Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 6603–6607, August 1991
Biochemistry

Mutations in 16S rRNA that affect UGA (stop codon)-directed translation termination

(*Escherichia coli*/site-directed mutagenesis/nonsense suppressor/stop codon recognition)

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Communicated by Bernard D. Davis, April 10, 1991 (received for review September 27, 1990)

ABSTRACT **Site-directed mutagenesis was performed on a sequence motif within the 3′ major domain of *Escherichia coli* 16S rRNA shown previously to be important for peptide chain termination. Analysis of stop codon suppression by the various mutants showed an exclusive response to UGA stop signals, which was correlated directly with the continuity of one or the other of two tandem complementary UCA sequences (bases 1199–1204). Since no other structural features of the mutated ribosomes were hampered and the translation initiation and elongation events functioned properly, we propose that a direct interaction occurs between the UGA stop codon on the mRNA and the 16S rRNA UCA motif as one of the initial events of UGA-dependent peptide chain termination. These results provide evidence that base pairing between rRNA and mRNA plays a direct role in termination, as it has already been shown to do for initiation and elongation.**

The direct involvement of rRNA sequence motifs in the assembly and the function of the ribosomal particle is now evident from a large variety of experimental findings (1). Especially since the discovery of various RNA molecules with intrinsic catalytic activities (2–4), it is no longer an iconoclastic hypothesis to view the ribosome as an RNA-driven enzyme. From what we know mechanistically about functional steps and functional domains of the translational machinery, RNA·RNA interactions are a fundamental principle in protein biosynthesis. Base-pairing interactions between rRNA sequences and the mRNA molecule can be identified at the initiation and the elongation steps of translation (5–8). An analogous event has also been proposed for the termination of peptide synthesis, based on the isolation of a translational suppressor, a 16S rRNA mutant (9, 10). The mutant, selected as a spontaneously occurring UGA suppressor, resulted from a deletion of a cytidine residue at position 1054, which is contained in helix 34 within the 3′ major domain of the 16S rRNA molecule (11). Helix 34 is located, at a three-dimensional level, in proximity to the 1400 region, known to be part of the decoding domain on the 30S subunit (12). Site-directed mutagenesis of position 1054 demonstrated that a C → G base change also confers UGA read-through, although to a lesser extent when compared with the deletion (13).

Attempts to explain the suppressor phenotype of the ΔC1054 and 1054G mutations have led to a rRNA·mRNA base-pairing hypothesis. Based on the observation that helix 34 contains two consecutive phylogenetically conserved and partly single-stranded 5′-UCA-3′ sequence elements (bases 1199–1204), an antiparallel base pairing between the UGA stop codon and one of the complementary UCA sequences was suggested (9, 10). Such base pairing could prevent tRNA molecules from misreading the UGA stop signal and could, therefore, be one of the initial events of the termination process. Lang *et al.* (14) have shown that the UGA stop codon can be crosslinked to the 16S rRNA molecule in the presence of release factor 2 (RF-2), whereas a direct interaction between the factor and the stop codon in the absence of ribosomes was not observed. However, the crosslinking site on the 16S rRNA molecule could not be identified in these experiments.

In this paper we describe a set of mutations in helix 34 that systematically tests the above proposed 16S rRNA·mRNA base-pairing model. The major prediction of the model is that changing the UCA sequences will disrupt base pairing with UGA but not with UAA or UAG stop codons, thereby permitting read-through of UGA stop codons in mRNAs. Using site-directed mutagenesis, we have destroyed selectively the two complementary UCA sequence motifs and show here that the results support the model for termination.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. All bacterial strains used in this study were *Escherichia coli* K-12 derivatives. Strain HB101 (15) was the host for noninducible plasmids and XL1-Blue (16) was host for phage M13 and its derivatives. The bacteria used to assay read-through levels of the nonsense codons were derivatives of strain KL16 (*Hfr thi-1 relA1 spoT1*) (17) and have the following pertinent genotypes: DEV1-KL16, *lacZ105* (UAG); DEV14-KL16, *lacZ659* (UAA); DEV15-KL16, *lacZ* (UGA). Strain CJ236 (*dut-1, ung-1, thi-1, relA-1*) with pCJ105 (Cm^r) (18) was used for the mutagenesis procedure. Strain pop2136 (19) contains the chromosomal *c*I857 allele and was used as the host for plasmids with the λ P_L promoter. SU1675 with pPY1025 and pKB4 (synRF-2) is an RF-2 protein overproducing strain.

Plasmid pKK3535 carries the entire *E. coli rrnB* operon (20). Mutated derivatives of pKK3535 were pKK1054 (deletion of base C1054), pKK1199C (U → C at position 1199), pKK1202C (U → C at position 1202), and pKK1192G (C → G at position 1192) (21) according to the base position mutated in the 16S RNA cistron. pNO2680 contains the *rrnB* operon cloned behind the repressible λ P_L promoter (22). Plasmids pNO138 and pNO214 are derived from pNO2680, with either T → C transitions at positions 1199 and 1202 (pNO138) or a T → C transition at position 1199 and a guanine insertion between the 16S RNA positions 1202 and 1203 (pNO214). Plasmid DNA preparation, transformations, media, and other genetic procedures were as described (23).

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Abbreviation: RF-2, release factor 2.

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Site-Directed Mutagenesis. Site-directed mutagenesis was performed using the basic mismatched oligodeoxynucleotide technique (18, 24). The single-stranded DNA template was isolated from a recombinant M13mp11 derivative, containing a 1050-base-pair *Eco*RI–*Xba* I insert containing the coding region of the 3′ domain of the 16S rRNA cistron. Mutants were screened by sequencing single-stranded DNA isolates from transformants (25). Each mutation was confirmed after back-cloning into the plasmid vector by restriction mapping (pNO214, *Nsi* I; pNO138 and pKK1202, *Nco* I; pKK1199, *Bgl* I), plasmid sequencing, and sequencing the 16S RNA by using primer-directed reverse transcriptase reactions (26).

β-Galactosidase Activity Determination. For assaying readthrough levels of *lacZ*–UGA, –UAA, and –UAG, β-galactosidase activity was measured (27). Whole-cell assays were carried out in a final volume of 1.0 ml of Z buffer (27) at 28°C and were started by the addition of 0.2 ml of *O*-nitrophenyl β-D-galactopyranoside (4 mg/ml). The termination of the reaction was done by adding 0.5 ml of 1 M Na_2CO_3. The assay tubes were centrifuged before reading the A_{420}. The whole-cell unit definition is β-galactosidase units = $[10^3 (A_{420})]/[OD_{600} \times \text{time (min)} \times \text{vol (ml)}]$.

***In Vivo* Labeling of RF-2 Protein.** [^{35}S]Methionine-labeled RF-2 protein was isolated essentially as described (13). The overproducing strain SU1675(pPY1025, pKB4) (synRF-2) was kindly provided by R. Weiss and L. Yang (University of Utah, Salt Lake City, UT).

Binding of RF-2 to 70S Ribosomes. The interaction between 70S ribosomes and RF-2 termination factor was standardly determined in 0.2 ml of TMK buffer (20 mM Tris·HCl, pH 7.5/20 mM $MgCl_2$/100 mM KCl) containing 20–50 pmol of heat-activated (40°C, 30 min) 70S ribosomes, 2.5 nmol of termination codon (UGA or UAA), and various amounts of [^{35}S]RF-2 protein (2.5–500 pmol) in the presence of 6% ethanol (28). Equilibrated termination complexes (0–4°C, 30 min) were analyzed by isokinetic centrifugation on linear sucrose gradients as described (13, 29).

Chemical Modification. The 30S subunits were modified according to the general procedure described by Moazed *et al.* (30). Modified bases were identified by primer extension with reverse transcriptase after gel electrophoresis of the cDNA fragments. Both strands of helix 34 were tested using two primer molecules (ATTGTAGCACGTGTGTAG, complementary to bases 1223–1240, and CGGGACTTAACCCAACAT, complementary to bases 1082–1099 in 16S RNA). Modification was done at 37°C (31) for 15 and 30 min with thermoactivated 30S subunits (30 min, 42°C) isolated from translationally active 70S ribosomes. Control samples were treated identically throughout, except for the omission of modification reagent. Autoradiograms were analyzed by densitometry at 525 nm.

RESULTS

Cell-Growth Phenotype of Mutants. Base changes within the 16S rRNA helical segment 34 were constructed by site-directed mutagenesis (18, 24). Fig. 1 summarizes the base changes created and the current secondary structure model of helix 34 according to Gutell *et al.* (32). Mutant 16S rRNA genes were cloned into the expression vector pKK3535, which contains the entire *E. coli rrnB* operon (20). The integrity of the mutations was verified by directly sequencing the altered region of the plasmids. The effect of the mutations on cell growth was measured after transformation into strain HB101. Cell doubling times for all three single mutants (1199C, 1202C, and ΔC1054) were only slightly increased when compared with bacteria bearing wild-type plasmids. However, the transition time between the lag and logarithmic phases of growth was drastically increased for all three point mutants (Table 1). This clearly indicated an effect on translation by the mutant ribosomes, especially at low growth rates.

16S RNA MUTANTS

BASE CHANGES	DESIGNATIONS
U1199 ► C	C1199
U1202 ► C	C1202
U1199 ► C + U1202 ► C	C1199/C1202
U1199 ► C + G INSERT AT U1202-C1203	C1199/G.INS-1202-1203
C1054 DELETION	ΔC1054

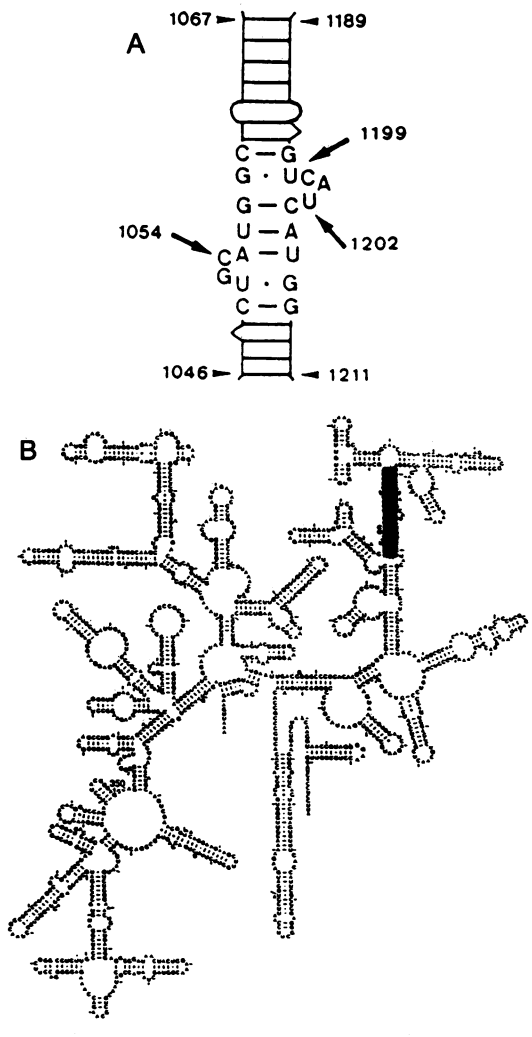

FIG. 1. Mutant 16S RNAs and the secondary structure of helix 34 in *E. coli* 16S rRNA. The presented model (*A*) is derived from phylogenetic sequence comparison studies (32). The numbering of the helical element was adopted from Maly and Brimacombe (11). (*B*) Secondary structure of the entire *E. coli* 16S rRNA molecule (32) with helix 34 shaded. A listing of the various base changes that were created in that stem structure is given.

Since it was not possible to obtain transformants of HB101 cells with either double-mutant construct (1199C/1202C or 1199C/$G_{ins.}$1202-03), we inferred that both mutations are lethal. To confirm this, we cloned the mutant genes into the conditional expression vector pNO2680 (22), where transcription is directed by the λ P_L promoter. Strain pop2136, containing the thermolabile *c*I857 repressor allele (19), was transformed with the double mutant plasmids. At 30°C no growth difference between wild-type and both mutant cell lines was apparent, whereas at 42°C the mutants failed to grow, confirming the deleterious effects of the mutations on HB101 cells.

Expression of Mutant 16S rRNAs and Incorporation into 70S Ribosomes. The transcription and subsequent processing of

Table 1. Growth behavior of cells containing episomal-coded mutants in helix 34 of 16S rRNA

Plasmid	Mutant designation	Doubling time, min	Transition time, min
pKK3535	Wild type	51 ± 2	86 ± 3
pKK1054	ΔC1054	59 ± 4	215 ± 10
pKK1199C	C1199	60 ± 8	255 ± 12
pKK1202C	C1202	63 ± 5	115 ± 9
pNO138	C1199/C1202	Lethal	Lethal
pNO214	C1199/$G_{ins.}$1202/03	Lethal	Lethal

Growth rates were determined in YT medium with ampicillin selection (33). The transition from lag to logarithmic phase growth was determined by a graphical approximation from the growth curves. Data are expressed as mean ± SD.

the mutant 16S rRNAs as well as their assembly into 70S ribosomes was investigated by primer-directed sequencing of total RNA and of rRNA isolated from 70S ribosomes. The ratio of mutant versus wild-type rRNA was determined by comparing the band intensities on autoradiograms of wild-type and mutant nucleotides. The results are summarized in Table 2. All mutant 16S rRNA molecules were present in total RNA isolates, and the expression levels varied from 47% to 75%, depending on the particular mutant. An essentially identical variation was found for the incorporation of mutated 16S rRNAs into 70S particles, ranging from 45% to 67%. This showed that the various mutant rRNA molecules were able to associate with the protein moiety of the 30S subunit and that the assembled subunits were able to form stable 70S ribosomes. Considering the relative errors of the expression/incorporation values (±10%, as determined from at least three experiments), the data reflected a roughly 1:1 cellular ratio of mutant and wild-type rRNA.

Stop Codon Suppression Analysis. All viable 16S rRNA mutants were screened for their ability to suppress the three termination signals on an identical mRNA molecule. This was achieved *in vivo* utilizing a system in which ribosomes must translate through a stop codon to synthesize functional β-galactosidase (17). After transformation of the mutant plasmids into the three test strains [DEV1, *lacZ* (UAG); DEV14, *lacZ* (UAA); DEV15, *lacZ* (UGA)], enzyme assays were performed to determine the relative level of translational read-through. Wild-type plasmid pKK3535 transformants were used as a control to estimate background levels of termination suppression. Plasmid pKK1192G, containing a C → G mutation at position 1192 in the 16S rRNA cistron (21), was used as an additional control to demonstrate that not every mutation in helix 34 confers stop codon read-through. All data are summarized in Fig. 2. Deletion mutant ΔC1054 exhibited UGA-specific suppression as demonstrated (9) by a different experimental approach. Its UGA suppression level was higher by a factor of 9 than that of the wild-type plasmid, whereas the recognition of UAG and UAA stop codons was not affected. Two single mutants, C1199 and C1202, also read through the UGA stop codon although to a lesser extent. The C1199 read-through was higher by a factor of 2.8 and C1202 was higher by a factor of 2.5 than wild-type read-through. However, both respond normally to UAA and UAG stop signals, thus showing the predicted specificity of the helix 34·mRNA interaction for only UGA stop signals.

Table 2. Expression/incorporation of helix 34 mutant 16S rRNAs in total RNA and 70S ribosomes *in vivo*

Mutant 16S RNA	Expression in total RNA, %	Incorporation into 70S ribosomes, %
ΔC1054	75	45
C1199	74	67
C1202	58	65
C1199/C1202	54*	45
	41†	38
C1199/$G_{ins.}$1202-03	47*	47
	31†	31

Amount of mutant 16S rRNA within a mixture of wild-type and mutant molecules was determined by primer-directed sequencing using avian myeloblastosis virus reverse transcriptase, essentially as described (26). The relative band intensities for both the wild-type and mutated nucleotide position was assessed by densitometry at 525 nm from nonsaturated autoradiograms. The values were normalized by comparison to neighboring bands. Relative errors were estimated from a minimum of three experiments and were in the range of 10%. The two mutants C1199/C1202 and C1199/$G_{ins.}$1202-03 had a lethal phenotype and could only be expressed using a conditional expression system (22). Expression at either 2 (*) or 5 (†) hr after induction of transcription is indicated.

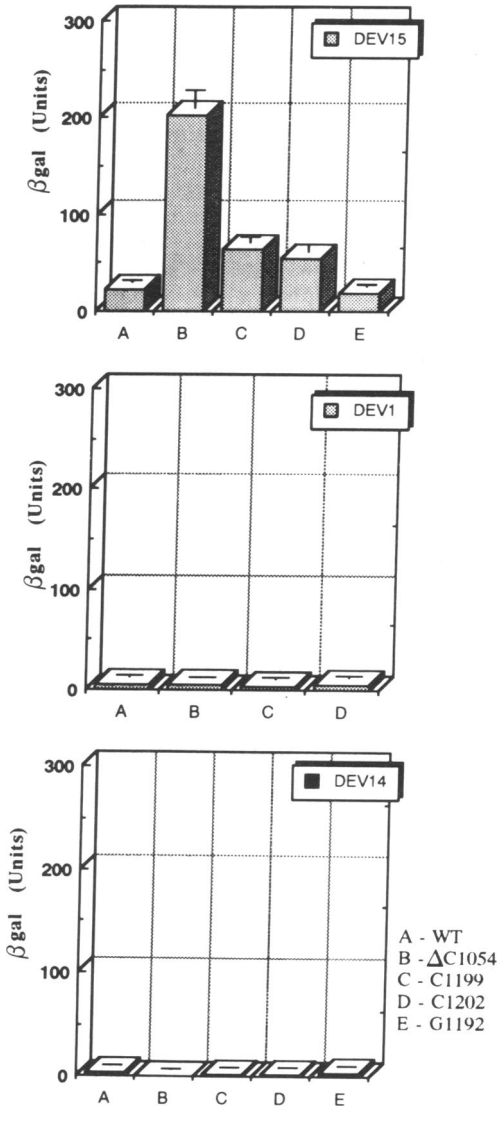

FIG. 2. Termination codon suppression of 16S RNA mutants *in vivo*. For assaying read-through levels of *lacZ*–UGA (DEV15), –UAA (DEV14), and –UAG (DEV1), β-galactosidase (βgal) activity was measured according to Miller (27). The DEV strains were a generous gift from D. Elsevier (New York Medical College, Valhalla, NY). Their genotypes are derived from strain KL16 (17). The UGA suppression values are normalized according to the percentage of mutated 16S RNA actually incorporated into 70S ribosomes (Table 2). The standard deviation of the measurements is indicated as error bars.

Protein Analysis of Mutant Ribosomes. To determine the specific protein composition of the mutated ribosomes, the protein of the 70S particles was isolated by acetic acid extraction and characterized by two-dimensional polyacrylamide gel electrophoresis. The intensity of the protein spots was quantitated using a two-dimensional laser scanning device. Ribosomes from the three single mutants (ΔC1054, C1199, and C1202) and the wild type showed identical stoichiometry for all ribosomal proteins (data not shown). The observed UGA suppression by the various mutants is, therefore, a consequence of the changes in the rRNA and not of an altered protein composition of the ribosomes. [Note: The previously reported (10) reduction of protein S5 from HB101(pKK1054) cells, analyzed by one-dimensional gel electrophoresis, could not be confirmed by this more precise quantitation approach.]

Structure Probing of Helix 34. The higher-order structure of helix 34 within the 30S subunit was probed by chemical modification (30) to determine whether the stop codon suppression effect caused by the mutants was accompanied by detectable structural changes within the "steady-state" conformation of helix 34. Mutant ΔC1054 was used because it showed the highest level of UGA read-through (Fig. 2). The four bases were monitored at defined atoms with kethoxal: guanine (N-1, N-2), 1-cyclohexyl-3(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate; uracil (N-3) > guanine (N-2), diethyl pyrocarbonate; adenine (N-7) and dimethyl sulfate; guanine (N-7) > adenine (N-1) > cytosine (N-3). The pattern of reactivity was visualized by oligonucleotide-directed reverse transcription and gel electrophoresis of the cDNA products. As outlined in Table 2, we were using a mixed population of 30S subunits containing ≈50% wild-type and 50% mutant 16S rRNA. Nucleotides U1049, A1067, U1189, A1196, A1197, A1201, and U1205 were accessible in both wild-type and mutant 30S subunits, but no differences were detected in the extent of modification at the various bases in wild-type and mutant subunits, with one exception. Base C1054 showed a 50% decrease in reactivity to the cytidine-specific probe dimethyl sulfate in the 30S pool containing the ΔC1054 mutant (data not shown). This demonstrated that the method was sensitive enough to detect structural differences within the mixed population of mutant and wild-type particles. We therefore conclude that the steady-state conformation of wild-type and of mutant helix 34 was isomorphic. Furthermore, the result demonstrated that the helix 34 binding proteins S2 and S3 (34) were associated with the rRNA helix in a manner indistinguishable from that in wild-type subunits.

RF-2 Binding of Mutant 70S Ribosomes. The codon-specific binding of termination factor RF-2 to 70S ribosomes was analyzed by a sucrose-gradient sedimentation assay (13, 29). Stable termination complexes were formed with a 70S ribosome, a termination codon, and a ^{35}S-labeled release factor protein in a partial reaction of the termination event, which requires the presence of 6% ethanol (28). The formation of the complex was dependent on the presence of the correct RF-2-specific termination codons (UGA or UAA), and nonspecific binding in the presence of UAG or in the absence of a termination triplet was subtracted as a background value. Binding data were collected for 70S ribosomes from the wild type and the three single mutants (C1199, C1202, and ΔC1054). Because the amount of active ribosomes and active RF-2 protein cannot be determined exactly (14), apparent association constants for the interaction of the three molecules were calculated as relative values (35) using a double reciprocal plot analysis. In the presence of the stop codon triplet UAA, the attraction between the three mutant 70S particles and the release factor protein was not significantly different when compared with wild type. Within the experimental error of the method, identical values were obtained for wild-type ribosomes and ribosomes from the mutants C1199 and C1202 using UGA as a stop codon (data not shown). In contrast, 70S particles from the ΔC1054 mutant showed a reduction of the relative apparent association constant when UGA was present (relative K_{app} = 0.4 [UGA] and 1.3 [UAA]). By taking into account that only 45% of the 70S population in this mutant contain the altered 16S rRNA molecule (Table 2), the change in the binding constant indicates an inability of the mutant ribosomes to interact specifically with RF-2 when a UGA stop codon is presented.

DISCUSSION

In the present paradigm for peptide chain termination, a translating ribosome stalls upon encountering a stop codon in the ribosomal A site. A protein release factor then interacts with the 70S ribosome·mRNA complex, modulating the peptidyltransferase center to activate a water molecule to hydrolyze the nascent peptide from peptidyl-tRNA (36, 37). Based on several lines of experimental evidence, two mechanisms for the initial step of termination have been proposed. The first, as described above, has the release factor and stop codon interacting in a sequence-specific manner, giving the factor a tRNA-like quality. In the second model, the release factor recognizes a structure resulting from a specific rRNA·mRNA interaction analogous to the Shine–Dalgarno base-pairing interaction during the initiation of translation. The two mechanisms are quite different, and until now strong evidence in support of either one was lacking.

Here we present evidence for a 16S rRNA·UGA stop codon interaction, based on termination suppression experiments with mutant 16S rRNA molecules created by site-directed mutagenesis. The two consecutive UCA sequences within helix 34 of the 16S rRNA, being complementary to UGA stop codons, were specifically changed, and the mutants were tested for their response to translational stop signals. Four such mutants were tested, along with two other mutants in helix 34 (ΔC1054 and 1192G). Three single-base mutants showed a specific and exclusive increase in UGA suppression. The two double mutants specified a lethal phenotype, suggestive of an extreme failure of UGA-mediated termination and consistent with the proposed base-pairing model.

Since the mutations appeared not to hamper the initiation and elongation steps of translation, rRNA processing, or subunit assembly, we attribute the defective translation termination to a direct effect of the mutations. Furthermore, the unaltered steady-state conformation of helix 34 in the mutant 16S rRNA argues strongly that the observed effects are due to the inability of this rRNA element to carry out its proper function. The unavailability of the UCA motifs in the mutant helix 34 that can be directly correlated with the level of UGA stop codon suppression indicates a direct interaction between the rRNA and mRNA molecules, presumably an antiparallel base pairing between the UCA and UGA sequences. The two double-mutant constructs (C1199/C1202 and C1199/G_{ins}1202-03) eliminate both UCA sequences, preventing proper base pairing with UGA stop codons; hence, they are lethal. Both of the single mutants (C1199 and C1202), which selectively destroy one or the other of the sequence motifs, result in UGA suppression. Therefore, either UCA sequence can apparently be used for termination, and the two may present a potential "safety device," to ensure efficient termination. Mutant ΔC1054 has both UCA sequences present, but in this case the RF-2 binding affinity is severely reduced.

Distal rRNA sequences as well as ribosomal proteins may provide additional structural features necessary for forming the termination domain on the 70S particle. In support of this suggestion, a point mutation at position 726 in 16S rRNA has UGA suppression activity (38). This region does not exhibit

any complementarity to the UGA stop codon, but the mutant does exhibit an increased binding affinity for RF-2, which presumably impairs its function. Ribosomes lacking protein S15, which is located near 726, also are able to suppress a UGA nonsense codon within a mutant *E. coli rpoH* cistron (39). Finally, UAA suppression in yeast mitochondria has been found due to a 15S rRNA mutation at a position equivalent to nucleotide 517 in *E. coli* (40). Again there is no apparent possibility for base pairing to the stop codon, but there is evidence that the 530 loop may be involved in allosterically induced conformational relationships with the decoding region (41, 42).

The topological arrangement between helix 34 and the UGA stop codon might be one of the recognition signals for factor RF-2 to bind to the ribosome. The reduced association constant of RF-2 for ΔC1054 ribosomes supports this hypothesis, although this type of experiment cannot distinguish between a direct interaction of the factor at the rRNA·mRNA contact site or an induced conformational change that promotes RF-2 binding at a different site. Chemical modification data demonstrate the partial accessibility and the high degree of conformational flexibility of helix 34 within the 70S ribosome (43). Since cross-linking data locate the protein factor near ribosomal proteins S6, S17, and S18 at the lower body of the 30S particle (28), an "induced fit" for RF-2 binding, at a rather distal location from the helix 34·stop codon interaction site, seems to be the most plausible scenario.

The tandem UCA sequences in helix 34 are conserved features in small subunit rRNAs (44). A notable exception to this conservation occurs in *Mycoplasma capricolum*, where the sequence between positions 1199 and 1204 is 5′-CUACUA-3′; but in that organism UGA codes for tryptophan (45). Further support for the base-pairing model comes from analogous observations with mitochondrial 16S-like rRNA and from a recent analysis of termination involving *Marchantia* chloroplast rRNA (46).

This paper is dedicated to the memory of Dr. Heinz-Günter Wittmann. We thank B. Kleuvers, S. Billig, K. Bousset, and A. Hänfler for excellent help with some of the experiments. D. Elseviers kindly provided the DEV strains, and L. Yang and R. Weiss provided the RF-2 protein-overproducing strain. We are grateful to C. Prescott, M. Zacharias, R. Brimacombe, and G. Q. Pennabble for helpful comments and critical reading of the manuscript. We thank P. Makosky for providing pKK1192G and C. T. Caskey and C.-C. Lee for stop codon triplets. This research was supported by grants from the National Institute of General Medical Sciences to A.E.D. (GM 19756) and to E.J.M. (GM 21499) and from the American Cancer Society to E.J.M. (NP-167).

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