Covariance of Complementary rRNA Loop Nucleotides Does Not Necessarily Represent Functional Pseudoknot Formation In Vivo

NATALYA S. CHERNYAEVA AND EMANUEL J. MURGOLA*

Department of Molecular Genetics, The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030

Received 31 March 2000/Accepted 19 July 2000

We examined mutationally a two-hairpin structure (nucleotides 57 to 70 and 76 to 110) in a region of domain I of *Escherichia coli* 23S rRNA that has been implicated in specific functions in protein synthesis by other studies. On the basis of the observed covariance of several nucleotides in each loop in *Bacteria*, *Archaea*, and chloroplasts, the two hairpins have been proposed to form a pseudoknot. Here, appropriate loop changes were introduced in vitro by site-directed mutagenesis to eliminate any possibility of base pairing between the loops. The bacterial cells containing each cloned mutant rRNA operon were then examined for cell growth, termination codon readthrough, and assembly of the mutant rRNAs into functional ribosomes. The results show that, under the conditions examined, the two hairpins do not form a pseudoknot structure that is required for the functioning of the ribosome in vivo and therefore that sequence covariance does not necessarily indicate the formation of a functional pseudoknot.

Discerning the functions and structure of the ribosome has been a challenge for more than 30 years. Much progress in the understanding of ribosome structure has been made recently, particularly by analyses using cryoelectron microscopy (1, 26) and X-ray crystallography (3, 5, 7, 8, 32), prompting the expectation that most of the fine-structure details will soon be revealed. On the other hand, the functions of many ribosome sites and structures are far from understood. This is especially true for 23S rRNA, the large RNA of the large ribosomal subunit, with regard to higher-order structures such as pseudoknots, that is, RNA structural motifs formed when a single-stranded loop region is involved in base pairing with a complementary region outside of that loop (23).

Potential pseudoknots have been identified with the aid of comparative sequence analyses on the basis of covariance of potentially base-pairing nucleotides. Accordingly, more than 15 pseudoknots have been proposed to exist in rRNA (13, 14, 17). Five of them have been tested by mutational analysis and found to be functionally significant (4, 16, 24, 25, 30). Here, we examined the functional significance of a proposed pseudoknot that could be formed between two hairpin structures in domain I of 23S rRNA (Fig. 1), one defined by nucleotides (nt) 57 to 70 and the other defined by nt 76 to 110. Covariance has been observed between nucleotides in the two loops, loop 60 (nt 60 to 67) and loop 90 (nt 88 to 94), in *Bacteria*, *Archaea*, and chloroplasts (13, 14, 17). The two hairpins are located in a part of domain I of 23S rRNA that has been implicated in specific functions in protein synthesis. In particular, it has been shown that the expression of a small fragment of *Escherichia coli* 23S rRNA (nt 74 to 136) or its antisense causes ribosomes to read through UGA nonsense mutations (2). Besides corresponding to a part of domain I, the fragment contains the nucleotides (nt 76 to 110) that form one of the two hairpins under study here. Furthermore, ribosomal protein L23, implicated in ribosome

formation (see Discussion), has been cross-linked to this region of 23S rRNA (22) and so could interact with it. Finally, the cytoplasmic ribosomes of *Eucarya* contain 5.8S rRNA, which corresponds to domain I of bacterial 23S rRNA. Studies of this rRNA have demonstrated that it plays an important role in eucaryal ribosome functions (9, 10, 31).

To address the question of whether these two hairpin loops form a functional pseudoknot by way of base pairing between specific nucleotides in each loop, we reasoned, as was done for other proposed pseudoknots, that if this kind of interaction between the loops is essential for ribosome function, then mutations that prevent the base pairing should result in impairment of cell growth and possibly other ribosome-associated properties. Conversely, compensatory mutations in the two loops should restore wild-type-like characteristics. Consequently, with site-directed mutagenesis, we introduced into a cloned rRNA operon mutations that destroyed the apparent complementarity and also, in one construct, mutations in both loops that recreated base-pairing possibilities. The bacterial cells containing each cloned mutant rRNA operon were then examined for cell growth, termination codon readthrough, and assembly of the mutant rRNAs into functional ribosomes.

MATERIALS AND METHODS

Strains and plasmids. *E. coli* strains with nonsense mutations in *trpA* were tested for suppression (stop codon readthrough) resulting in an active *trpA* protein (18). The *trpA* mutations, representing all three stop codons at each of five codon positions, 15, 102, 115, 211, and 243, have been described elsewhere (2, 19; F. T. Pagel and E. J. Murgola, unpublished results). Strain JM109 was used for most of the cloning experiments (33). The main plasmid used in this study for cloning mutations in rRNA was pNO2680, which is derived from pBR322 and contains the *rrnB* operon under the transcriptional control of the phage λ P₁ promoter (12). Media and other genetic procedures have been described elsewhere (18, 21) or are described in Results.

Construction of mutant rRNA plasmids. The rRNA mutations in pNO2680 are listed in Table 1. For production of these mutations, the segment between the *Xba*I and *Bss*HII restriction sites of pNO2680 (570 bp) was amplified by PCR and an *Xho*I restriction site was added immediately 3' to the *Bss*HII site. The segment between the *Xba*I and *Xho*I sites was inserted into the pBluescript II KS(+) vector. Site-directed changes were made by oligonucleotide-directed mutagenesis (15) using the following pairs of back-to-back primers, complementary to the wild-type segments (Fig. 1) indicated by the superscript numbers: for mutant S60, 5'-A⁷⁸CGCTTATCGCACTATAGCACG⁵⁷-3' and 5'-C⁷⁹GGTAA

* Corresponding author. Mailing address: Molecular Genetics (Box 11), M. D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030. Phone: (713) 792-8939. Fax: (713) 794-4295. E-mail: mannyj@mdanderson.org.

FIG. 1. Secondary structures of nt 50 to 119 of wild-type 23S rRNA and four mutant rRNAs. The mutational changes are indicated in boldface uppercase letters. Solid lines indicate potential base pairings between covariant nucleotides (13, 14, 17) in the two loops, loop 60 (nt 60 to 67) and loop 90 (nt 88 to 94). Broken lines indicate nucleotides that, in *E. coli*, could conceivably participate in the tertiary-structure base pairing indicated by the covariant nucleotides.

GGTGATATGAACCG^{98-3'}; for S60/S90, 5'-A⁷⁸CGCTTATCGCACTATAGC ACG^{57-3'} and 5'-C⁷⁹GGTAAGGCTAATGAACCGTTA^{101-3'}; for TL60, 5'-C⁶⁹GATAAGCGTCGGTAAGC^{86-3'} and 5'-AAC⁵⁸GTCCTTCATCGCCTC T^{42-3'}; and for TL90, 5'-AAA⁸⁷CCTTACCGACGCTTATCG^{69-3'} and 5'-CGA⁹⁵CCGTTATAACCGGCGATTT^{114-3'}. The mutational changes introduced by these primers in amplified fragments of the *mb* operon are shown in italics. The mutagenized *XbaI/BssHII* fragments were recloned into plasmids pNO2680 and pNO2680(A2058G); the A2058G mutation confers erythromycin resistance on plasmid-encoded ribosomes (29).

Restriction enzymes, ligase, and polynucleotide kinase were used according to the suppliers' recommendations. DNA sequence determinations, in which mutant plasmid preparations were subjected to automated sequence analysis using appropriate primers. Applied Biosystems reaction protocols, and an Applied Biosystems model 373A DNA sequencer, were performed at the M. D. Anderson Cancer Center automated DNA sequencing core facility.

Quantification of mutant rRNAs in subunits, monomers, and polysomes. Cell lysates were prepared from cells harboring pNO2680 and its rRNA mutant derivatives and were grown in Luria-Bertani (LB) broth with ampicillin (100

μg/ml). Sucrose gradient separation of polyribosomes, monomers (70S), and subunits (50S and 30S) was done as described by Godson and Sinsheimer (11). Purification of RNA from ribosome fractions and quantification of amounts of mutant RNA by primer extension analysis were performed essentially as described by Rosendahl et al. (25). The proportions of mutant 23S rRNA in 50S subunits, 70S ribosomes, and polysomes were estimated using the G2058 marker in 23S rRNA (25), either by itself as a control or together with each of the mutant loops described in Table 1. The oligodeoxyribonucleotide used for primer extension analysis was the icosamer 5'-TAGTAAAGGTTTCACGGGGTC-3', complementary to nt 2061 to 2080 in 23S rRNA. The relative amounts of extension products were quantified with a PhosphorImager S1 (Molecular Dynamics).

RESULTS

Plasmids encoding mutant 23S rRNAs. The two adjacent hairpins under investigation here, located in domain I of 23S rRNA, are shown in Fig. 1 (center), along with the potential

TABLE 1. Mutations constructed for this study

Mutant designation	Nucleotide sequence changes in pNO2680-encoded 23S rRNA
S60	A64U, U65A, C66G ^a
S60/S90	A64U, U65A, C66G/G88C, A89U, U90A ^a
TL60	Replacement of nt 59–68 (UGCUAUAGUG) with a UUCG tetraloop
TL90	Replacement of nt 88–94 (GAUAUGA) with a UUCG tetraloop

^a The wild-type nucleotide precedes the number, and the mutant nucleotide, in bold, follows the number.

base pairings proposed to lead to the formation of a functional pseudoknot (13, 14, 17). In a mutational test of the existence of a functional pseudoknot, we introduced base substitution mutations (Table 1 and Fig. 1) into loop 60 (mutant S60; 3-base substitution) and into both loop 60 and loop 90 (double mutant S60/S90, with complementary, potentially compensatory changes). We also replaced each of the loops separately with a UUCG tetraloop (mutants TL60 and TL90). This tetraloop motif occurs naturally in rRNA and has a unique conformation that contributes to the stability of helical structures (6, 27). Except for the pair of 3-base complementary substitutions (S60/S90), the changes disrupted the potential base pairings indicated in Fig. 1 for the wild-type structure.

Mutant rRNAs in translating ribosomes. Since the growth of the mutants was equivalent to that of the wild-type control (see below), it was necessary to verify that the mutant rRNAs were incorporated into translating ribosomes (polysomes). This was accomplished in two ways. The first, and perhaps the more meaningful way, is described below (growth in the presence of erythromycin). In the second way, the amount of each mutant rRNA in ribosome fractions was quantified by primer extension analysis (see Materials and Methods) (Table 2). The rRNA with the S60 base substitutions and the rRNA with the pair of compensatory changes, S60/S90, entered the pool of translating ribosomes efficiently but less so than the control 23S rRNA, without mutations in the loops (see below and Discussion). The directly relevant observation, however, is that the level of incorporation of S60 rRNA was the same as that of S60/S90 rRNA, with compensatory changes that restore the potential base pairings.

The rRNA in which loop 90 has been replaced with a UUCG tetraloop (mutant TL90) was incorporated into active ribosomes as effectively as the control construct (which contains the G2058 marker but not changes in the loops). However, replacement of loop 60 with the tetraloop motif (mutant TL60) virtually abolished the incorporation of the mutant rRNA into translating ribosomes (Table 2). Consequently, while the results obtained with mutants TL90, S60, and S60/S90 clearly indicate that a pseudoknot structure is not required for incorporation into polysomes, it appears that the TL60 alteration disrupted an interaction of loop 60 with some other region of rRNA or with ribosomal proteins, an interaction presumably necessary for ribosome assembly (see Discussion).

Cell growth and suppression of nonsense mutations. We examined the growth of cells containing the pNO2680 constructs at three temperatures, 42, 37, and 31°C, both by monitoring the doubling time of liquid cultures (LB broth) and by making colony diameter measurements on solid medium (LB agar). No growth differences were observed between the strain containing wild-type pNO2680 and those containing the mutant plasmids.

To examine the effects on cell growth of the mutations when present in homogeneous populations of translating ribosomes,

all of the mutant constructs were cloned into a pNO2680 derivative that contained the 23S rRNA mutation A2058G, which confers erythromycin resistance (29). Consequently, the addition of erythromycin (45 µg/ml) to the growth medium inhibited protein synthesis by wild-type ribosomes, forcing growing cells to use resistant ribosomes, which contain plasmid-encoded 23S RNA that has both a hairpin loop mutation (made in this study) and the G2058 mutation. Under these conditions, the S60, S60/S90, and TL90 mutations had no effect on cell growth in either the absence or the presence of erythromycin. The wild-type-like growth phenotype of cells harboring ribosomes with mutations was indicative of normal translational activity. On the other hand, the alteration in TL60, which exhibits no growth defect when grown in the absence of erythromycin, was nevertheless lethal for the cells when grown in the presence of the drug. This result is consistent with the results of primer extension analysis (Table 2), which showed that the TL60 alteration prevented the incorporation of the RNA into ribosomes, and indicates that this mutant rRNA does not participate in protein synthesis.

Nonsense suppression is a property of mutations in several highly conserved rRNA structures (some of which are reviewed in reference 20) and particularly is exhibited by overexpression of an rRNA fragment that contains one of the hairpin loops studied here (2; see introductory material). Therefore, we tested whether the mutations in the proposed pseudoknot caused readthrough of any of the three termination codons present at each of five codon positions in *trpA*, 15, 102, 115, 211, and 243. None did.

DISCUSSION

We tested mutationally the proposal that a two-hairpin structure (nt 57 to 70 and 76 to 110) in a region of domain I of *E. coli* 23S rRNA that has been shown to be involved in specific aspects of protein synthesis forms a pseudoknot in vivo. That proposal (13, 14, 17) was based on the observed covariance of several nucleotides in each loop in *Bacteria*, *Archaea*, and chloroplasts. Here, appropriate changes were introduced in vitro by site-directed mutagenesis of either loop (mutants S60 and TL90) to eliminate any possibility of base pairing between the loops and in both loops (mutant S60/S90) to recreate base-pairing possibilities. The bacterial cells containing each cloned mutant rRNA operon were then examined for cell growth and termination codon readthrough. In both parameters, the mutants were observed to be equivalent to the wild type. The lack of mutant phenotypes of those mutants was not explainable by a failure of the mutant rRNA to enter functional ribosomes, as shown both by primer extension analysis (Table 2) and by growth of erythromycin-resistant derivatives in the presence of

TABLE 2. Quantification of the distribution of pNO2680-encoded mutant rRNAs among ribosome fractions^a

Loop mutation ^b	% Mutant 23S rRNA (mean ± SD) in ^c :		
	50S subunits	70S monomers	Polysomes
None	57 ± 2.5	54 ± 1.9	54 ± 1.9
S60	39 ± 1.9	31 ± 2.8	32 ± 1.7
S60/S90	38 ± 2.8	31 ± 3.1	33 ± 1.9
TL90	57 ± 1.4	53 ± 0.9	53 ± 2.1
TL60	8 ± 1.2	0	0

^a See Materials and Methods for preparation of ribosome fractions and details of primer extension analysis.

^b See Table 1.

^c Each value is the average of three determinations.

erythromycin (see Results). These results indicate that, under the conditions examined, the two loops do not form a pseudoknot structure that is required for the functioning of ribosomes *in vivo* and therefore that sequence covariance does not necessarily indicate the formation of a functional pseudoknot.

Several earlier studies used the mutational approach to test the existence of functional pseudoknots (4, 16, 24, 25, 30). In those investigations, mutations that disrupted pairing in the proposed pseudoknot structure impaired cell growth, while compensatory changes that restored pairing restored wild-type-like growth. On the other hand, in this study, we demonstrated that mutations that would disrupt a proposed pseudoknot structure did not noticeably decrease the translational activity of the ribosomes, showing that nucleotide covariance does not necessarily indicate the formation of a pseudoknot essential for ribosomal functions. The reason for this difference between our results and the previous ones may be found in comparative sequence analyses. Specifically, the major difference between the pseudoknots tested previously (4, 16, 24, 25, 30) and the hairpin structures that we examined here seems to be that the latter are not conserved across all phylogenetic domains, the nucleotide covariances having been observed in *Bacteria* and *Archaea* but not in *Eucarya* (13, 14, 17). Therefore, it appears that the mutational results seen with the other tested structures were obtained because of the phylogenetic conservation of the structures rather than simply because of the particular nucleotide covariances.

Our results showed that mutations involving loop 60 of 23S rRNA affected the incorporation of that RNA into ribosomes (Table 2). The most dramatic effect was seen with TL60 RNA, very little of which was incorporated into 50S subunits and essentially none of which was incorporated into 70S ribosomes or polysomes. Only a slight effect was seen with the two loop 60 base substitution mutants. Specifically, S60 RNA, with three nucleotide changes in loop 60 that would disrupt a potential pseudoknot, and S60/S90 RNA, with the same loop 60 nucleotide changes but also containing compensatory mutations in loop 90, exhibited the same levels of incorporation into active ribosomes, but slightly less than the control.

Large ribosomal subunit protein L23, a core protein required for ribosome assembly (28), has been cross-linked to loop 60, a result consistent with the possibility that the loop is a site of rRNA interaction with L23. If such an interaction between loop 60 and L23 occurs, it is possible that the mutations in loop 60, especially the replacement of the loop by the UUCG tetraloop, interfere with that interaction, thereby affecting ribosome assembly and incorporation of the mutant RNA into ribosomes. Alternatively, the dramatic effect of TL60 on the incorporation of 23S RNA into ribosomes may be due to the fact that it is the only one of the tested mutants whose mutations disrupt a possible base-pairing interaction between covariant nt 67 and 74 (14).

Even though we did indeed examine the mutants under a few different conditions, for example, low and high temperatures, glucose minimal medium and rich medium, and liquid and solid growth media, we cannot rule out the possibility that the existence of a functional pseudoknot might be revealed (in the mutants) only under special stress conditions (such as starvation or increased salt or iron concentrations) or during stationary-phase growth.

ACKNOWLEDGMENTS

We are grateful to K. Hedenstierna for suggesting the introduction of the tetraloops, to K. Hedenstierna and F. Pagel for helpful technical advice and discussions, and to W. J. Pagel for expert editorial consultation. We thank an anonymous reviewer for the suggestion that an

interaction between nt 67 and 74 may play a role in 23S rRNA incorporation into ribosomes.

This work was supported by a grant to E.J.M. from the National Institute of General Medical Sciences (GM21499). The automated DNA sequencing core facility at the M. D. Anderson Cancer Center was supported by grant CA16672 from the National Cancer Institute.

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