Mutations in RNAs of both ribosomal subunits cause defects in translation termination

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Mutations in RNAs of both subunits of the *Escherichia coli* ribosome caused defects in catalysis of peptidyl-tRNA hydrolysis in a realistic *in vitro* termination system. Assaying the two codon-dependent cytoplasmic proteins that drive termination, RF1 and RF2, we observed large defects with RF2 but not with RF1, a result consistent with the *in vivo* properties of the mutants. Our study presents the first direct *in vitro* evidence demonstrating the involvement of RNAs from both the large and the small ribosomal subunits in catalysis of peptidyl-tRNA hydrolysis during termination of protein biosynthesis. The results and conclusions are of general significance since the rRNA nucleotides studied have been virtually universally conserved throughout evolution. Our findings suggest a novel role for rRNAs of both subunits as molecular transmitters of a signal for termination.

Keywords: *in vitro* termination/mutant rRNAs/protein synthesis/RF2 defect

Introduction

RNAs from both large and small subunits of the ribosome have been implicated in various steps of translation (reviewed in Dahlberg, 1989; Noller, 1991; Noller *et al.*, 1996), supporting the idea that the rRNAs function directly in protein biosynthesis (Woese, 1980; Noller and Woese, 1981). One of the strongest supports for this idea was the observation that *Thermus aquaticus* ribosomes, depleted of 95% of their ribosomal protein component, were able to catalyze peptide bond formation efficiently (Noller *et al.*, 1992).

The involvement of rRNA in the last step of translation, peptide chain termination, was suspected on the basis of *in vivo* studies (reviewed in Murgola, 1996). In *Escherichia coli*, the C1054A mutation in helix 34 of 16S rRNA, the RNA of the small ribosomal subunit (Figure 1A), isolated as a suppressor of nonsense mutations (Murgola *et al.*, 1988), was found under certain conditions to cause read-through specifically of UGA, one of the three stop codons (Murgola *et al.*, 1988; Pagel *et al.*, 1997). Similarly, the G1093A mutation, in the 23S rRNA of the *E.coli* large ribosomal subunit, was isolated and characterized as a

nonsense suppressor causing UGA-specific readthrough (Jemiolo *et al.*, 1995). Nucleotide 1093 is situated in the GTPase center, a region involved in elongation factor G-dependent hydrolysis of GTP (Thompson, 1996; see Figure 1B). Although the mutations caused UGA read-through at a certain position of the *trpA* reporter gene, they did not exhibit mistranslation of UGG at the same position. Therefore, it was suggested that both mutants were defective in some step of peptide chain termination rather than just in general accuracy during translation (Murgola *et al.*, 1988; Jemiolo *et al.*, 1995; Xu and Murgola, 1996; Pagel *et al.*, 1997).

Two *in vitro* studies have implicated *E.coli* 16S rRNA as being directly involved in peptide chain termination (Caskey *et al.*, 1977; Brown *et al.*, 1993), but both utilized in many aspects a non-natural termination assay (Caskey *et al.*, 1968). The assay employed a very high, non-physiological concentration of Mg²⁺, 30 mM, and two trinucleotides instead of a continuous mRNA. Both studies suggested the involvement of 16S rRNA in release factor (RF) binding (Caskey *et al.*, 1977; Brown *et al.*, 1993). In *E.coli* there are two RFs, RF1 and RF2, that trigger the hydrolysis of peptidyl-tRNA during termination. RF1 works at UAA and UAG stop codons, and RF2 functions at UAA and UGA stop codons (reviewed in Nakamura *et al.*, 1996; Tate and Mannering, 1996; Tate *et al.*, 1996a,b). No clear evidence was obtained, however, implicating 16S rRNA in catalysis of peptidyl-tRNA hydrolysis rather than in factor binding.

No direct evidence for the involvement of the 23S rRNA (large subunit) in termination of protein synthesis, either in catalysis or factor binding, has been presented to date. Nevertheless, the possible involvement of this rRNA in termination has been proposed (Tate and Brown, 1992). Indeed, there is indirect evidence that peptidyl transferase may be involved in termination (Vogel *et al.*, 1969; Caskey *et al.*, 1971; Caskey and Beaudet, 1972), and 23S rRNA has been shown to be the central element of peptidyl transferase (Noller *et al.*, 1992; Garrett and Rodriguez-Fonseca, 1996). Therefore, it is conceivable that 23S rRNA is important for termination. However, the identification of the RNA component of peptidyl transferase has been limited so far to domains IV and V in the secondary structure of 23S rRNA and those domains are quite distant from domain II, in which is located the site of the G1093A mutation (Figure 1B). Therefore, it was of great importance to analyze that mutation in an *in vitro* termination assay, since, if it did cause a defect in termination, it would not only provide the first direct demonstration of the involvement of rRNA from the large subunit of the ribosome in termination but would also identify domain II as an entirely new contributor to the termination process.

In this study, we present the first direct *in vitro* evidence

Fig. 1. Nature and location of the mutations, in rRNAs from ribosomal subunits, analyzed in this study. (A) Top: the secondary structure of 16S rRNA (small subunit) with helix 34 indicated in bold. Bottom: detail of helix 34, with the C1054A change indicated (arrow). Nucleotide 1192 is encircled. (B) Top: the secondary structure of 23S rRNA (large subunit) with the GTPase center indicated in bold. The general location of the RNA component of peptidyl transferase in domains IV and V of the rRNA (Garrett and Rodriguez-Fonseca, 1996) is indicated (PT). Bottom: detail of the GTPase center with the G1093A change indicated (arrow).

for the involvement of *E. coli* rRNAs from both subunits in catalysis of peptidyl-tRNA hydrolysis during peptide chain termination. In particular, mutations C1054A in helix 34 of 16S rRNA (Figure 1A) and G1093A in the GTPase center of 23S rRNA (Figure 1B) caused a defect in that catalysis in the presence of RF2. Moreover, the evidence was obtained using a new realistic *in vitro* assay of termination, one that more closely reproduces the conditions *in vivo*. Finally, our results were consistent with the results previously obtained with the mutants *in vivo*.

Results

Hydrolysis of peptidyl-tRNA determined for ribosomes isolated from mutant strains

The conditions used in the 'classical' *in vitro* termination assay (Caskey *et al.*, 1968) are far from physiological. Apart from being dependent on a high concentration of Mg^{2+} , the assay uses two trinucleotides, AUG and a stop codon trinucleotide, instead of mRNA. The termination event is monitored by measuring the rate of hydrolysis of the initiator fMet-tRNA^{fMet}, which normally does not serve as a substrate for peptide chain termination *in vivo*. Finally, simply changing the concentration of the trinucleotides or cation was sufficient to produce conflicting results (Goldstein and Caskey, 1970).

To determine if the rRNA mutations, C1054A in 16S rRNA (Figure 1A) and G1093A in 23S rRNA (Figure 1B), caused a defect in termination *in vitro*, purified ribosomes from the mutant *E. coli* strains were tested for catalytic efficiency of peptidyl-tRNA hydrolysis in a recently developed realistic assay (Figure 2; Freistroffer *et al.*, 1997). The new assay measures the rate of hydrolysis of ribosome-bound tetrapeptidyl-tRNA (release complex)

that has been pre-formed in a fully purified *in vitro* translation system. Synthesis of the tetrapeptidyl-tRNA is initiated from a model mRNA at AUG in the presence of initiation factors (Figure 2). After several cycles of elongation, the translating ribosome, with the peptidyl-tRNA in the P-site, encounters a stop codon, UAA, UAG or UGA, in the decoding site (A-site). Since the translation is run without RFs, the ribosomes stall at the stop codons. The release complex is purified by gel filtration and the catalytic rate of peptidyl-tRNA hydrolysis is measured after the addition of saturating amounts of RFs (Figure 2). The *in vitro* translation as well as the termination assay are carried out in the buffer system referred to as polymix, which has been optimized for fast and accurate translation (Jelenc and Kurland, 1979). The termination assay used in our study was an attempt to approximate the *in vivo* conditions for termination as closely as possible.

Since the time course experiments were carried out in the presence of saturating amounts of RF1 and RF2, they measured the catalytic rate of the hydrolysis rather than binding of RFs to ribosomes (see legend to Figure 3). Our results show, therefore, that both mutants were defective in the catalysis of peptidyl-tRNA hydrolysis in the presence of RF2 (Figure 3C and D). Statistical analysis of the data from experiments with RF2 (Figure 3C and D) showed that values obtained for mutant ribosomes were significantly different from those for wild-type ribosomes. Even though statistical analysis for almost all experiments with RF1 indicated that the mutant ribosomes were significantly slower than the wild-type ribosomes in catalysis, values for mutant ribosomes were close to those for wild-type ribosomes (Figure 3A and B). It should be mentioned that it is possible that either or both mutants, in addition to their defect in RF2 catalysis, are also defective in RF

Fig. 2. Diagram of the essential features of the realistic *in vitro* termination system used in this study. The system has been described (Freistroffer *et al.*, 1997) and was used here with slight modifications. Purified ribosomes were engaged in translation of a model mRNA coding for the tetrapeptide (fMet-Phe-Thr-Ile) in a fully purified *in vitro* translation system. The mRNA was transcribed *in vitro* with T7 RNA polymerase and purified as described (Freistroffer *et al.*, 1997). The sequence of the mRNA has been reported (Freistroffer *et al.*, 1997). The mRNA had natural features such as a Shine-Dalgarno sequence upstream of the open reading frame that started with AUG and ended with one of the three stop codons, UAA, UAG or UGA. Translational initiation was carried out in the presence of pure initiation factors and fMet-tRNA^{fMet} as described in Materials and methods. Peptide elongation took place after elongation factors (EF-G, EF-Ts and EF-Tu), together with Phe-, Thr- and Ile-tRNA synthetases, tRNAs and the amino acids Phe, Thr and [¹⁴C]-Ile, were added to the initiation reaction mixture. The ribosomes with [¹⁴C]-labeled tetrapeptidyl-tRNA stalled at the stop codons of the mRNAs (release complexes) were purified on a gel filtration column. Release complexes were incubated with release factors, and at each time point the reaction was stopped with cold 5% TCA. The samples were filtered through glass fiber filters and the amount of unhydrolyzed tetrapeptidyl-tRNA retained on the filters was determined by a liquid scintillation counter.

binding. However, the experiments reported here did not measure binding.

Estimation of defects in peptidyl-tRNA hydrolysis of a pure population of mutant ribosomes

Since *E. coli* has seven chromosomal copies of the wild-type rRNA operon, it is impossible to isolate a pure population of mutant ribosomes. However, using the mixed population of the ribosomes (mutant + wild-type), we have demonstrated that the rRNA mutations analyzed in this study caused a defect in catalysis of peptidyl-tRNA hydrolysis in the presence of RF2 (see previous section).

Here we attempted to model the kinetic behavior of a pure population of mutant ribosomes (Figure 4 and Table I). First, the contribution of wild-type ribosomes in each experiment with ribosomes from a mutant bacterial strain, as presented in Figure 3, was estimated by multiplying the experimental values for the ribosomes obtained from the wild-type strain by the fraction of wild-type ribosomes contained in the mixed populations of ribosomes from the mutant strains (see legend to Figure 4). Then, the contribution of the wild-type ribosomes, calculated for each mutant, was subtracted from the values for mutants presented in Figure 3. Figure 4 shows the semi-logarithmic plots of calculated values for mutants that estimate the contribution of just the mutant ribosomes in peptidyl-tRNA hydrolysis. Table I shows the estimated k_{cat} values for peptidyl-tRNA hydrolysis calculated from the data presented in Figure 4. Based on the estimated k_{cat} values for the RF2-dependent reactions, the C1054A mutant ribosomes were three times slower at UAA and UGA than the wild-type ribosomes (Table I). On the other hand, the G1093A mutant ribosomes were five and 17 times slower in the presence of UAA and UGA, respectively, in the RF2-dependent reactions. For the RF1-dependent hydrolysis, estimated k_{cat} values for the mutant ribosomes were close to those for the wild-type ribosomes (Table I).

Discussion

Here we present the first direct *in vitro* evidence for the involvement of *E. coli* rRNAs from both subunits in catalysis of peptidyl-tRNA hydrolysis during peptide chain termination. In particular, mutations in both 16S and 23S rRNAs caused substantial defects in catalysis of peptidyl-tRNA hydrolysis in the presence of RF2 but not RF1. Our finding that the mutations affect preferentially RF2-driven catalysis is a very interesting fact on its own since it points to the difference in action of RF1 and RF2 which are structurally similar proteins that drive the same reaction of peptidyl-tRNA hydrolysis. Our results were also consistent with the results previously obtained *in vivo*.

Comparison of *in vitro* and previous *in vivo* results

In vivo results have demonstrated that the mutations analyzed in this study, C1054A and G1093A, cause readthrough of UGA, but not of UAA or UAG, on glucose minimal medium (Jemiolo *et al.*, 1995; Pagel *et al.*, 1997). Since RF2 is the only release factor that functions at UGA, the UGA-specific readthrough observed *in vivo* in the presence of these two mutations can now be explained by the defects in RF2-dependent termination seen in this *in vitro* study. Failure to detect UAA and UAG readthrough *in vivo*, under the same conditions as where UGA readthrough is detected, can be explained by close to normal catalytic function of RF1 (which works at both UAA and UAG) as shown here *in vitro*. Consistent with this explanation is the fact that different mutations in *E. coli* RF2 caused readthrough of UGA in two positions of a fused *lacI-lacZ* gene but not readthrough of UAA or UAG at the same positions of that gene *in vivo* (Mikuni *et al.*, 1991). Results of a study from Tate's laboratory indicated that the C1192U and C1192G mutations in helix 34 of *E. coli* 16S rRNA (Figure 1A) caused defects in binding of both RF1 and RF2 to the ribosome *in vitro*

Fig. 3. Hydrolysis of peptidyl-tRNA determined with ribosomes isolated from mutant and wild-type strains. The ribosomes isolated from mutant strains were a mixture of mutant and wild-type ribosomes since the genome of the *E. coli* cells had seven chromosomal copies of the wild-type rRNA operon in addition to the mutant copy expressed from the plasmid. Mutant ribosomes constituted 44.2 and 32.6% of the total ribosomal population for the G1093A and C1054A mutants respectively, as determined by primer extension analysis (see Materials and methods). Release complexes (1.2–1.5 pmol) were mixed with 80 pmol of pure RF1 or ~16 pmol of partially purified RF2 in 100 μ l of polymix. These amounts of RF were determined to be very high in the range of saturation (in that range, no increase in the rate of hydrolysis was observed), with ribosomes from the two mutant strains, as well as with wild-type ribosomes. Consequently, the assays measured catalysis rather than binding of RFs. Hydrolysis was allowed to proceed, and at a given time the reaction was stopped with cold TCA (see Materials and methods). %S is the percentage of unhydrolyzed peptidyl-tRNA at a given time point measured as described in Materials and methods. The data were plotted after subtraction of background values for the number of inactive ribosomes, in the wild-type as well as the mutant ribosome preparations. The values, which ranged from 20 to 28% of the starting values for mutants and from 15 to 18% for the wild-type, were determined as the amounts of tetrapeptidyl-tRNA that could not participate in the ribosome-dependent peptidyl transfer reaction with saturating amounts of puromycin (see Materials and methods). %S was plotted as a function of time for RF1-driven reactions at UAA (A) and UAG (B), and for RF2-driven reactions at UAA (C) and UGA (D). $\square$, G1093A [23S]; $\diamond$, C1054A [16S]; $\circ$, wild-type. In (A), (B) and (C), the average values from 2–4 experiments for almost every time for each mutant and wild-type are presented. In (D), the averages of two experiments at 4 s are presented for each mutant and the wild-type. The mutant ribosomes showed a similar defect in hydrolysis at UGA in another independent time course experiment with less RF2 (data not shown). In the latter experiment, the saturating amount of RF2 was used as it had been in (D). For both RF1- and RF2-driven reactions, the same phase of the reactions is presented. Therefore, the time scale for reactions with RF1 is different from that for reactions with RF2. The last time for each reaction was 12 min. By that time, mutant %S values were 0.5–7.9% and wild-type %S values were 0–3.3%.

(Brown *et al.*, 1993). However, the defects in binding of RF2 to the mutant ribosomes were larger than the defects in that of RF1. That study, therefore, is consistent with ours, demonstrating that mutations in another position in helix 34, a position close to the 1054 residue, cause a major defect in RF2 function.

There are some conditions, however, under which the mutations analyzed in the present study caused readthrough of all stop codons *in vivo* (Moine and Dahlberg, 1994; Pagel *et al.*, 1997; W.Xu and E.J.Murgola, unpublished data). Those conditions are either low incubation temperature (W.Xu and E.J.Murgola, unpublished data),

enriched media (Moine and Dahlberg, 1994; Pagel *et al.*, 1997) or a combination of both (W.Xu and E.J.Murgola, unpublished data). One can speculate that the low temperature and certain nutrients in the enriched media, such as amino acids (Yarus, 1993), affect the conformation of rRNA around the mutant sites. The conformational change may result in better ribosome binding to tRNAs that can misread stop codons, and this, in turn, may cause the increased readthrough of all stop codons. Consistent with this proposed effect on tRNAs is the finding that C1054A increases the efficiency of GAG reading by a missense suppressor tRNA^{Gly} mutant (Pagel *et al.*, 1997). Further-

Fig. 4. Estimation of defects of mutant ribosomes in peptidyl-tRNA hydrolysis. The contributions of mutant ribosomes from mixed populations of wild-type and mutant ribosomes to peptidyl-tRNA hydrolysis were calculated as follows. For each point of the time course experiments presented in Figure 3, as well as for the 120 s point for RF1-driven terminations and the 30, 60 and 120 s points for RF2-driven terminations, averages of experimental values for the wild-type ribosomes were multiplied by the fraction of wild-type ribosomes that were present in the mixed population of ribosomes isolated from each mutant strain (0.558 for the G1093A [23S] mutant and 0.674 for the C1054A [16S] mutant). The fraction of wild-type ribosomes was determined by primer extension analysis as described in Materials and methods. For example, for calculation of the contribution of C1054A ribosomes in RF1-dependent hydrolysis at UAA, the wild-type values from Figure 3A were multiplied by 0.674 at each time and the results of the multiplication were subtracted from the experimental values for C1054A at each time from 0 to 120 s. The calculated C1054A mutant values were normalized, so the value at zero time became 100%, and the normalized values were transformed into natural logarithmic (ln) values. The ln values for mutant ribosomes for other experiments were obtained in a similar way. The experimental values for the wild-type ribosomes were transformed into ln values as well. The ln values for a given mutant or for wild-type were plotted against time, and those that declined linearly from the start of the reaction were presented in this figure and used for calculation of the slope (regression coefficient) of the best-fitting line using SPSS software for MS WINDOWS, Release 6.1. The slopes of the lines were the estimates for k_{cat} of hydrolysis (see Table I). The best-fitting lines for mutants and wild-type on the semi-logarithmic plots are presented for RF1-driven reactions at UAA (A) and UAG (B), and for RF2-driven reactions at UAA (C) and UGA (D). Averages of the ln values were transformed back into %S and plotted as a function of time. $\square$, G1093A [23S]; $\diamond$, C1054A [16S]; $\circ$, wild-type. In (D), for mutant G1093A, one additional value, at 30 s (not shown), was considered for calculation of the slope.

Table I. The k_{cat} estimates for peptidyl-tRNA hydrolysis by mutant and wild-type ribosomes in the RF1- and RF2-driven reactions

rRNA mutations	RF1		RF2	
	UAA	UAG	UAA	UGA
G1093A [23S]	5.5 ± 0.5 (11) ^a	6.7 ± 0.7 (13)	4.9 ± 0.6 (13)	2.3 ± 0.5 (6)
C1054A [16S]	6.6 ± 1.1 (9)	6.4 ± 0.9 (13)	7.5 ± 0.9 (11)	11.2 ± 1.1 (4)
Wild-type	9.4 ± 0.3 (11)	10.0 ± 0.3 (13)	25 ± 2 (7)	38 ± 2 (3)

^aData are $(k_{\text{cat}} \pm \text{SE}) \times 10^2/\text{s}$, where SE stands for standard error. k_{cat} values are slopes of the lines plotted in Figure 4. k_{cat} and SE were calculated from the linear regression analysis of the data presented in Figure 4 with the help of the SPSS software for MS WINDOWS, Release 6.1. The number of values obtained for each regression analysis is given in parentheses.

more, two nucleotides that are close to the 1093 residue, in the GTPase center of 23S rRNA, have been shown to be protected from chemicals by A-site-bound tRNA (Moazed and Noller, 1989), indicating the proximity of the GTPase center to aminoacyl-tRNA which recognizes a codon in the A-site of the ribosome. Therefore, it is conceivable that a conformational change in the GTPase center may also increase misreading of stop codons by tRNAs.

How might the rRNA mutations affect peptidyl-tRNA hydrolysis?

There is indirect evidence that the peptidyl transferase of the ribosome, which is located in the large ribosomal subunit, also catalyzes peptidyl-tRNA hydrolysis (Vogel *et al.*, 1969; Caskey *et al.*, 1971; Caskey and Beaudet, 1972). An important discovery was made by Caskey *et al.* (1971), who showed that both bacterial and mammalian ribosomes can carry out the hydrolysis of peptidyl-tRNA without RFs in the presence of 30% acetone and tRNA. Some antibiotics, inhibitors of peptidyl transferase, inhibited such a hydrolysis. Other antibiotics inhibited the peptidyl-transferase activity but stimulated the hydrolysis. These results suggested that (i) the ribosome alone catalyzed the hydrolysis of peptidyl-tRNA and (ii) the catalytic site of the ribosomal hydrolytic activity is either the same as the ribosome site that carries out peptidyl transfer or is situated very close to the peptidyl transferase. The close proximity of the two ribosome sites (one for peptidyl transfer and the other for peptidyl-tRNA hydrolysis) or the existence of a common catalytic site for both reactions is very easy to imagine. In both reactions, the same peptidyl-tRNA ester bond needs to be cleaved, either leading to a new peptide bond during peptidyl transfer or resulting in release of a free polypeptide from the ribosome during peptide chain termination.

The rRNA mutations analyzed in this study caused major defects in the RF2-dependent peptidyl-tRNA hydrolysis. Since hydrolysis was almost normal in RF1-dependent reactions, we suggest that the rRNA sites at which the mutations are located do not constitute the ribosomal site that directly cleaves the ester bond in a peptidyl-tRNA during termination. (We assume that there is one catalytic site in the ribosome that directly hydrolyzes peptidyl-tRNA in response to both RF1 and RF2.) These mutations may rather affect RF2's ability to activate that catalytic site. One can imagine that these mutations prevent the correct positioning of RF2 so that it cannot reach the catalytic site and 'switch it on'. Another possibility is that the mutations prohibit a conformational change of the RF2 that is necessary for triggering hydrolysis. It is also possible that RF2 induces conformational changes in helix 34 and the GTPase center and, after those changes have occurred, the rRNA regions activate the catalytic site for hydrolysis. It is possible, therefore, that the mutations may interfere with the conformational changes in helix 34 and the GTPase center. In other words, helix 34 and the GTPase center may be parts of a signal transduction network that, in the presence of RF2, transmits a signal (namely the presence of a stop codon in mRNA) from the small subunit portion of the A-site to the catalytic site for hydrolysis, most likely situated in the large subunit, at or near the peptidyl transferase.

Ribosomal sites that are involved in transmission of the termination signal in the presence of RF1 may be very close to those for RF2. For example, as discussed earlier, mutations in position 1192 of the *E.coli* 16S rRNA in helix 34 (Figure 1A) caused defects in both RF1- and RF2-dependent binding of stop codons to the ribosomes *in vitro* (Brown *et al.*, 1993). Therefore the results of that study (Brown *et al.*, 1993) indicated the involvement of helix 34 in the binding of both RFs to the ribosome. Similarly, thiostrepton, an antibiotic that binds specifically to the GTPase region of the *E.coli* 23S rRNA (Thompson and Cundliffe, 1991), was shown to inhibit both RF1- and RF2-dependent terminations *in vitro* (Brot *et al.*, 1974). Furthermore, a study from Douthwaite's laboratory strongly implicated two nucleotides in the GTPase center (Figure 1B), A1067 and A1095, as being in contact with thiostrepton (Rosendahl and Douthwaite, 1994). Since A1095 is just two nucleotides 3' to the G1093A change analyzed in our study, a ribosomal site that is involved in RF1-specific catalysis may also be very close to G1093.

Our demonstration, with this new *in vitro* termination assay (Freistroffer *et al.*, 1997), of defects in the catalysis of ribosome-dependent peptidyl-tRNA hydrolysis associated with the mutants A1054 (16S rRNA) and A1093 (23S rRNA) supports the validity of a previously proposed strategy for finding termination-defective rRNA mutants, namely, to look for rRNA nonsense suppressors that, at least under some conditions, are specific for one or two of the three termination codons (Murgola *et al.*, 1995). Conversely, our results verify the 'realistic' nature of the *in vitro* assay by their correspondence with and reflection of the mutant properties *in vivo* (Murgola *et al.*, 1988, 1995; Jemiolo *et al.*, 1995; Xu and Murgola, 1996; Pagel *et al.*, 1997).

Both C1054 and G1093 are virtually universally conserved in both prokaryotes and eukaryotes (Maidak *et al.*, 1996; Van de Peer *et al.*, 1996). Moreover, it has been shown for a eukaryote, *Saccharomyces cerevisiae*, that a change of C to either A or G at the position in 18S rRNA corresponding to nucleotide 1054 of *E.coli* 16S rRNA causes readthrough of stop codons (Chernoff *et al.*, 1996). The nucleotide conservation and the results of Chernoff *et al.* (1996) as well as of our present study suggest the universal importance of the rRNAs from both ribosomal subunits in translation termination.

Materials and methods

Isolation of ribosomes

Ribosomes were isolated from *E.coli* strain AL1 [*glyV55 recA*/F'*trpA*(UGA115)] by gel filtration on Sephacryl S-300 essentially as described (Jelenc, 1980). The isolated ribosomes were stored in polymix buffer at -80°C. The polymix was prepared as described (Jelenc, 1980) and contained 5 mM magnesium acetate, 0.5 mM CaCl₂, 95 mM KCl, 5 mM NH₄Cl, 8 mM putrescine, 1 mM spermidine, 5 mM potassium phosphate and 1 mM dithioerythritol (DTE), pH 7.5. In AL1, the mutant rRNAs were expressed from the λ PL promoter in the plasmid pNO2680 (Gourse *et al.*, 1985). Since *E.coli* has seven chromosomal copies of the wild-type rRNA operon, it is impossible to isolate a pure population of mutant ribosomes. Furthermore, we found that the analyzed mutations were heat-sensitive when highly expressed in pNO2680 (Murgola *et al.*, 1995; Pagel *et al.*, 1997). Therefore, in order to obtain a large amount of mutant ribosomes and not to compromise cell growth, bacteria were grown at 31°C, a growth-permissive temperature for the mutants. In a 15 l fermentor, ~12 l of TY-2 medium (see below) were inoculated with each bacterial strain, and the cells were harvested after 14–18 h of

growth, while they were growing exponentially. The composition of the TY-2 medium was according to Holme *et al.* (1970) except for trace elements. A stock solution of trace elements was prepared by dissolving 72 mg of $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 60 mg of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and 60 mg of citric acid in 100 ml of water. One ml of the stock solution was added for each 1000 ml of the TY-2 medium.

Isolation of ribosomal complexes stalled at a stop codon

The substrate for the *in vitro* termination reaction (release complex) is factor-free ribosomes with mRNA bound (the A-site codon is a stop codon) and fMet-Phe-Thr-Ile-tRNA^{Ile} bound to the P-site. This substrate is made by initiating and elongating the peptide using a fully purified system of components and then purifying the ribosome fraction away from proteins, RNA, nucleotides, etc., on a gel filtration column (Freistroffer *et al.*, 1997). Purification of translation factors that are used for generating release complexes was done as described (Freistroffer *et al.*, 1997). To generate the release complex, translational initiation was carried out in the presence of 10 nmol of ribosomes, 7.7 nmol of mRNA, initiation factors 1, 2 and 3 (IF1, 2 and 3) in the amounts of 2, 0.35 and 2 nmol respectively, 5.5 nmol of initiator fMet-tRNA^{fMet} and 1 mM GTP in 400–450 μl of the polymix buffer for 40 min at 28°C. After initiation, the initiation reaction mixture was added to an elongation mix (EM) that had been pre-incubated for 20 min at 28°C. The EM was prepared in 678 μl of polymix and contained 10 mM phosphoenolpyruvate, 1 mM ATP, 1 mM GTP, 150 μM phenylalanine, 150 μM threonine, 12 μM [¹⁴C]isoleucine (475 c.p.m./pmol), elongation factors EF-G, EF-Ts and EF-Tu in the amounts of 2, 4 and 6 nmol respectively, 6 μg of myokinase, 100 μg of pyruvate kinase, 300 nmol of total *E. coli* tRNA and 500 U each of phenylalanyl-, threonyl- and isoleucyl-tRNA synthetases as defined in Ehrenberg *et al.* (1990). After 1 min of elongation, the reaction mixture was ice-cooled. The ribosomes with [¹⁴C]-labeled tetrapeptidyl-tRNA stalled at the stop codons of the mRNAs were purified on a gel filtration column (Sephacryl S-300HR, 5 cm² × 45 cm, Pharmacia) and eluted in polymix at 4°C as described (Freistroffer *et al.*, 1997).

In vitro termination assay

Release complexes (1.2–1.5 pmol) in 50 μl of polymix were mixed with 80 pmol of pure RF1 or ~16 pmol of partially purified RF2 that had been pre-incubated in 50 μl of polymix for 10 min at 28°C. Hydrolysis was allowed to proceed, and at a given time the reaction was stopped with 5 ml of cold 5% trichloroacetic acid (TCA) containing 0.75% casamino acids. The samples were filtered through glass fiber filters and the amount of unhydrolyzed tetrapeptidyl-tRNA retained on the filters was determined by a liquid scintillation counter, Quickszint 701 (Zinser Analytic). Therefore, the signal measured in our assays is peptide remaining as peptidyl-tRNA after exposure to release factors. The unhydrolyzed peptidyl-tRNA retained on the filters is detected by a [¹⁴C] label on the final amino acid in the peptide, isoleucine. RFs were purified as described (Freistroffer *et al.*, 1997). The concentration of RF2 in the partially purified RF2 fraction was measured as described (Freistroffer *et al.*, 1997).

Determination of fractions of wild-type and mutant ribosomes by primer extension analysis

For a given mutant strain, the fractions of both wild-type and mutant ribosomes that could form release complexes and participate in reactions of hydrolysis *in vitro* were assumed to be the same as the amounts of the wild-type and mutant monoribosomes (monosomes), respectively, isolated from that mutant strain. For isolation of monosomes, bacteria were grown at 31°C to late log phase in TY-2 medium and then lysed as described (Godson and Sinsheimer, 1967). The lysate was loaded onto a 10–40% w/v linear sucrose density gradient made up in 20 mM Tris-HCl (pH 7.8), 10 mM MgSO_4 and 60 mM KCl and centrifuged at 35 000 r.p.m. for 5 h at 4°C in a Beckman SW 40 rotor. The monosome gradient fraction was precipitated in ethanol. The fractions of wild-type and mutant monosomes were determined by measuring the fractions of wild-type and mutant rRNAs in the monosomes. The rRNA was extracted sequentially with phenol, phenol-chloroform and chloroform, and then precipitated with ethanol. Fractions of wild-type and mutant rRNA were determined by primer extension analysis essentially as described (Sigmund *et al.*, 1988). Previous experiments detected no reduction of mutant ribosomes in polysomes *in vivo* caused by either C1054A (Pagel *et al.*, 1997) or G1093A (A.L. Arkov and E.J. Murgola, unpublished data) in comparison with the amount of mutant ribosomes present in the fraction of monosomes. That was interpreted to indicate that mutant ribosomes were able to translate mRNA efficiently after initiation.

Determination of background values in termination reactions with puromycin

Puromycin was added to 1.2–1.5 pmol of release complexes, pre-incubated for 40 s in 100 μl of polymix at 28°C, so that the final concentration of puromycin was 500 μM . After 4 min of incubation of release complexes with puromycin at 28°C, 5 ml of cold 5% TCA containing 0.75% casamino acids was added to the reaction mixture. The samples were filtered through glass fiber filters and the amount of tetrapeptidyl-tRNA retained on the filters was determined by a liquid scintillation counter, Quickszint 701 (Zinser Analytic). The product of the puromycin reaction, namely the tetrapeptidyl portion of tetrapeptidyl-tRNA covalently bound to puromycin, passed through the filters.

Statistical analyses of the data

The significance of the differences between values for ribosomes isolated from mutant strains and those isolated from the wild-type strain was estimated using analysis of variance procedures (Zar, 1974). The averages of those values are presented in Figure 3. The mutant values were compared with the wild-type ones as follows. For both RF1-dependent termination at UAG and RF2-dependent termination at UAA (Figure 3B and C), the comparison was made for the values at the times when values for wild-type decreased exponentially during the first (fastest) phase of the reaction. That phase accounted for the hydrolysis of ~75% of the peptidyl-tRNA bound to the wild-type ribosomes (Figure 4B and C). For RF1-dependent reactions at UAA (Figure 3A), the values for mutants were compared with those for the wild-type at the four times (4–12 s). The values at the last time point, 15 s, when the wild-type reaction was still in the first, exponential phase, were not considered for the comparison since that phase for the C1054A mutant occurred in the range of 4–12 s (Figure 4A) but not at 15 s. For RF2-dependent reactions at UGA (Figure 3D), 4 s values for mutants were compared with those for the wild-type. The significance of the differences between mutant and wild-type values both for all of the RF1-dependent reactions and for the RF2-dependent reaction at UAA (Figure 3A–C) were estimated with Model I two-factor analysis of variance (Zar, 1974). The calculations for the two-factor analysis of variance were made with the SPSS software for MS WINDOWS, Release 6.1. For the RF2-dependent hydrolysis at UGA (Figure 3D), the significance of the differences between values for mutants and those for wild-type were calculated with the procedure for Model I single factor analysis of variance (Zar, 1974). The significance level used for the evaluation of a difference's significance was $\alpha = 0.05$.

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