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Genetic Analysis of a Structural Motif Within the Conserved 530 Stem-Loop of *Escherichia coli* 16S rRNA

SZATKIEWICZ, JIN P.[1], HYUNDAE CHO[1], SANG-MI RYOU[2], JONG-MYUNG KIM[2], PHILIP R. CUNNINGHAM[1], AND KANGSEOK LEE[2]*

[1]*Department of Biological Sciences, Wayne State University, Detroit, MI, 48202, U.S.A.*
[2]*Department of Life Science, Chung-Ang University, Seoul 156-756, Korea*

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Abstract The 530 stem-loop is a 46 nucleotide stem-loop structure found in all small-subunit ribosomal RNAs. Phylogenetic and mutational studies by others suggest the requirement for Watson-Crick interactions between the nucleotides 505–507 and 524–526 (530 pseudoknot), which are highly conserved. To examine the nature and functional significance of these interactions, a random mutagenesis experiment was conducted in which the nucleotides in the proposed pseudoknot were simultaneously mutated and functional mutants were selected and analyzed. Genetic analysis revealed that the particular nucleotide present at each position except 524 was not exclusively critical to the selection of functional mutants. It also indicated that base-pairing interactions between the positions 505–507 and 524–526 were required for ribosomal function, and much weaker base-pairing interactions than those of the wild-type also allowed high ribosomal function. Our results support the hypothesis that the 530 pseudoknot structure may undergo a "conformational switch" between folded and unfolded states during certain stages of the protein synthesis process by interacting with other ligands present in its environment.

Key words: rRNA, 530 stem-loop, instant evolution, pseudoknot

It is now well accepted that ribosomal RNA is the catalytic portion of the ribosome, which makes it the most complex ribozyme known [8, 15]. Several regions of the small subunit RNA are highly conserved and are believed to play a fundamental role in the protein synthesis process of all organisms [4, 16, 26, 36]. One of these conserved regions is commonly referred to as the 530 stem-loop (Fig. 1) [10, 11].

*Corresponding author
Phone: 82-2-820-5241 ; Fax: 82-2-822-5241 ;
E-mail: kangseok@cau.ac.kr

This stem-loop contains two modified nucleosides, Ψ516 and m^7G527, and a pseudoknot structure between residues 524–526 and 505–507 that contributes to the complexity of its structure [2, 10, 28, 35, 38]. The function or role of nucleotides in this hairpin has been implicated in tRNA binding [21, 23, 24, 37], translational fidelity [22, 27, 30, 34], streptomycin resistance [9, 20, 28, 29], and protein binding [3, 5, 6, 25, 31, 33]. The nucleotides involved in the 530 pseudoknot are protected from chemical attack by assembly of ribosomal protein S12 [32], whereas strains harboring a mutant ribosomal protein S4, that leads to a ribosomal ambiguity (*ram*) or "error-prone" ribosome, exhibit

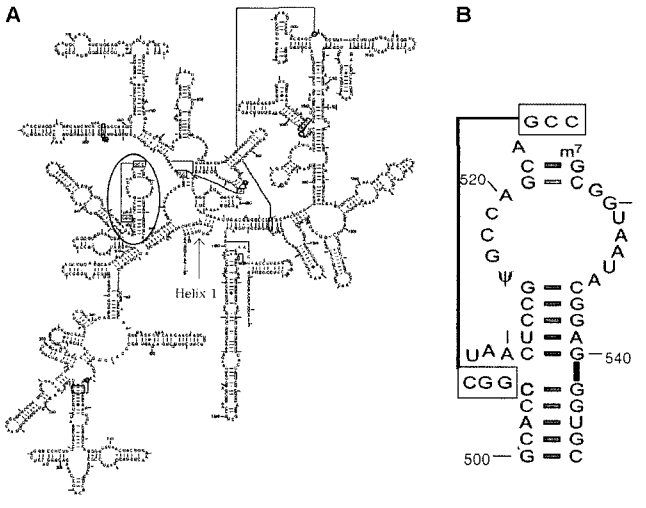

Fig. 1. A. A secondary structure of *E. coli* 16S rRNA. The location of the 530 stem-loop and the helix 1 are indicated with an oval and an arrow, respectively. **B.** The secondary structure of the 530 stem-loop.
A proposed pseudoknot is indicated between nucleotides 505–507 and 524–526.

an increased chemical reactivity in two RNA sites at the interface between the central pseudoknot (positions between 17–19 and 916–918) present in helix 1 (H1) and the 530 pseudoknot [1].

As a first step in our studies on rRNA structure/function, we examined Watson-Crick base-pairings between the nucleotides 505–507 and 524–526 to know (1) whether the interactions do indeed exist and (2) what functional constraints exist at each position of the proposed interaction.

Materials and Methods

Bacterial Strains and Media

All plasmids were maintained and expressed in *Escherichia coli* DH5 (*supE44*, *hsdR17*, *recA1*, *endA1*, *gyrA96*, *thi-1*, *relA1*) [12]. Cultures were maintained in LB containing 100 μg/ml ampicillin (LB-Ap100). To induce the synthesis of plasmid-derived rRNA from the *lacUV5* promoter, IPTG was added to a final concentration of 1 mM. Unless otherwise indicated, transformants were grown in SOC medium.

Plasmids and Mutagenesis

The construction scheme for p16ST containing the randomized 530 stem-loop was similar to the one described by Lee *et al.* [19]. Briefly, random mutations were introduced at the positions 505–507 and 524–526 in 16S rRNA in p16ST by replacing the wild-type sequence between AvrII and BglII with a fragment containing PCR-directed random mutations. p16ST is a pUC19 derivative containing all the features of pRNA122 [18, 19], except that the entire 23S rRNA was deleted. It has an advantage over pRNA122 in introducing mutations into 16S rRNA, since it provides more unique restriction enzyme sites and more plasmid DNA for sequencing.

All PCR-directed mutagenesis were performed essentially by the method of Higuchi [13] and the primers used were 16S-AvrII (5' ACGTCGCAAGACCAAAGAGG), 16S-BglII (5' CTCTCAAATTTTCGCAACAC), 16S-537F (5' GGAGGGTGCAAGCGTTAATC), and 530-6N (5' TTCC-GATTAACGCTTGCACCCTCCGTATTACCGCNNNTG CTGGCACGGAGTTANNNGGTGCTTCT). Transformants were incubated in SOC medium containing 1 mM IPTG for four hours to induce rRNA synthesis and then plated on LB agar containing ampicillin (100 μg/ml) and 1 mM IPTG with or without chloramphenicol (100 μg/ml). Next, 54 transformants were randomly selected and sequenced.

Minimum Inhibitory Concentration (MIC)

MICs were determined by standard methods in microtiter plates. Overnight grown cultures in LB-Ap100 were diluted and induced in the same medium containing 1 mM IPTG for 2–3 hours. Approximately 10^4 of the induced cells were then added to wells containing LB-Ap100+IPTG (1 mM) and chloramphenicol at increasing concentrations. Cultures were grown for 24 h, and the lowest concentration of chloramphenicol that completely inhibited growth was designated as the MIC.

Results

Genetic Analysis of the Proposed Watson-Crick Interactions in the 530 Stem-Loop by Instant Evolution

We adopted a novel genetic approach [18, 19] to test the existence and to examine the nature of the proposed interactions by randomly mutating the nucleotides 505–507 and 524–526 involved and then by selecting alternative functional sequences. In this specialized *in vivo* system, the chloramphenicol acetyltransferase (CAT) reporter message was translated exclusively by plasmid-derived ribosomes that cannot translate normal cellular messages (Fig. 2) [17–19]. Thus, the level of function of mutant plasmid-derived ribosomes could be assayed *in vivo* by growing clones in the growth media containing different levels of chloramphenicol and determining the minimal inhibitory concentration (MIC): Nonfunctional mutations in plasmid-encoded ribosomes result in lower chloramphenicol resistance of the cell, whereas compensatory changes that restore base-pairing should result in functional ribosomes. Sequence analysis of cloned functional mutants revealed nucleotides that play a direct role in function (invariant) and those that play an indirect structural role (covarying positions). This approach to discriminate between nucleotides involved in the rRNA structure and function is termed "instant evolution" [18].

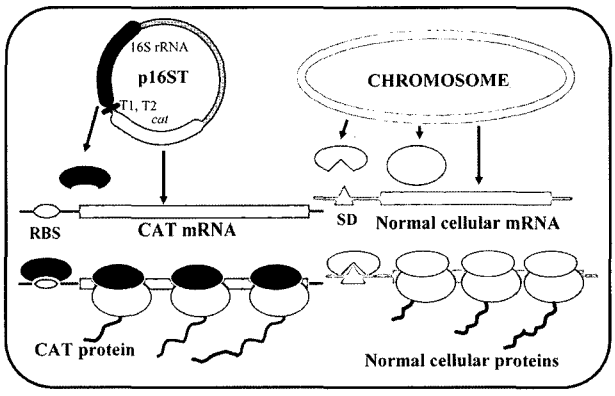

Fig. 2. A system for the genetic analysis of rRNA function.
Black ovals and white ovals indicate plasmid-encoded 30S ribosomal subunits and chromosome-encoded ribosomes, respectively. Transcription of 16S rRNA is driven by the inducible *lacUV5* promoter. CAT gene (*cam*) transcription is constitutive. The message binding sequence (MBS) of 16S rRNA and rRNA binding sequence (RBS) of the CAT message were changed to allow targeting of CAT mRNA specifically to plasmid-encoded ribosomes [17, 19]. In the absence of inducer (IPTG), cells carrying p16ST are resistant to 75 μg/ml chloramphenicol (MIC=100 μg/ml) because of low-level transcription of the 16S rRNA. Induced cells (1 mM IPTG) are resistant to 700 μg/ml (MIC=750 μg/ml). T1 and T2, *rrnB* transcriptional terminators. SD, Shine-Dalgarno sequence.

Table 1. Phylogenetic variation of 530 peudoknot nucleotides.

Base -pair	G C	C G	U A	A U	G U	U G	A A	A C	A G	C A	C C	C U	G A	G G	U C	U U	Gap
505:526	98.6	0.2	0.2	0.1	0.4	0	0	0	0	0	0	0	0.1	0.1	0.1	0	0.1
506:525	97.6	0	0.1	1.1	0	0	0	0.5	0	0	0.1	0	0	0	0.1	0	0.2
507:524	0	90	8.7	0	0	0.5	0	0	0.2	0.1	0.5	0.1	0	0	0	0	0.1

8,513 small subunit rRNA sequences from all known organisms were used to analyze phylogenetic variation of 530 peudoknot nucleotides [4]. The 5' nucleotide (with a lower position number) appears first in each pair, and the 3' nucleotide (higher position number) appears second. The frequencies appearing in the columns are calculated as: [(number of sequences containing the noted base-pair) / (total number of sequences)]×100%. Gap indicates the percentage of sequences with deletions at one or both positions for the given base-pair.

Sequence Analysis of Functional Mutants

The interaction involving the nucleotides 505–507 and 524–526 in 16S rRNA, proposed by Woese and Gutell [35], is shown in Fig. 1, and phylogenetic variations in these positions are shown in Table 1. Six positions (505–07 and 524–26) were simultaneously mutated in the 530 region. Functional mutants were selected in *E. coli* DH5 cells and analyzed for the mutated sequences and their effect on ribosome function. A total of 680,000 transformants were obtained to ensure that all of the 4,096 (4^6) mutant sequences were represented in a selected pool with >99.9% confidence [7]. Approximately 5.3% of plated cells survived on media containing 100 μg/ml chloramphenicol. Fifty-four chloramphenicol-resistant functional mutants were randomly selected and sequenced. Among them, three wild-type sequences were present, and eleven duplicate and two triplicate sequences were excluded from further statistical analyses. A total of 39 unique mutant sequences were obtained and analyzed (Table 2). Although nucleotide preferences in the selected mutants (Table 2) were similar to those observed in the phylogenetic data (Table 1), the mutant sequences selected in this study showed much more variability than those found in nature. This may be because all of the positions in the pseudoknot were mutated simultaneously, allowing normally deleterious mutations in one position to be compensated by mutations at other positions, which is unlikely to occur in nature.

Nucleotide Distribution

To determine if selection of nucleotides in the chloramphenicol-resistant clones at each of the positions was random, the distribution of nucleotides at each position was examined for goodness of fit to an even distribution among possible substitutions. Results of these analyses are presented in Fig. 3. At each mutated position, we observed nonrandomness and alternative mutations as well.

The Effect of Nucleotide Identity upon Translational Efficiency

Nonrandom distribution of nucleotides among the selected functional clones suggests that nucleotide identity within the mutated sequences might affect the CAT translation or the level of ribosome function. To determine the effect of nucleotide identity upon translational efficiency, the mean activities (MICs) of ribosomes containing all mutations at a given position were compared by single-factor analysis of variance of the effect of nucleotide identity on the level of ribosome function at each mutated position for the chloramphenicol-resistant isolates. As shown in Table 3, no significant functional differences were observed among the mutants at each position, with the only exception being at the position 524 ($P<0.05$). Thus, it appears that the particular nucleotide present at each position, except 524, was not exclusively critical to the selection of functional mutants. These data are consistent with the current paradigm that the six nucleotides might play a primary structural role.

Covariation of Nucleotides Involved in the Proposed Interaction

Since the interactions of the mutated sequences may also significantly affect ribosome function, covariations within each mutated nucleotide were determined to examine whether the presence of a particular nucleotide at one position affected nucleotide selection at other mutated positions. These analyses are shown in Table 4. Significant covariations were observed only between the proposed base-pairing positions ($P<0.001$). These data indicate that the 530 stem-loop is structurally constrained.

To test the nature of the covariation, the distribution of Watson-Crick base-pairs, wobble base-pairs and mismatches at each proposed interaction were examined for goodness of fit to an even distribution among possible substitutions. We observed nonrandomness for each of the interactions, and a significant base-pair preference after selection was shown (Table 4). Interestingly, five of the selected mutant sequences carried a G-C base-pair at the positions 507 and 524, although it is not present in nature (Table 1). However, all the mutant sequences carrying a G-C base-pair at the positions 507 and 524 also contained additional mutations at other positions, indicating that a G-C base-pair at these positions is deleterious for ribosome function and is compensated for by mutations at other positions in the selected mutants.

Selection of functional mutants by simultaneous mutagenesis resulted in extensive pairings between the mutated positions. To examine the potential effect of mutations on

Table 2. Sequences of functional isolates.

Sequence					MIC	ΔG	Sequence					MIC	ΔG
5' 3'	505 526	506 525	507 524	3' 5'	μg/ml	kcal/mol	5' 3'	505 526	506 525	507 524	3' 5'	μg/ml	kcal/mol
wt	G C	G C	C G		750	−2.8		C G	G C	G C		550	−2
	G C	G C	C U		750	1.6		G C	C C	U G		500	−0.3
	G C	G C	A G		750	0.8		A U	G C	G C		550	−1.2
	U A	U A	C G		750	1.1		G C	A U	A U		550	2.0
	G C	C G	C G		750	−2.8		G U	G U	C C		500	2.8
	A C	G C	C G		700	−0.5		G C	C G	G U		500	−0.5
	G C	C G	G C		700	−2.1		C U	G C	C G		500	−1.0
	G C	U A	A U		700	1.8		C A	G C	C G		450	−1.5
	G C	U U	C G		700	5.1		A U	U A	C G		450	0.9
	G C	C G	A U		700	−0.2		A U	C G	G C		450	−0.4
	G C	G C	U U		700	0.6		C G	G C	A G		400	1.7
	G C	U C	C G		650	2.1		A U	G C	U A		400	1.5
	G A	G C	C G		650	−1.2		U C	U C	C G		450	4.8
	A C	G C	G G		650	−0.1		U G	U A	C A		400	2.9
	G C	G G	C G		600	0.4		G C	C U	C G		400	2.5
	G C	G C	C C		600	0.4		G C	G U	G U		300	0.8
	U A	C G	C G		600	−1.3		G C	A U	U U		200	1.9
	G C	U U	G C		600	0.8		U A	C G	U A		200	1.3
	G C	G C	U G		600	−0.8		A G	U U	C A		200	4.1
	U C	G C	C G		550	−0.3		U U	G C	C G		200	−0.7

Mutated nucleotides are underlined. Watson-Crick and G-U Wobble base-pairs are indicated as point. ΔG is predicted using the Mfold program by calculating the minimum folding energy of RNA sequences containing 505-507UAAA524-526.

Table 3. Effects of the nucleotide identity upon translation.

Position	P-value
505	0.310
506	0.376
507	0.01
524	0.011*
525	0.587
526	0.109

Data from Table 1 were analyzed to determine the effect of nucleotide identity upon CAT translation by analysis of variance (ANOVA). *, P<0.05.

folding of the hairpin, the minimum-energy secondary structure of each mutant was predicted using Mfold [14]. The free energy data of all the selected functional mutants ranged from −2.8 to +5.1. To assess the relationship between free energy and ribosome function (MICs), a regression analysis was performed. However, the correlation coefficient between ΔG and MIC was not significant (r^2=0.0094).

DISCUSSION

We conducted an "instant evolution" [18] experiment, and analyzed functional sequences of the random 530 stem-loop to examine the hypothesis of the pseudoknot structure in the conserved 530 stem-loop. This genetic approach allowed us to identify a tertiary motif in functional ribosome involving the nucleotides 505–507 and 524–526. It appeared that the 530 stem-loop could accommodate sequence variants while retaining this motif and ribosomal function. Preference for the wild-type nucleotides was observed; however, many mutants that do not violate the constraints of the preference were also highly functional.

Our genetic analysis revealed that base-pairing interactions between the positions 505–507 and 524–526 were required

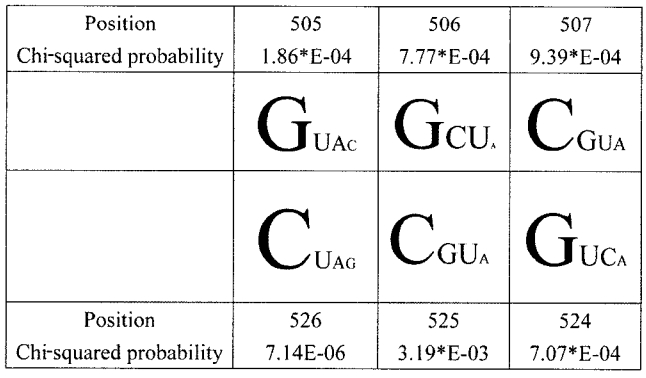

Fig. 3. Analysis of 530 stem-loop mutants. Nucleotide preferences for each mutated position are shown and scaled to reflect the frequencies in Table 2. Chi-squared analysis was used to test the null hypothesis that distribution at each position occurred randomly.

Table 4. Paired distribution of selected nucleotides.

Covariation	505/526	506/525	507/524
Chi-squared P	***	***	***
A/U, U/A	7	7	5
C/G, G/C	22	23	22
G/U, U/G	2	2	3
Mismatch	9	8	10
Chi-squared P (Base-pair/Mismatch)	***	***	*

To determine if the presence of a particular nucleotide at one position affected the selection of nucleotides at another position, we used Chi-square analysis to test the null hypothesis that nucleotide identity at one position did not affect distribution of nucleotides at other positions. Base-pair and mismatch distribution among the selected nucleotides are also presented. The Chi-squared statistic for the pair is indicated along with the probability: *, P<0.05; **, P<0.01; ***, P<0.001.

for ribosome function. However, much weaker base-pairing interactions than those of wild-type also allowed high ribosomal function, indicating that the pseudoknot structure may be flexible and may even undergo a "conformational switch" between folded and unfolded states during certain stages of the protein synthesis process. The crystal structure of 30S from *Thermus thermophilus* supports this hypothesis, which showed that the environment of the 530 stem-loop is composed of the 16S rRNA helix 1, ribosomal proteins S4, S5, and S12, and the S4-S5, and that the central pseudoknot (H1)-530 pseudoknot interaction surfaces are contiguous [6]. These structural data indicate that this hybrid surface may move during protein synthesis, and S4-S5 interaction may possibly modulate the interaction of the central pseudoknot with the 530 pseudoknot.

Folding of the wild-type sequence and most functional mutant sequences revealed that the minimum-energy structure contained the base-pairs, although a very low correlation coefficient was observed between ΔG and MIC. One possible explanation is that base-pairing is facilitated by other ligands such as the central pseudoknot or ribosomal proteins S4, S5, or S12. Three mutants with the lowest ribosome activity (MIC 200 μg/ml) were predicted to form minimum-energy secondary structures. It is possible that, for some of the less functional mutants, decreased function could be caused by the violation of the minimum requirement of base preferences, since the 530 region is highly conserved. It also needs to be pointed out that the stability of model pseudoknot structure is no more than the stability of the most stable component helix.

All the phylogenetic, structural modeling, and genetic and biochemical evidence indicated a dynamic and crucial role of the 530 pseudoknot, and we are currently in a process to characterize partially functional mutant ribosomes containing mutations in the 530 pseudoknot to understand in which specific aspect of ribosome function it plays a role.

Acknowledgments

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