

The importance of base pairing in the penultimate stem of *Escherichia coli* 16S rRNA for ribosomal subunit association

Matthew A. Firpo⁺ and Albert E. Dahlberg*

Department of Molecular and Cell Biology and Biochemistry, Box G, Brown University, Providence, RI 02912, USA

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ABSTRACT

The influence of base pairing in the penultimate stem of *Escherichia coli* 16S rRNA (defined as nt 1409–1491) on ribosome function has been addressed by the construction of mutations in this region of rRNA. Two sets of mutations were made on either side of a structurally conserved region in the penultimate stem that disrupted base pairing, while a third set of mutations replaced the wild-type sequence with other base pair combinations. The effects of these mutations were analyzed *in vivo* and *in vitro*. The mutations that disrupted base pairing caused significant increases in cell doubling times as well as a severe subunit association defect and a modest increase in frame shifting and stop codon read-through. Restoration of base pairing restored wild-type growth rates, decoding and subunit association, indicating that base pairing in this region is essential for proper ribosome function.

INTRODUCTION

The conserved 3' minor domain of small subunit rRNA contains RNA elements involved in mRNA decoding, initiation of translation and interaction with the large subunit (1–4). In *Escherichia coli* 16S rRNA this structure consists of three conserved single-stranded regions separated by two conserved stem-loop structures (Fig. 1). The region encompassing bases 1395–1408 and 1492–1505 has been shown to be important for decoding, while the terminal dimethyl adenosine stem (bases 1506–1529) and the 3' single-stranded region are involved in translational initiation events (1–3). The penultimate stem between bases 1409 and 1491 (Fig. 1), although not conserved at the sequence level, is a conserved structure (5,6), which suggests that the presence of the helix is important for translation. Based on phylogenetic comparisons the penultimate stem can be divided into three sub-domains: the base helix (bases 1409–1416/1484–1491), which is structurally conserved in all classes of ribosomes; the central helix (bases 1419–1430/1470–1481), which is structurally conserved in all classes except mitochondrial ribosomes; and a region of variable structure (bases 1431–1469) (6).

Our understanding of the function of the penultimate stem has come primarily from genetic studies. There is evidence to suggest that the stem is involved in decoding, interaction with antibiotics (7–10) and subunit association (11,12). Various mutations at the base of the helix (nt 1409 and 1491) caused either increased or decreased translational fidelity (7) and conferred resistance to aminoglycoside antibiotics (10). Fidelity effects have also been seen with ribosomal ambiguity mutations at positions 1469 and 1483 (9,13). In models of the 70S ribosome the penultimate stem has been placed at the interface between the 30S and 50S subunits (14–16). Deletion of the helix resulted in aberrant subunit association (17), as did a mutation at position 1416 (11,12). Footprinting studies have implicated the penultimate stem in ribosomal protein S20 binding (18) and subunit association was impaired when S20 was deleted (19,20). Like the mutations at positions 1469 and 1483, this S20 deletion strain also had an increased level of miscoding errors.

Most of the functional studies of the penultimate stem have focused on the highly conserved base helix. In the present report we have used compensatory base change analysis to investigate the function of the central helix of the penultimate stem. We have constructed mutations on either side of the helix that introduced mismatched bases into the helix. The mutant ribosomes had a decrease in growth rate, translational fidelity and ability to associate with 50S subunits. Compensatory mutations that restored base pairing in the helix restored ribosomal functions to wild-type or near wild-type levels, indicating that the secondary structure is essential for proper ribosome function.

MATERIALS AND METHODS

Bacterial strains and plasmids

Strain SU1675 [(F' *lacI*^q Tn::5 *kan*^r) *recA* Δ(*lac-pro*) *thi* *ara*], a derivative of CSH26 (21), was used for plasmid propagation and analysis. The low copy plasmid pM011 (22) contained the entire *E.coli* *rrnB* operon with the wild-type P₁P₂ promoters on a pSC101-based replicon. Plasmid pMM1192 was derived from plasmid pM011 and contained a C to U base change at position 1192 in 16S rRNA that conferred resistance to spectinomycin. Strain MC140 [F⁻ *thi* Δ(*lac-pro*) *recA*], containing the temperature-sensitive λ *cI* repressor on plasmid pLG857 (23), was used for

*To whom correspondence should be addressed. Tel: +1 401 863 2223; Fax: +1 401 863 1182; Email: albert_dahlberg@brown.edu

⁺Present address: Department of Biochemistry, School of Medicine, 50 North Medical Drive, Salt Lake City, UT 84112, USA

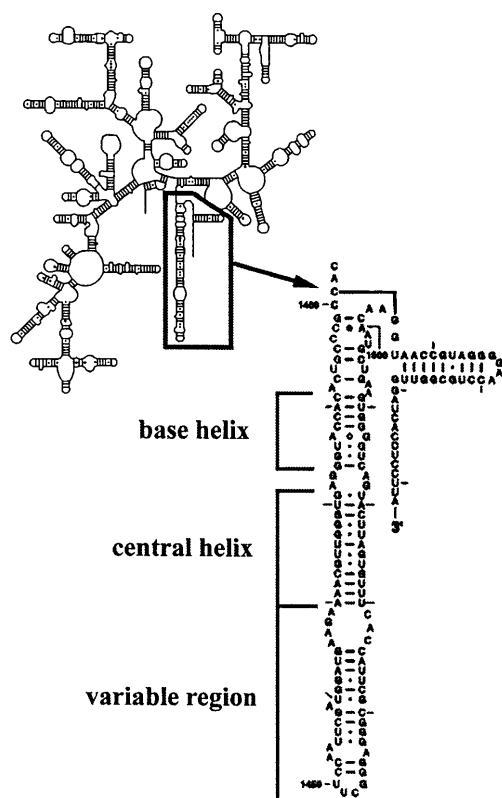


Figure 1. Secondary structure map of *E. coli* 16S rRNA (36) showing an enlargement of the 3' minor domain. The sub-domains of the penultimate stem, as described in the text, are the base helix (bases 1410–1416/1484–1490), the central helix (bases 1419–1430/1470–1481) and the variable helix (bases 1431–1469).

propagation and analysis of pNO2680-based plasmids carrying the *rmB* operon under control of the inducible λ leftward promoter (24). Plasmid p Δ H1192 was a derivative of pNO2680 that contained the C1192U mutation. Plasmid pKK3535 (25,26) was a pBR322-derived plasmid containing the entire *rmB* operon. Plasmids containing *lacZ* genes (pSG series), used in fidelity assays, have been described elsewhere (7,22).

Construction of rRNA mutations

Multiple base substitutions in 16S rRNA (U1472A, U1474A, G1475U and U1477A) were made as described (27) by oligonucleotide-directed mutagenesis on a single-stranded uracil-containing M13 substrate. The M13 substrate also carried the C1192U mutation. A second oligonucleotide was used to introduce compensatory mutations (G1423U, U1425G, G1426U and A1428U) on the same substrate. Mutations on both sides of the penultimate helix were made with the second oligonucleotide using single-stranded M13 DNA containing the first set of mutations (Fig. 2). All three mutant constructs were initially subcloned into pMM1192 and then transferred into pKK3535 and p Δ H1192. Mutant plasmids were named using the first mutated nucleotide in the following manner: pMFM72 contained mutations A1472, A1474, U1475 and A1477; pMFM23 had U1423, G1425, U1426 and U1428; pMFM23/72 carried both sets of mutations. The corresponding p Δ H1192-based plasmids were

	a	b	c	d
	wt	1472	1423	23/72
	I G A A G	I G A A G	I G A A G	I G A A G
	G·U	G·U	G·U	G·U
	1420-U-A-1480	U-A	U-A	U-A
	G·C	G·C	G·C	G·C
	G·U	G·U	G·U	G·U
	G·U	G·U	G·U	G·U
	U-A	U-A	U-A	U-A
	U·G	U·G	U·G	U·G
	C·G	C·G	C·G	C·G
	A·U	A·U	A·U	A·U
	1430-A-U-1470	A·U	A·U	A·U
	A G	A G	A G	A G
	I A	I A	I A	I A
D.T.	57±4	71±5	73±8	57±6

Figure 2. Mutations in the central helix of the penultimate stem. Italicized nucleotides represent the base changes introduced into the three mutant plasmids. Doubling times (D.T.) for strains containing wild-type plasmid (a) or the three mutant plasmids (b–d) are indicated beneath each structure. (a) Wild-type sequence and structure; (b) sequence and potential structure of single-strand mutant 1472, indicating base changes at positions 1472, 1474, 1475 and 1477; (c) sequence and potential structure of single-strand mutant 1423, indicating base changes at positions 1423, 1425, 1426 and 1428; (d) sequence and potential structure of double mutant 23/72.

pMFL72, pMFL23 and pMFL23/72. Growth rates in Luria–Bertani (LB) broth were determined using a Klett–Summerson colorimeter.

Preparation of ribosomes and ribosomal subunits

Cells containing plasmid pMM1192, pMFM23, pMFM72 or pMFM23/72 were grown in LB broth supplemented with 34 μ g/ml chloramphenicol. At late logarithmic stage growth cells were harvested and ribosomes were prepared as described (28). Free subunits were prepared from ribosomal aliquots by dialysis in dissociation buffer (20 mM Tris–HCl, pH 7.6, 60 mM KCl, 1.5 mM MgCl₂, 1 mM DTT) for 6 h at 4°C with two buffer changes. Dissociated subunits were either used directly in subunit re-association assays or separated into subunits by centrifugation in 10–30% sucrose gradients in dissociation buffer for 20 h at 21 000 r.p.m. in a SW 28 rotor at 4°C. Subunit fractions were pooled and pelleted. Polyribosomes were prepared as described (29) and separated on 10–30% sucrose gradients containing 20 mM Tris–HCl, pH 7.6, 100 mM KCl, 15 mM MgCl₂, 1 mM DTT centrifuged for 15 h at 21 000 r.p.m. at 4°C. Fractions were collected and precipitated with 2.5 vol 95% ethanol at –20°C.

Primer extension analysis

The proportion of plasmid-encoded rRNA in total cellular RNA, 30S subunits, 70S ribosomes and polyribosome preparations was quantified by primer extension analysis (30) using a ³²P 5'-end-labeled primer (5'-TCGTAAGGCCATGATGACTTGAC) complementary to bases 1193–1216 in 16S rRNA. In the presence of dTTP, dGTP, dCTP and ddATP, extension of the primer by reverse transcriptase proceeded to the first adenosine residue and was halted by incorporation of ddATP. Subsequent analysis on sequencing gels allowed quantification of wild-type and mutant rRNA. Autoradiograms were scanned using an LKB Ultrascan XL laser densitometer.

Subunit re-association assays

The ability of mutant subunits to form 70S ribosomes was analyzed in subunit re-association assays. Aliquots of dissociated subunits (4 mg) in 100 ml dissociation buffer, but with 1.5, 5, 10 or 15 mM MgCl₂, were incubated at 37°C for 50 min and then layered onto 10–30% sucrose gradients at the corresponding MgCl₂ concentration. Gradients were centrifuged in a SW28 rotor for 18 h at 21 000 r.p.m. (4°C) and fractionated. Peaks corresponding to 30S subunits and 70S ribosomes were precipitated in ethanol and the rRNA analyzed by primer extension.

Assay for β-galactosidase activity

Cultures of strain MC140 carrying pLG857, one of the pSG series plasmids, and one of the pLΔH plasmids expressing either wild-type or mutated rRNA were grown to saturation at 30°C in LB supplemented with 50 μg/ml neomycin, 12.5 μg/ml tetracycline and 200 μg/ml ampicillin. The cultures were diluted 1:50, incubated for 150 min at 42°C and then assayed as described (21).

RESULTS

Construction and expression of penultimate stem mutations

Oligonucleotide-directed mutagenesis was used to introduce four base changes on one side of the penultimate stem (A1472, A1474, U1475 and A1477; Fig. 2b), generating a single-strand mutant referred to as 1472. A second rRNA mutant (designated 1423) was made with base changes complementary to the 1472 mutations (U1423, G1425, U1426 and U1428; Fig. 2c). A third mutant (designated 23/72) was generated with both regions mutated such that base pairing was restored (Fig. 2d). Note that the structure of the double mutant restored a non-canonical U-G pair at 1425/1475 but replaced non-canonical G-U pairs at 1423/1477 and 1426/1474 with U-A pairs. These mutants were initially subcloned into the high copy vector pKK3535. However, in this vector expression of the 1423 and 1472 mutants resulted in an unstable growth phenotype yielding both large and small colonies and reversion to faster growing clones, suggesting that these rRNA mutants had deleterious effects on ribosome function when expressed at high levels. Therefore the mutants were subcloned into pMM1192, a low copy number plasmid, and pLΔH1192, a plasmid with an inducible promoter, for more stable expression.

The effects of the three mutants on growth rates were determined in strain SU1675 using the low copy plasmid. Expression of the two single-strand mutants 1423 and 1472 resulted in an increased doubling time (73 and 71 min respectively, compared with a wild-type doubling time of 57 min), indicating that disruption of the penultimate stem was deleterious to the cell (Fig. 2). However, the double-strand mutant 23/72 showed a wild-type doubling time (57 min). This suggested that secondary structure of the stem, not primary sequence, was the more important element in this region for proper ribosome function.

Effects of penultimate stem mutations on translational fidelity

Since mutations in the base helix and at positions 1469 and 1483 had been reported to have significant effects on translational fidelity (7,9,13), we examined the effect of mutations in the

central helix on accuracy of decoding. Using plasmids containing the *rrnB* operon transcribed from the inducible λ P_L promoter we analyzed the ability of mutant ribosomes to cause frameshifts and read-through of stop codons in a series of *lacZ* reporter genes (Table 1). A modest increase (50%) in stop codon read-through was observed for the single-strand mutant 1423, while the 1472 mutant had less of an effect on stop codon read-through. Both caused a modest (28%) increase in frameshifting. The double-strand mutant 23/72 displayed levels of frameshifting and read-through virtually identical to wild-type levels. This result, coupled with the recovery in growth rate observed for the double-strand mutant, indicated that the base paired stem structure was important for optimal decoding.

Table 1. Effect of penultimate stem mutations on translational fidelity

<i>lacZ</i> plasmids	β-Galactosidase activity (Miller units)			
	1192 (wt)	1472	1423	23/72
Wild-type				
pSG25	5500 ± 199	5400 ± 135	5532 ± 224	5153 ± 169
Stop codon mutations				
pSG163 (UAG)	56.2 ± 5.6	60.0 ± 2.7	83.5 ± 4.5	55.2 ± 3.7
pSG3/4 (UGA)	138.5 ± 7.9	153.8 ± 7.6	216.0 ± 7.7	134.7 ± 8.1
pSG853 (UAA)	17.5 ± 1.4	22.1 ± 1.4	25.7 ± 1.3	17.4 ± 1.1
Frameshift mutations				
pSG12dP (-1)	72.3 ± 1.7	91.9 ± 3.4	90.6 ± 3.6	70.0 ± 1.7
pSGCAUGGA (-1)	10.3 ± 0.5	12.0 ± 0.4	12.9 ± 0.5	10.5 ± 0.9
pSGCCCU (+1)	19.5 ± 0.7	23.4 ± 1.1	25.1 ± 1.4	18.3 ± 2.0
pSGlac7 (+1)	61.0 ± 2.4	77.9 ± 1.1	74.1 ± 1.9	60.5 ± 1.2

Values represent the average of five to ten independent measurements ± 1 SD. β-Galactosidase activities (in Miller units) were measured after induction of transcription of plasmid-encoded rRNA at 42°C for 150 min. The level of enzyme synthesized from the wild-type plasmid is not a good quantitative measure of mutant ribosome activity since the mRNA is not limiting.

Distribution of plasmid-encoded rRNA in 30S subunits, 70S ribosomes and polyribosomes

The distribution of plasmid-encoded rRNA in 30S subunits, 70S ribosomes and polysomes was determined in strain SU1675 carrying the low copy pMM1192-derived plasmids. Cells pelleted from exponentially growing cultures were lysed gently with detergent and ribosomes and polysomes were fractionated by sucrose density gradient centrifugation. The results of primer extension analysis of plasmid-encoded 16S rRNA from 30S, 70S and polyribosome fractions are shown in Table 2. Plasmid encoded 16S rRNA was present in polysomes, indicating that all three mutant rRNAs were processed and assembled into functional ribosomes. However, the fraction of all three mutants in 70S ribosomes was reduced, indicating that mutant subunits were less stable as 70S ribosomes. It was surprising that the double mutant also showed this defect, given that the restoration of base pairing restored both growth rate and accuracy to wild-type levels. This suggests that the primary sequence, in addition to secondary structure of the penultimate stem, may have a role in ribosome function.

Table 2. Distribution of plasmid-encoded rRNA in 30S subunits, 70S ribosomes and polysomes

Plasmid	30S	70S	Polysomes
pMM1192	58.6 ± 4.8	58.0 ± 2.9	54.0 ± 1.5
pMF72	61.1 ± 8.4	40.7 ± 0.4	66.7 ± 4.7
pMF23	78.8 ± 4.5	47.1 ± 1.5	68.4 ± 7.9
pMF23/72	67.7 ± 6.2	48.9 ± 2.7	70.6 ± 2.6

Sucrose gradient fractions of 30S subunits, 70S ribosomes and polysomes were analyzed by primer extension as described in Materials and Methods. Numbers represent percentages of plasmid-encoded rRNA in each fraction ± 1 SD and were determined by the formula (plasmid-encoded rRNA/plasmid-encoded rRNA + host-encoded rRNA) × 100. Values are percent mutant ribosomes in each peak and cannot be added to get total percent mutant RNA since most of the total rRNA is in the 70S peak.

Subunit re-association of mutant ribosomes

To investigate the apparent subunit association defect further the ability of dissociated subunits to re-form 70S ribosomes was analyzed. Equimolar amounts of 30S and 50S subunits containing the host- and plasmid-encoded rRNA were incubated in 5, 10 or 15 mM MgCl₂ and subjected to sucrose density gradient centrifugation. The gradient profiles were similar (data not shown), but primer extension analysis of 16S rRNA from each 30S and 70S fraction (Table 3) showed that only small percentages of the 1472 and 1423 single-strand mutant rRNAs were located in the 70S fractions, indicating that subunits containing mutated rRNA could not associate with 50S subunits to form 70S ribosomes. The same was true of the 23/72 double-strand mutant at 5 mM MgCl₂, but at higher MgCl₂ concentrations the wild-type level of association was restored in this mutant. Thus, disruption of base pairing resulted in decreased ability of the mutant 30S subunits to interact with 50S subunits and this defect was corrected upon restoration of base pairing in the helix

Table 3. *In vivo* subunit re-association: percent of plasmid-encoded 30S subunits incorporated into 70S ribosomes

Plasmid	MgCl ₂ (mM)		
	5	10	15
pMM1192	54.0 ± 4.1	69.5 ± 0.8	86.1 ± 5.5
pMF72	12.5 ± 3.5	6.9 ± 2.6	24.1 ± 5.6
pMF23	6.7 ± 2.4	8.6 ± 3.0	16.1 ± 1.5
pMF23/72	13.7 ± 5.1	82.9 ± 1.6	91.5 ± 6.3

Sucrose gradient fractions of 30S subunits and 70S ribosomes were analyzed by primer extension as described in Materials and Methods and in the legend to Table 2.

30S subunits lacking ribosomal protein S20, which interacts with the penultimate stem, have a subunit association defect (19,20). This defect was shown to be associated with a decrease in methylation of several bases of 16S rRNA which are normally modified. The decrease in methylation was most notable for the dimethyl adenine residues at positions 1518 and 1519. In order to determine if the subunit association defect we observed for subunits containing mutated rRNA was also associated with

under-modification we analyzed the methylation state of mutant subunits at the dimethyl adenines (bases 1518 and 1519) by primer extension analysis. In these assays the presence of a methylated nucleotide inhibits extension by reverse transcriptase, resulting in a truncated extension product. We used as controls rRNA isolated from a *ksgA* strain that lacked methylations at 1518 and 1519. No difference in the length of primer extension products was observed for rRNA isolated from ribosomes containing wild-type or mutated rRNA, indicating that expression of mutated rRNA did not result in hypomethylation (data not shown).

DISCUSSION

The central helix of the penultimate stem of 16S rRNA is essential for proper ribosome function. Disruption of base pairing by mutations in either stem (1423 or 1472) affected cell growth and decreased the accuracy of translation. Restoration of base pairing in the double-strand mutant (23/72) restored growth rate and accuracy to wild-type levels. The single-strand mutants also caused a decrease in the amount of 70S ribosomes *in vivo*, suggesting that base pairing had an effect on subunit association. This was confirmed by *in vitro* studies. Restoration of base pairing in the 23/72 mutant did not restore normal subunit association completely, as the 70S peak was still small *in vivo* and the amount of this mutant rRNA incorporated into 70S ribosomes at 5 mM MgCl₂ *in vitro* was low. Thus, while base pairing was restored in the 23/72 mutant, substitution of AU for GU pairs was not without effect.

All three mutant rRNAs were functional and were found in polysomes at wild-type levels. Thus they did not appear to affect initiation of translation, although they did make errors during elongation. The mutations also appeared to reduce stability of the 70S ribosomes. While most mutations in the 3'-end of 16S rRNA affect the initiation phase of translation, this does not appear to be the case with mutations in the central helix of the penultimate stem.

Tertiary structure models of 30S subunits place the penultimate stem of 16S rRNA at the subunit interface in the body of the 30S subunit (14–16). Cross-links between positions in the region 1408–1411 and domain IV of 23S rRNA indicate that the penultimate stem and the 50S subunit are in close proximity (31). Further evidence for proximity of the 50S subunit and the penultimate stem is seen in the protection from chemical probes of nucleotides A1413 and G1487 by the 50S subunit (32). Another chemical protection study showed that nucleotide 1435 in the variable helix was protected from chemical attack upon 50S subunit binding to the 30S subunit, but other nucleotides in the variable helix had enhanced reactivity (33), suggesting a conformational change in the variable helix upon 50S binding. Our data extend the evidence for proximity of the penultimate stem and the 50S subunit by showing that the central helix is important for association with 50S ribosomal subunits.

A sequence in the 3'-strand of the central helix (bases 1469–1483) has been proposed to base pair with 'downstream box' sequences in certain mRNAs, thus enhancing their translational efficiency (34,35). Disruption of this rRNA sequence without a corresponding effect on growth rate in the 23/72 mutant indicates, however, that the downstream box interaction with 16S rRNA is not essential for normal cell growth.

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