotic ribosomes showed that the mRNA cavity on the small subunit covered ~30 nt of mRNA (28, 29), corroborating the report that the length of mRNA protected from RNase digestion by a eukaryotic translating ribosome was about 30 nt (3, 6). The only available experimental data on stacked ribosomes came from RNase protection experiments which showed a ladder of protected RNA fragments, with the mRNA protected by a ribosome calculated to be 27–29 nt in WGE (8). However, no experimental study had reported on the exact intervals of the stacked ribosomes in terms of codons.

The anticodon end of a PtR can be positioned either in the small subunit P-site, the A-site-to-A-site distance would be eight codons. Available data from the literature are limited to evaluate these two possibilities; however, an A-site-to-A-site distance of eight codons seems too small for the length of mRNA that is “held” by a ribosome (3, 6, 8). Thus, the most likely interpretation of our data is that PtR(V85) on the stacked second ribosome was located in the small subunit A-site, giving an A-site-to-A-site distance of nine codons between the arrested and stacked second ribosomes (Fig. 7A). Given that the PtR(V85) on the stacked second ribosome is located in the small subunit A-site, the same logic applies to the distance between the stacked second and third ribosomes, and the third ribosome would bear its PtR(A76) in the A-site of the small subunit (Fig. 7A).

Puromycin Reactivity of the AdoMet-arrested Ribosome—Puromycin reactivity has been used to monitor the PTC function in a number of ribosome arrest systems (27, 30–36). In all these systems, in which toeprint analysis was applied, PtR was normalized to that at time 0. Average ± S.D. (n = 3) are shown.
shown to be located in the P-site. In these systems, puromycin reaction was slow, which was interpreted as PTC activity was inhibited and/or puromycin could not readily access the PTC A-site. In the case of arginine attenuator peptide of Neurospora crassa (15, 24), PTC function was suggested to be interfered by the nascent peptide in the P-site and the presence of arginine (27). So, how can we explain the slow puromycin reaction in the CGS1 system? PTC catalyzes the puromycin reaction when PtR resides in the A-site and puromycin comes into the A-site, whereas the AdoMet-arrested ribosome is stalled at the pre-translocation step and PtR resides in the A-site. Although no reports have examined puromycin reactivity of the A-site-bound PtR in eukaryotes, studies in prokaryotes have shown that puromycin reacts 1,000–3,000 times slower with pre-bound PtR in eukaryotes, studies in prokaryotes have shown that puromycin reacts 1,000–3,000 times slower with pre-bound PtR in eukaryotes. As such, the A-site of the ribosome will pass through the post-translocation state, during which puromycin reaction is efficient, whereas resumption of translation could be blocked on the nonstop RNA. If this assumption is granted, the $k_{\text{app}}$ of $S94\text{ns}^{\text{AdoMet}}$ of $4.2 \times 10^{-4}$ ± $0.5 \times 10^{-4}$ s⁻¹ represents the puromycin reactivity of pre-translocation state ribosomes, which we will refer to $k_{\text{app}}$ (Pre). Corroborating the report in the purified E. coli in vitro translation system mentioned above (21), $k_{\text{app}}$ (Pre) is ~1,400 times smaller than $k_{\text{app}}$ (Post).

When M8:Ex1(S94ns) RNA was translated in the absence of AdoMet, $k_{\text{app}}$ ($S94\text{ns}^{\text{AdoMet}}$) was intermediate between $k_{\text{app}}$ (Pre) and $k_{\text{app}}$ (Post). In E. coli, puromycin reactivity of the A/P hybrid state ribosomes was ~10-fold lower than that of the post-translocation state ones (21, 37, 38). This coincidence suggests that $k_{\text{app}}$ ($S94\text{ns}^{\text{AdoMet}}$) of $3.1 \times 10^{-2}$ ± $0.3 \times 10^{-2}$ s⁻¹ represents the puromycin reactivity of the A/P hybrid state ribosomes.

Puromycin Reactivity of PtR on the Stacked Second and Third Ribosomes—Puromycin reactivity of the stacked second and third ribosomes showed that the ribosomes are composed of two fractions: (i) The majority (~60%) of the ribosomes exhibited $k_{\text{app}}$ ($V85\text{slow}$) and (ii) ~40% exhibited $k_{\text{app}}$ ($V85\text{fast}$). Let us
consider the majority first. The $k_{app}(V85_{slow})$ was similar to but significantly larger than $k_{app}(Pre)$ (Table 2). During translocation, a ribosome undergoes several conformation changes, which include rotation of the small subunit relative to the large subunit (39, 40), rotation of the head of the small subunit (40, 41), and conformation changes of the ribosomal protein L1 stalk to facilitate tRNA release from the E-site (40, 42). Notably, upon transition to the hybrid state, rotation of the small subunit occurs (41). When a ribosome collides with the preceding one, some of these conformation changes might be hindered, and the ribosome may stall during translocation either in the P/E or A/P hybrid state. As discussed above, $k_{app}(S94_{ns}/H11002\text{AdoMet})$ likely represents the puromycin reactivity of the A/P hybrid state, which is significantly larger than $k_{app}(V85_{slow})$ ($p < 0.05$, Welch’s t test). We can reasonably expect that puromycin reactivity of the P/E hybrid state is as low as that of the pre-translocation step, because the peptidyl-tRNA occupies the A-site as in the pre-translocation step. In E. coli, experiments using viomycin, which inhibits translocation at the P/E hybrid state, showed that puromycin reactivity of the P/E hybrid state was ~2-fold higher than in the pre-translocation state (21). Therefore, we may infer that the majority of the stacked second and third ribosomes are in the P/E hybrid state (Fig. 7A).

Next, consider the ~40% of the stacked ribosomes. The $k_{app}(V85_{fast})$ was not significantly different from $k_{app}(Post)$ (Table 2), suggesting that these ribosomes are in the post-translocation state. As discussed in the previous section, occupation of PtR(V85) in the small subunit P-site would be too short of a distance between the arrested and stacked ribosomes. Because 10% of the PtR(S94) are likely in the post-translocation state, a corresponding fraction of PtR(V85) is stacked behind the P-site-arrested ribosome, resulting in a nine-codon interval (Fig. 7B). Then, we must address the remainder of the PtR(V85). For the stacked ribosomes to stall at the post-translocation state and are prevented from proceeding to the peptidyl-transfer step, either the release of eEF-2 or the incorporation of aminoacyl-tRNA must be inhibited (21, 43). In prokaryotes, post-translocation state ribosomes with associated EF-G exhibit full puromycin reactivity, although entry of aminoacyl-tRNA is possible only after dissociation of EF-G (21, 38). This logic reveals an explanation for the remaining PtR(V85): the leading ribosome had initially arrested at Ser-94 and the second ribosome stacked at Val-85, but the leading ribosome has resumed translation (and thus was no longer detected at the Ser-94 position) before the start of puromycin reaction (Fig. 7C). In this scenario, the second ribosome sitting at Val-85 has gone one round of translocation to be in the post-translocation state. The prokaryotic L7/L12 (eukaryotic P0/P1) stalk, which is located near the factor binding site of the large subunit, is thought to function in accommodating translation factors including aminoacyl-tRNA (44, 45, 46). Ribosomal collision may hinder one of these functions.

Ribosome Stacking and CGS1 mRNA Degradation—In the present study, we carried out precise mapping of the 5'-ends of the degradation intermediates (Fig. 6). DI-2 and DI-3 are separated by 27 nt, or nine codons, corroborating the distance...
between the PtR codons of the stacked second and third ribosomes (Fig. 7A). Our previous polysome profiling experiment showed that DI-1 is predominantly found in the monosome fraction, and DI-2 and DI-3 are found in two- and three-ribosome fractions, respectively (12). The nine-codon distance between DI-2 and DI-3 strongly suggests that the stacked second and third ribosomes are responsible for determining the positions of DI-2 and DI-3, respectively (Fig. 7A). Notably, DI-2 was detected most strongly, suggesting that ribosome collision has a role in inducing the mRNA degradation. Understanding mRNA cleavage defined by stacked ribosomes will shed light on the important regulatory roles that stacked ribosomes play in the control of mRNA degradation.

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