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Monitoring translation with modified mRNAs strategically labeled with isomorphic fluorescent guanosine mimetics

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Abstract

Here we examine three mRNAs, site-specifically modified at codon positions 4, 5, and 6 with a new emissive and responsive isosteric guanosine mimetic (thG), with the goal of developing real time assays for monitoring translation-related events at nucleotide resolution. All three emissive mRNAs tested form initiation (70SIC), pretranslocation (PRE) and posttranslocation (POST) complexes. In most cases spectral differences are seen on binding of the mRNA to the ribosome during 70SIC formation, and on conversion of 70SIC to PRE complexes and PRE complexes to POST complexes. These differences allow measurement of the kinetics of such conversions by changes in the fluorescence of labeled mRNAs. Such measurements directly identify a specific step during PRE complex formation, provisionally assigned to codon:anticodon-loop base pair formation, that follows aa-tRNA.EF-Tu.GTP ternary complex binding to the ribosome and precedes aa-tRNA accommodation into the A-site of the ribosome. These observations not only demonstrate the functionality of mRNAs modified with the emissive guanosine mimetic, but also the potential this mimetic offers for observing the formation and disappearance of discrete intermediates during the polypeptide elongation cycle.

The past dozen years or so have witnessed huge progress in our understanding of ribosome function, fueled by high resolution structures^{1,2} and by increasingly sophisticated biochemical, biophysical, and computational studies that probe the detailed workings of this complex machine.^{3–7} Many of the studies directed toward understanding of the dynamics of ribosome function rely heavily on both ensemble and single molecule time-resolved observations made with functional, fluorescent-labeled derivatives of components of the protein synthesis machinery, for the most part including tRNAs, ribosomal proteins, and protein auxiliary factors such as EF-Tu and EF-G.^{4,8–10} For example, changes in the fluorescence of labeled tRNAs and labeled ribosomal proteins, combined with several other assays measuring GTP hydrolysis, dipeptide formation, P_i release, and peptidyl-tRNA reactivity toward puromycin have resulted in the formulation of quantitative kinetic schemes for the two major steps within the elongation cycle: i) ternary complex (TC, consisting of aminoacyl (aa)-tRNA.EF-Tu.GTP) binding to a posttranslocation (POST) or 70S initiation complex (70SIC) followed by accommodation of aa-tRNA within the A-site of the ribosome and peptide bond formation, leading to pretranslocation (PRE) complex formation^{5,11–12}, and ii) EF-G.GTP-catalyzed translocation of tRNAs bound in the A- and P-sites to the P- and

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ASSOCIATED CONTENT

Supporting Information. Supplementary figures and table. This material is available free of charge via the Internet at <http://pubs.acs.org>.

E-sites, respectively, along with mRNA to which they are bound, leading to conversion of PRE complex to POST complex^{9, 13–16}, thus completing the cycle.

Despite the central role of mRNA in protein synthesis, the use of fluorescent mRNAs has, by contrast, been quite limited, confined almost exclusively to mRNAs derivatized at the 3'-end with fluorophores that monitor the movement of this terminus to a position at the entrance of the mRNA channel during translocation.^{14, 16–18} Although it would be of obvious interest to monitor mRNA codons that interact directly with the anticodon loops of tRNAs bound within the A, P, and E sites of the ribosome, progress in this direction has been held back by the expectation that bulky fluorophores attached to codon nucleotides within the narrow confines of the mRNA channel would either abolish or distort mRNA activity, making such derivatives unreliable probes of normal mechanism. Recently, however, new emissive and responsive nucleoside mimetics based on thieno[3,4-d]pyrimidine, which maintain normal Watson-Crick base pairing, have been described for all four RNA nucleosides,^{19–21} that either are isosteric with the nucleoside being mimicked [thGuanosine (thG, Figure 1a) or thAdenosine] or have the effect of converting pyrimidines into isosteres of purines (thUridine and thCytosine).

Here we describe the preparation of three functional mRNAs containing thG at the 5', middle, and 3'-positions of mRNA codons that display easily measurable fluorescence changes at different stages of protein synthesis. Such changes permit monitoring of the conversion of a 70SIC first to a pretranslocation (PRE) complex and then to posttranslocation (POST) complex, at nucleotide resolution. We use such monitoring, in conjunction with other fluorescence assays, to directly identify a specific step during PRE complex formation, that follows aa-tRNA.EF-Tu.GTP ternary complex binding to the ribosome and precedes aa-tRNA accommodation into the A-site of the ribosome, and that we provisionally assign to codon:anticodon-loop base pair formation.

RESULTS AND DISCUSSION

thG containing mRNAs

Three short mRNAs containing a single thG within the second codon that follows the AUG initiation codon were prepared, and named as thG4, thG5 and thG6, respectively, according to the positions of the thG at the 5', middle, and 3'-positions (Figure 1b). The thG4 and thG6 mRNAs encode Val at the second position, whereas thG5 encodes Arg. Two additional mRNAs containing no modified nucleotides were also prepared and named as MVF, corresponding to thG4 or thG6, and MRF, corresponding to thG5.

thG4, thG5 and thG6 form functional initiation and elongation complexes

thG4, thG5 and thG6 mRNAs were tested for their activities in different stages of translation: 70S initiation complex (70SIC) formation (Figure 2a and 2b), as measured by fMet-tRNA^{fMet} co-sedimentation with a 70S ribosome; pretranslocation (PRE) complex formation (Figure 2c and 2d), as measured by Val-tRNA^{Val} (thG4, thG6) or Arg-tRNA^{Arg} (thG5) co-sedimentation with a 70SIC; and EF-G.GTP dependent translocation (Figure 2e and 2f), as measured by fMetVal-puromycin (thG4, thG6) or fMetArg-puromycin (thG5) formation. The results in Figure 2 demonstrate that substantial functionality is retained on thG substitution at positions 4, 5, or 6. The lower relative activity of thG4- and thG6-programmed ribosomes with respect to PRE complex formation, measured at a single TC concentration, is due to lower affinity and/or more labile binding of TC to 70SIC, as compared with MRF-programmed ribosomes. The decoding center forms a tight “mould” that strongly constrains the mRNA^{22–24}, so that such weakening of TC binding could result from small structural perturbations arising from both the substitution of the larger sulfur for carbon (atomic radii

1.0 Å and 0.7 Å, respectively) at purine position 8, and the loss of polar interactions resulting from carbon for nitrogen substitution at purine positions 7 and 9.

thG Fluorescence changes on functional complex formation

thG is a highly emissive ($\phi = 0.46$) and responsive fluorescent G mimetic.¹⁹ Although incorporation into oligonucleotides generally lowers the quantum yield of emissive nucleosides,²⁵ we anticipated that thG-containing oligonucleotides would retain a high level of responsiveness that would allow one to monitor mRNA binding, movement and subtle environmental changes within the ribosome during the elongation cycle. thG4, thG5 and thG6 mRNAs each have a broad excitation maximum at 340–350 nm (Supplementary Figure S1a). To minimize background fluorescence from the ribosome, 360 nm was chosen as the excitation wavelength for experiments displayed in Figures 3–5.

Each of the three mRNAs shows a small bathochromic shift on binding to the ribosome as part of a 70SIC (Supplementary Figure S1b), presumably arising from the altered environment of thG on the ribosome compared to solution. Conversion of 70SIC to PRE complex via addition of cognate aminoacyl-tRNA.EF-Tu.GTP ternary complex (aa-tRNA.EF-Tu.GTP, denoted TC) results in Watson-Crick base pairing of the thG present in the first elongator tRNA codon, so that observed changes reflect both generalized environmental effects of converting a 70SIC into the first PRE complex and the specific effects of codon:anticodon H-bonding at the A-site. No such changes are seen on addition of non-cognate TCs in place of cognate TCs (Supplementary Figure S2) to 70SICs programmed with each of the three modified mRNAs. Conversion of thG4-programmed 70SIC to PRE complex proceeds with a small hypsochromic shift and an increase in fluorescence intensity (Figure 3a,d). In contrast, neither the thG5- nor thG6-programmed 70SICs shows a significant change in the fluorescence maximum on conversion to PRE complex but both show decreases in fluorescence intensity (Figure 3b–d). Conversion of a PRE complex to a POST complex via EF-G.GTP addition proceeds with maintenance of the anticodon-loop: codon Watson-Crick base pairs. For the thG4- and thG6-programmed PRE complexes, such conversions result in fluorescence changes that either completely (thG4) or partially (thG6) reverse the changes seen on conversion of 70SIC to PRE complex (Figure 3a, c, d). In contrast, conversion of the thG5-programmed PRE complex to POST complex proceeds with virtually no fluorescence change (Figure 3b, d). We are unable to attribute the fluorescence intensity changes shown in Figure 3 to specific changes in the immediate surroundings of the modified base, since they may include contributions from multiple effects, including changes in polarity, viscosity and H bonding, as well as ground and excited state processes brought about by neighboring nucleobases. Nevertheless, they provide sensitive spectroscopic handles, allowing us to monitor changes in the microenvironments of specific codon nucleotides at the decoding center during the first cycle of polypeptide elongation.

Kinetics of PRE complex formation

We used the changes seen in Figure 3 to determine rates of PRE complex formation. Earlier work showed that 1) proflavin(prf)-labeled aa-tRNA can be used to monitor a two phase reaction of ternary complex (aa-tRNA.EF-Tu.GTP, TC) with 70SIC, initial TC binding followed by aa-tRNA accommodation into the A-site¹¹, that results in PRE complex formation; and 2) fMet-tRNA^{fMet} (prf) bound in the P-site monitors accommodation of aa-tRNA^{aa} within the ribosome in an essentially single-phase reaction.²⁶ Rates of thG fluorescence change, which in all three cases also proceed in an essentially single-phase reaction, were determined in three parallel experiments, using either i) unlabeled fMet-tRNA^{fMet} and unlabeled aa-tRNA^{aa}, ii) unlabeled fMet-tRNA^{fMet} and aa-tRNA^{aa}(prf), or iii) fMet-tRNA^{fMet}(prf) and unlabeled aa-tRNA^{aa} (Figure 4). In ii) and iii), parallel

measurements were made for the rates of proflavin fluorescence change. Rates of proflavin fluorescence change were also determined when MVF and MRF were substituted for thG4 or thG6, and thG5, respectively.

thG4- and thG6-programmed ribosomes

Below we distinguish *apparent* rate constants, which are determined by fitting the kinetic data in Figures 4a and 4c to either a double exponential [aa-tRNA^{aa}(prf) fluorescence intensity changes] or to a single exponential [both fMet-tRNA^{fMet}(prf) and thG fluorescence changes], from *microscopic* rate constants, which are determined by globally fitting all of the kinetic data obtained with a given mRNA to a posited kinetic mechanism. For both mRNAs, the measured apparent rate constants fall in the order aa-tRNA^{aa}(prf)-Phase 1 (denoted k_{1app}) $\gg$ thG (denoted k_{2app}) $>$ aa-tRNA^{aa}(prf)-Phase 2 $\sim$ fMet-tRNA^{fMet}(prf) (both denoted k_{3app}) (Supplementary Table S1). That k_{app2} values, derived from thG fluorescence changes, differ from both k_{app1} and k_{app3} values, derived from prf-labeled tRNA fluorescence changes, demonstrates that thG-labeled mRNA provides evidence for a step in PRE complex formation not previously seen with prf-labeled tRNAs. k_{2app} values for thG4 and thG6 fluorescence change were very similar to each other and in both cases were little affected by prf labeling of either fMet-tRNA^{fMet} or Val-tRNA^{Val}. Similarly, k_{1app} values were unaffected by thG substitution for unmodified G, although k_{3app} values were reduced by about one-third.

Microscopic rate constants for the two mRNAs were determined by globally fitting each set of rate measurements in Figures 4a and 4c to a simplified three-step kinetic scheme for the mixing of 70SIC and TC that results in PRE complex formation (Scheme 1, Table 1). Such fittings were carried out ignoring the quite minor changes resulting from carrying out experiments with unlabeled vs. prf-labeled forms of fMet-tRNA^{fMet} or Val-tRNA^{Val}. Interpreted in the light of earlier results,^{26–27} steps 1 and 3 should correspond, respectively, to an initial binding of TC to 70SIC that doesn't involve codon-anticodon base pairing, and to accommodation of aa-tRNA^{aa} into the A-site that is followed by peptide bond formation and EF-Tu.GDP release from the ribosome. The thG fluorescence change provides a new probe for the intervening step 2, that we presume corresponds to the base pairing step between the codon and the aa-tRNA anticodon loop. Importantly, the rate constant values k_1 – k_3 obtained with thG4- and thG6-programmed ribosomes are virtually identical to one another.

thG5-programmed ribosomes

Results with thG5-programmed ribosomes (Figure 4b) differed from those discussed above in showing an apparent interaction between Arg-tRNA^{Arg}(prf) and thG5 that slowed PRE complex formation, as manifested by $\sim$ 2- fold increased values of k_{1app} when MRF replaces thG5 and of k_{2app} and k_{3app} when Arg-tRNA^{Arg} replaces Arg-tRNA^{Arg}(prf) (Supplementary Table S1). As a result, we separately fit results obtained with thG5-programmed ribosomes to Scheme 1 according to whether they were obtained with Arg-tRNA^{Arg}(prf) or unlabeled Arg-tRNA^{Arg} (Table 1).

The values obtained lead to two observations concerning thG5-programmed ribosomes. First, that k_2 and k_3 using unlabeled Arg-tRNA^{Arg} are quite similar to the values found for thG4- and thG6-programmed ribosomes, using either prf-labeled or unlabeled Val-tRNA^{Val}. It is worth emphasizing that the similarity in the values of k_2 for all three thG-labeled mRNAs supports the notion that the changes in thG fluorescence intensity seen in Fig. 4 are monitoring a common event, the formation of anticodon-codon base pairs. The second observation is that labeling Arg-tRNA^{Arg} with proflavin has major ($\sim$ 3–4-fold) and minor (1.6-fold) inhibitory effects on k_2 and k_3 , respectively. We speculate that these rate

effects might result from small perturbations in tRNA binding to the ribosome resulting from proflavin substitution in the D-loop of aa-tRNA that are propagated to the anticodon loop. That such effects are only seen for thG in the middle position of the codon triplet is consistent with the observation that misreading by the ribosome occurs least frequently at the middle codon nucleotide,²⁸ and the tight structural constraints to which codon-anticodon base pairing at the middle position is subject within the ribosomal decoding center.^{22–24}

Kinetics of POST complex formation

The results in Figure 3a and 3c demonstrate that thG fluorescence change can be used to monitor conversion of PRE to POST complex. Previous work demonstrated that fluorescence changes resulting from rapid mixing of PRE complexes containing fMet-aa-tRNA^{aa}(prf) with EF-G. GTP measures the rate of translocation of fMet-aa-tRNA^{aa}(prf) from the A- to the P-site of the ribosome.^{13–14} When PRE complexes containing thG4-labeled mRNA and fMet-Val-tRNA^{Val}(prf) are rapidly mixed with EF-G.GTP, the changes in both thG and proflavin fluorescence intensity are well fit by a single k_{app} value ($4.7 \pm 0.3 \text{ s}^{-1}$, Figure 5a), indicating that both fluorophores are monitoring the same process. Similar results are obtained for the PRE complex containing thG6-labeled mRNA, with a slightly lower k_{app} value ($3.4 \pm 0.2 \text{ s}^{-1}$, Figure 5b). The PRE complex programmed with thG5 had a rate constant of $6.2 \pm 1.6 \text{ s}^{-1}$ for fMet-Arg-tRNA^{Arg}(prf) fluorescence change, but showed, as expected from the results presented in Figure 3b, almost no change in thG fluorescence. These results demonstrate that thG-programmed ribosomes are fully functional in the translocation step that converts PRE complex to POST complex, and that in some cases this step can be monitored by changes in thG fluorescence.

SUMMARY

Herein we report the preparation of functional modified mRNAs containing thG, an emissive guanosine mimetic, and their application in elucidating the dynamics of mRNA:tRNA pairs during the decoding and translocation steps of the elongation cycle of active ribosomes. Our results indicate that a thG present at the 5', middle, or 3'-positions of an mRNA codon is an effective probe codon:anticodon interaction at the ribosomal decoding center as well as for mRNA translocation within the ribosome. Such nucleoside analogues could also serve as direct reporters of various changes during translation, including mRNA:tRNA interaction during frame shifting events, or of the melting (e. g., of a downstream pseudo-knot) or transient formation (e. g., of an internal Shine-Dalgarno sequence with the 3'-end of 16S rRNA) of secondary structure.

METHODS

Materials

mRNAs were prepared using standard solid-phase phosphoramidite synthesis on an Expedite 8909 synthesizer on a 500 Å CPG (1 μmol scale) column. Commercially available reagents and phosphoramidites (Glen Research), and a thG-phosphoramidite, prepared as described,¹⁹ were employed. Cleavage from the solid support and deprotection were accomplished with 50:50 mixture of MeNH₂ in water (40 wt. %) and in ethanol (33 wt. %) at 35 °C for 6 hours. The 2'-TOM group was removed by TEA·3HF at 65 °C for 3 hours and the residue was desalted by precipitation (Glen Report 19–20, Glen Research). All oligonucleotides were purified by preparative polyacrylamide gel electrophoresis (PAGE) using the crush and soak method; the desired band was cut out, pulverized, extracted with 50 mM TEAA (pH 7.0) for a minimum of 12 hours (while shaking) and decanted. The buffer containing the purified oligonucleotide was lyophilized and the residue was taken up in 0.2 M TEAB (pH 7.0) buffer and desalted on a Sep-pak C-18 (Waters). The oligonucleotides

were eluted with 40 % acetonitrile in water. All oligonucleotides were >98% pure as determined by analytical high resolution PAGE (Supplementary Figure 3). The structures were confirmed by MALDI-TOF mass spectrometry (thG4, calculated M 9576.6, found 9600.2 [M+Na-H]⁺; thG5, calculated M 9536.6, found 9577.0 [M+K-H]⁺; thG6, calculated M 9576.6, found 9603.5 [M+Na-H]⁺). Purified oligonucleotides were quantified by UV absorbance at 260 nm at 70 °C.

GTP and puromycin were obtained from Sigma. *Escherichia. coli* tRNA^{fMet}, tRNA^{Val} and tRNA^{Arg} were obtained from Chemical Block (Moscow). Previously reported procedures were used to prepare tight-coupled 70S ribosomes from *E.coli* MRE600 cells²⁶ and cloned *E.coli* His-tagged proteins IF1, IF2, IF3 and EF-G,²⁶ fMet-tRNA^{fMet}, Val-tRNA^{Val}, and Arg-tRNA^{Arg}.¹³ Proflavin (prf) labeled tRNAs were prepared similarly to described procedures¹³ except that tRNAs, reduced with NaBH₄, were aminoacylated (and, in the case of fMet-tRNA^{fMet}, formylated) and purified on FPLC (MonoQ) before being labeled with prf.⁸

EF-Tu was prepared in a manner similar to that described²⁹ using C-terminal His-tagged *E. coli* wild type EF-Tu cloned into pET15b as an expression vector. Transformed BL21 cells were grown in LB medium at 37 °C and harvested 5 h after induction of protein expression by IPTG (1 mM). Cells (6 g) were resuspended in buffer (50 mM Tris-HCl, pH 7.6, 60 mM NH₄Cl, 7 mM MgCl₂, 7 mM 2-mercaptoethanol, 15 μM GDP, 15% glycerol) and were broken by French press. EF-Tu was purified on a Co(II)-Sephacrose (Talon) column (bed volume of 3 mL) and stored in frozen aliquots (15 mg mL⁻¹) in buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM MgCl₂, 15 μM GDP).

Complex preparation and quantification

All complexes were prepared in buffer A (50 mM Tris-HCl [pH7.5], 70 mM NH₄Cl, 30 mM KCl, 7 mM MgCl₂ and 1 mM DTT) at 37 °C.

70SIC was prepared by incubating 70S ribosomes (1–4 μM) with mRNA (half of 70S concentration for experiments presented in Figure 2; 2-fold excess over 70S for all other experiments), IF1–3 (each 1.5-fold excess over 70S), GTP (1 mM) and [³⁵S]- fMet-tRNA^{fMet} (1.5-fold excess over 70S) for 25 min. Purified 70SIC was prepared by a subsequent step of ultracentrifugation through a 1.1 M sucrose cushion (SORVALL S120-AT2 rotor, 110K rpm, 40 min, 4 °C) in buffer A. Purified 70SIC was quantified (Figure 2a and 2b) as co-sedimenting [³⁵S]-fMet-tRNA^{fMet} per mRNA, where mRNA stoichiometry was assumed to be half of 70S. All experiments reported in this paper used purified 70SIC except for those shown in Figure 2c and 2d, for which the ultracentrifugation step was omitted.

TC was formed by incubating [³H]-labeled aminoacyl-tRNA (0.8–8 μM) with EF-Tu (4-fold ratio to aa-tRNA), GTP (1 mM), phosphoenolpyruvate (1.5 mM) and pyruvate kinase (0.5 mg mL⁻¹) for 10 min.

PRE complex was formed by incubating 70SIC (0.5–2 μM, as measured by A₂₆₀ of 70S) with TC (2-fold ratio to 70S) for 1 min. Purified PRE complex was prepared by ultracentrifugation through a 1.1 M sucrose cushion in buffer B (buffer A with 20 mM MgCl₂), performed as above, and quantified (Figure 2c and 2d) as co-sedimenting [³H]-Val (thG4- or thG6-programmed ribosomes) or [³H]-Arg (thG5-programmed ribosomes). All experiments reported in this paper used purified PRE complexes, except for those shown in Figure 2e, for which the ultracentrifugation step was omitted.

POST complex was prepared and quantified (Figure 2e and 2f) using two different procedures. PRE complexes programmed with thG4, thG6, or MVF (0.8 μM, as measured by A₂₆₀ of 70S) were incubated with EF-G.GTP (2 μM) and puromycin (2.5 mM) at 37 °C for 30 s, the reaction was quenched with 1 volumes of 0.3 M NaOAc, pH 5.0, fMet-[³H]-Val-puromycin was quantitatively extracted with ethyl acetate and the radioactivity in the extract was determined. A different procedure was required for PRE complexes programmed with thG5 or MRF because of the insolubility of fMet-[³H]-Arg-puromycin in ethyl acetate. Here PRE complex (5 μM, as measured by A₂₆₀ of 70S) was incubated with EF-G.GTP (10 μM) and puromycin (15 μM) at 20 °C for 30 s, and the reaction was quenched by adding 5 volumes of Buffer A adjusted to pH 5. These conditions were sufficient for full puromycin reaction with translocated fMet-[³H]-Arg-tRNA^{Arg}, resulting in fMetArg-puromycin formation. The resulting mixture was subject to ultracentrifugation through a 1.1 M sucrose cushion in Buffer B, performed as above. fMet-Arg-puromycin remained in the supernatant during centrifugation. Unreactive fMet-Arg-tRNA^{Arg} co-sedimented with the ribosome. Radioactivity in the pellet was determined.

The relative amount (RA) of EF-G- and mRNA-dependent POST complex formation was obtained by correcting the results obtained for differences in the efficiencies of formation of the 70SIC (Figure 2e) and PRE (Figure 2e and 2f) complexes and normalizing to the value found for unmodified mRNA. This was accomplished by measuring the amounts of [³H]-Val or [³H]-Arg found in either the ethyl acetate extract (thG4, thG6, or MVF) or sucrose cushion pellet (thG5 or MRF) in four different samples formed in the presence (a) or absence (c) of EF-G in the presence of mRNA, or in the presence (b) or absence (d) of EF-G, in the absence of mRNA, and calculating the efficiencies using eqs 1a (thG4, thG6, or MVF) or 1b (thG5 or MRF).

$$RA(^{th}G4, ^{th}G6) = 1 - [(c-d)/(a-b)] \text{ (1a)}; RA(^{th}G5) = 1 - [(a-b)/(c-d)] \text{ (1b)}$$

Steady-state fluorescence

Steady-state fluorescence was measured on a Fluorolog-3 spectrofluorometer (Horiba Jobin Yvon). Emission and excitation spectra were determined using excitation at 360 nm and monitoring emission at 450 nm, respectively.

Kinetic experiments

Kinetic experiments were performed in buffer A at 25 °C. Stopped-flow experiments were carried out on KinTek SF-300X stopped-flow spectrofluorometer. The excitation wavelengths and filters used are as followings: 360 nm and 450 ± 10-nm band-pass filter for thG; 462 nm and 515 ± 15-nm band-pass filter for proflavin. In each independent kinetic experiment, traces of fluorescence intensity changes were obtained as an average of at least 4 shots. Each experiment was performed twice. Apparent rate constants (*k*_{app}s) were obtained by either single (equation 2) or double (equation 3) exponential fitting of each independent experiment using Origin (OriginLab), respectively. Global fittings of data sets to Scheme 1 were carried out using Scientist (MicroMath Research), resulting in coefficients of determination of 0.98, 0.97, 0.97 and 0.85 for thG4, thG6, thG5 [with tRNA^{Arg}(prf)], and thG5 [with tRNA^{fMet}(prf)], respectively.

$$F = F_0 + F_1 e^{-k'_{1app} t} \quad (2)$$

$$F = F_0 + F_1 e^{-k_{1app} t} + F_2 e^{-k_{2app} t} \quad (3)$$

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

1. Schmeing TM, Voorhees RM, Kelley AC, Gao Y, Murphy FV, Weir JR, Ramakrishnan V. The crystal structure of the ribosome bound to EF-Tu and aminoacyl-tRNA. *Science*. 2009; 326:688–694. [PubMed: 19833920]
2. Wilson DN, Doudna Cate JH. The structure and function of the eukaryotic ribosome. *Cold Spring Harb. Perspect. Biol.* 2012; 4:a011536. [PubMed: 22550233]
3. Dever TE, Green R. The elongation, termination, and recycling phases of translation in eukaryotes. *Cold Spring Harb. Perspect. Biol.* 2012; 4:a013706. [PubMed: 22751155]
4. Frank J Jr, Gonzalez RL. Structure and dynamics of a processive Brownian motor: the translating ribosome. *Annu. Rev. Biochem.* 2010; 79:381–412. [PubMed: 20235828]
5. Wohlgenuth I, Pohl C, Mittelstaet J, Konevega AL, Rodnina MV. Evolutionary optimization of speed and accuracy of decoding on the ribosome. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 2011; 366:2979–2986. [PubMed: 21930591]
6. Rodnina MV, Wintermeyer W. The ribosome as a molecular machine: the mechanism of tRNA-mRNA movement in translocation. *Biochem. Soc. Trans.* 2011; 39:658–662. [PubMed: 21428957]
7. Sanbonmatsu KY. Computational studies of molecular machines: the ribosome. *Curr. Opin. Struct. Biol.* 2012; 22:168–174. [PubMed: 22336622]
8. Pan D, Qin H, Cooperman BS. Synthesis and functional activity of tRNAs labeled with fluorescent hydrazides in the D-loop. *RNA*. 2009; 15:346–354. [PubMed: 19118261]
9. Chen C, Stevens B, Kaur J, Cabral D, Liu H, Wang Y, Zhang H, Rosenblum G, Smilansky Z, Goldman YE, Cooperman BS. Single-molecule fluorescence measurements of ribosomal translocation dynamics. *Mol. Cell*. 2011; 42:367–377. [PubMed: 21549313]
10. Cooperman, BS.; Goldman, YE.; Chen, C.; Farrell, I.; Kaur, J.; Liu, H.; Liu, W.; Rosenblum, G.; Smilansky, Z.; Stevens, B.; Zhang, H. Mechanism and dynamics of the elongation cycle. In: Rodnina, MV., et al., editors. *Ribosomes: structure, function and dynamics*. New York: SpringerWien; 2011. p. 339-348.
11. Pape T, Wintermeyer W, Rodnina MV. Complete kinetic mechanism of elongation factor Tu-dependent binding of aminoacyl-tRNA to the A site of the *E. coli* ribosome. *EMBO J.* 1998; 17:7490–7497. [PubMed: 9857203]
12. Geggier P, Dave R, Feldman MB, Terry DS, Altman RB, Munro JB, Blanchard SC. Conformational sampling of aminoacyl-tRNA during selection on the bacterial ribosome. *J. Mol. Biol.* 2010; 399:576–595. [PubMed: 20434456]
13. Pan D, Kirillov SV, Cooperman BS. Kinetically competent intermediates in the translocation step of protein synthesis. *Mol. Cell*. 2007; 25:519–525. [PubMed: 17317625]
14. Savelsbergh A, Katunin VI, Mohr D, Peske F, Rodnina MV, Wintermeyer W. An elongation factor G-induced ribosome rearrangement precedes tRNA-mRNA translocation. *Mol. Cell*. 2003; 11:1517–1523. [PubMed: 12820965]
15. Munro JB, Wasserman MR, Altman RB, Wang L, Blanchard SC. Correlated conformational events in EF-G and the ribosome regulate translocation. *Nat. Struct. Mol. Biol.* 2010; 17:1470–1477. [PubMed: 21057527]
16. Cunha CE, Belardinelli R, Peske F, Holtkamp W, Wintermeyer W, Rodnina MV. Dual use of GTP hydrolysis by elongation factor G on the ribosome. *Translation*. 2013; 1:e24315.
17. Studer SM, Feinberg JS, Joseph S. Rapid kinetic analysis of EF-G-dependent mRNA translocation in the ribosome. *J. Mol. Biol.* 2003; 327:369–381. [PubMed: 12628244]

18. Liu H, Pan D, Pech M, Cooperman BS. Interrupted catalysis: the EF4 (LepA) effect on back-translocation. *J. Mol. Bio.* 2010; 396:1043–1052. [PubMed: 20045415]
19. Shin D, Sinkeldam RW, Tor Y. Emissive RNA alphabet. *J. Am. Chem. Soc.* 2011; 133:14912–14915. [PubMed: 21866967]
20. Samanta PK, Manna AK, Pati SK. Thieno analogues of RNA nucleosides: a detailed theoretical study. *J. Phys. Chem. B.* 2012; 116:7618–7626. [PubMed: 22671305]
21. Gedik M, Brown A. Computational study of the excited state properties of modified RNA nucleobases. *J. PhotoChem. Photobiol. A.* 2013; 259:25–32.
22. Ogle JM, Brodersen DE, Clemons WM Jr, Tarry MJ, Carter AP, Ramakrishnan V. Recognition of cognate transfer RNA by the 30S ribosomal subunit. *Science.* 2001; 292:897–902. [PubMed: 11340196]
23. Demeshkina N, Jenner L, Westhof E, Yusupov M, Yusupova G. A new understanding of the decoding principle on the ribosome. *Nature.* 2012; 484:256–259. [PubMed: 22437501]
24. Demeshkina N, Jenner L, Westhof E, Yusupov M, Yusupova G. New structural insights into the decoding mechanism: Translation infidelity via a G-U pair with Watson-Crick geometry. *FEBS Lett.* 2013 Epub May 23 2013.
25. Sinkeldam RW, Greco NJ, Tor Y. Fluorescent analogs of biomolecular building blocks: design, properties and applications. *Chem. Rev.* 2010; 110:2579–2619. [PubMed: 20205430]
26. Pan D, Zhang C, Kirillov S, Hou Y, Cooperman BS. Perturbation of the tRNA tertiary core differentially affects specific steps of the elongation cycle. *J. Biol. Chem.* 2008; 283:18431–18440. [PubMed: 18448426]
27. Gromadski KB, Rodnina MV. Kinetic determinants of high-fidelity tRNA discrimination on the ribosome. *Mol. Cell.* 2004; 13:191–200. [PubMed: 14759365]
28. Szaflarski W, Vesper O, Teraoka Y, Plitta B, Wilson DN, Nierhaus KH. New features of the ribosome and ribosomal inhibitors: non-enzymatic recycling, misreading and back-translocation. *J. Mol. Biol.* 2008; 380:193–205. [PubMed: 18508080]
29. Perla-Kajan J, Lin X, Cooperman BS, Goldman E, Jakubowski H, Knudsen CR, Mandeck W. Properties of *Escherichia Coli* EF-Tu mutants designed for fluorescence resonance energy transfer from tRNA molecules. *Protein Eng. Des. Sel.* 2010; 23:129–136. [PubMed: 20083494]

a **b**

thG4 : **+4**
 UUA AUA AGG AUA CAU ACU AUG **G**UG UUC CGU
 fMet Val Phe Arg

thG5 : **+5**
 UUA AUA AGG AUA CAU ACU AUG **C**GU UUC CGU
 fMet Arg Phe Arg

thG6 : **+6**
 UUA AUA AGG AUA CAU ACU AUG GUG **G** UUC CGU
 fMet Val Phe Arg

Figure 1.
 mRNAs labeled with isomorphous fluorescent guanosine mimetics. (a) thG structure. (b)
 Modified mRNA sequences, with thG positions shown in red.

Figure 2.

Activities of mRNAs containing thG. Formation of various complexes with ribosomes programmed with thG4 or thG6 (a, c, e) or thG5 (b, d, f). (a) and (b) 70SIC formation, measured as fMet-tRNA^{fMet} co-sedimented per mRNA added: ribosomes, 1.0 μ M, mRNA 0.5 μ M. (c) and (d) Normalized PRE complex formation, with background in the absence of mRNA subtracted: ribosomes, 0.8 μ M. (e) and (f) Normalized POST complex formation. thG4, thG6: ribosomes, 2 μ M. thG5: ribosomes 4.8 ± 0.2 μ M. Control experiments were performed, as indicated, with either no added mRNA, or with added unmodified mRNA, or with no added EF-G. Values reported in (c) and (d) to the corresponding unmodified mRNAs (MVF for G4 and G6; MRF for G5) after subtraction of background

obtained with no mRNA and normalized to the value obtained for unmodified mRNA. Values reported in (e) and (f) were obtained from eqs. 1a and 1b and normalized to the value obtained for unmodified mRNA.

Figure 3.

Fluorescence changes of thG-containing mRNAs. (a)–(c) Traces are shown for 70SIC (black); PRE complex (red); POST complex (green). 70S IC traces are for purified samples. PRE complex traces shown are the average of two independently determined traces: i) PRE complex prepared *in situ* by addition of TC to 70SIC; ii) purified PRE complex used in the preparation of POST complex. To account for small concentration differences arising from the two preparation methods, the traces for purified PRE complexes were normalized to those for *in situ* generated PRE complexes, using an 8 nm window covering the fluorescence maximum to generate a normalization factor. The resulting two very similar traces were then averaged. POST complexes were prepared *in situ* from purified PRE complexes by incubation with EF-G.GTP (2.5 μM for 20 s), and traces were normalized using the same normalization factors as above. For all traces and prior to any normalization, background fluorescence of the corresponding complex made with unlabeled mRNA was subtracted from the observed fluorescence of each of the labeled ribosome complexes. Intensities are normalized to the peak intensity value of 70SIC for each mRNA. (d) Fluorescence intensity changes were obtained by subtracting the spectrum of a purified complex (70SIC or PRE) from the spectrum of a complex prepared *in situ* (PRE or POST, respectively) and then normalizing the intensity differences to the peak intensity value of purified complex (70SIC or PRE, respectively).

Figure 4.

Kinetics of PRE complex formation. Fluorescence changes on rapid mixing of 70SICs (0.1 μM) programmed with $^{\text{th}}\text{G}$ -containing mRNAs and cognate TCs (0.4 μM) compared with fluorescence changes of aa-tRNA(prf) (dark blue), present as part of the TC, or of fMet-tRNA^{fMet}(prf) (cyan), present as part of the 70SIC. Rates of $^{\text{th}}\text{G}$ fluorescence change were determined using either unlabeled fMet-tRNA^{fMet} and unlabeled aa-tRNA^{aa} (green), unlabeled fMet-tRNA^{fMet} and aa-tRNA^{aa}(prf) (red), or fMet-tRNA^{fMet}(prf) and unlabeled aa-tRNA^{aa} (magenta). (a) $^{\text{th}}\text{G4}$; (b) $^{\text{th}}\text{G5}$; (c) $^{\text{th}}\text{G6}$. Traces shown are the average of two independently determined traces, which, in each case were quite similar to one another. Solid black lines are global fittings to Scheme 1 values, using rate constant values summarized in Table 1. The red curve in part b was fit with the following values (in s^{-1}): k_1 65 ± 4 , k_2 6.6 ± 0.2 , k_3 9.9 ± 0.4 .

Figure 5.

Kinetics of POST complex formation. Fluorescence changes on rapid mixing of PRE complexes (0.1 μ M) programmed with ^{th}G -containing mRNAs (black) and EF-G.GTP (2 μ M) compared with fluorescence changes of fMetVal-tRNA^{Val}(prf) (red), present as part of the PRE complex. (a) $^{th}G4$; (b) $^{th}G6$. Solid black lines are single exponential fits of both the ^{th}G and proflavin fluorescence intensity changes, with k_{app} of 4.7 ± 0.3 and 3.4 ± 0.2 sec^{-1} for $^{th}G4$ and $^{th}G6$, respectively. Average deviations are given for the two independent experiments.

Scheme 1.

Table 1Microscopic rate constants (s^{-1}) for 70SIC reaction with TC^a

mRNA	k_1	k_2	k_3
th G4	82 ± 9	7.2 ± 0.2	15.2 ± 0.7
th G5 ^b	45 ± 10 (45–85) ^c	1.8 ± 0.2 7.5 – 6.0	6.1 ± 1.1 9.9 ± 0.4
th G6	80 ± 11	8.4 ± 0.4	13.7 ± 1.3

^aValues presented are for results in Fig. 4 that are fit to Scheme 1 (see text), using data obtained at a single TC concentration of 0.4 μ M).Fluorescence changes in steps 1, 2, and 3, are, respectively: for thG4, unchanged, increased, unchanged; for thG5 and thG6, unchanged, decreased, unchanged; for aa-tRNA^{aa}(prf), increased, unchanged, decreased; for fMet-tRNA^{fMet}, unchanged, unchanged, decreased.^bUpper values: Fit using fluorescence changes of Arg-tRNA^{Arg}(prf) and thG5 mRNA. Lower values: Fit using results obtained both with thG5 mRNA as the only fluorescent component and with thG5 mRNA and fMet-tRNA^{fMet}(prf) present together. In this fit k_1 was not well-determined, but the variance increased markedly outside of the limits indicated. Values for k_2 and k_3 were, respectively, weakly dependent and independent of the value chosen for k_1 .