The EMBO Journal vol.7 no.5 pp.1503-1507, 1988

Reading frame switch caused by base-pair formation between the 3′ end of 16S rRNA and the mRNA during elongation of protein synthesis in *Escherichia coli*

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Communicated by J.F.Atkins

Watson-Crick base pairing is shown to occur between the mRNA and nucleotides near the 3′ end of 16S rRNA during the elongation phase of protein synthesis in *Escherichia coli*. This base-pairing is similar to the mRNA-rRNA interaction formed during initiation of protein synthesis between the Shine and Dalgarno (S-D) nucleotides of ribosome binding sites and their complements in the 1540-1535 region of 16S rRNA. mRNA-rRNA hybrid formation during elongation had been postulated to explain the dependence of an efficient ribosomal frameshift on S-D nucleotides precisely spaced 5′ on the mRNA from the frameshift site. Here we show that disruption of the postulated base pairs by single nucleotide substitutions, either in the S-D sequence required for shifting or in nucleotide 1538 of 16S rRNA, decrease the amount of shifting, and that this defect is corrected by restoring complementary base pairing. This result implies that the 3′ end of 16S rRNA scans the mRNA very close to the decoding sites during elongation.
Key words: ribosomal frameshifting/16S rRNA/Shine-Dalgarno nucleotides

Introduction

The polypyrimidine tract of nucleotides near the 3′ end of prokaryotic 16S rRNA participates in protein synthesis initiation by forming several Watson-Crick base-pairs with mRNA nucleotides located 5′ of the AUG start codon, the so-called Shine-Dalgarno (S-D) sequence (Shine and Dalgarno, 1974). This base-pairing has been demonstrated *in vitro* (Steitz and Jakes, 1975), inferred from mutations in ribosome binding sites (Dunn *et al.*, 1978) and recently shown directly *in vivo* utilizing mutant 16S rRNA genes (Hui and de Boer, 1987; Jacob *et al.*, 1987). The *in vivo* demonstration of base-pair formation during initiation relies on the correction of S-D nucleotide ribosome binding site defects by supplying the cell with ribosomes containing compensating nucleotide changes in the 1535-1540 region of 16S rRNA (Hui and de Boer, 1987). The 16S rRNA molecules with the compensating changes are produced from a cloned copy of the *rrnB* operon under P_L control; this production scheme is necessitated by the observation that high-level expression of 16S rRNA with changes in the 1535-1540 region is lethal (Jacob *et al.*, 1987). Although the interaction with ribosome binding sites is clear, the role of the 3′ end of 16S rRNA after progression of the ribosome from the initiation to the elongation phase of protein synthesis is not known.

Recently, base-pair formation between nucleotides 1535-1540 of 16S rRNA and the mRNA during elongation has been proposed (Weiss *et al.*, 1987) to be a key component in the mechanism of programmed ribosomal frameshifting within the *RF2* gene of *E.coli* (Craigen *et al.*, 1985). Saturation mutagenesis of a synthetic *RF2* frameshift 'window' constructed in a dispensable region of *lacZ* has defined a minimum sequence of 15 mRNA nucleotides (Weiss *et al.*, 1987; also see Figure 1) necessary and sufficient for the observed high-level (~30% efficient) of ribosomal frameshifting (Craigen and Caskey, 1986). These 15 nucleotides (5′-AGG-GGG-UAU-CUU-UGA-3′) comprise two distinct elements: the frameshift site (CUU-UGA) at which a CUU-decoding leucine tRNA slips into the +1 frame and an upstream element (AGG-GGG) whose sequence and spacing from the shift site are critical for high-level frameshifting. The frameshift site can be considered a 'shifty stop', which has been defined as a string of repetitive nucleotides (purines or pyrimidines) directly preceding a stop codon (Weiss *et al.*, 1987); such sequences cause the tRNA decoding the string to shift by −2, −1 or +1 nucleotides, at a high rate. The requirement for a tRNA which is capable of base pairing in alternate frames is important, and the level of frameshifting on the string of repetitive nucleotides is, in general, elevated 10-fold by placing a stop codon 3′ next to the string. The pyrimidine string in the synthetic *RF2* frameshift window (CUU-UGA) can be replaced by a purine string (GGG-UGA) without loss of frameshifting efficiency (a glycine tRNA now shifts +1 at this sequence); however, non-string substitutions (e.g. CUA-UGA) disrupt frameshifting completely (Weiss *et al.*, 1987). The other element within the 15-nucleotide minimal sequence is a 5′-AGG-GGG-3′ segment. Single base substitutions within this segment decrease frameshifting by 5-fold or more, and single base insertions between the AGG-GGG sequence and the CUU-UGA shift site decrease frameshifting by 15-fold or more (Weiss *et al.*, 1987). Also, the effect of the AGGGGG element on the amount of +1 frameshifting does not require the stop codon next to the shift site (Weiss *et al.*, 1987). It has been noted that the AGG-GGG element resembles an S-D sequence; i.e. it is complementary to nucleotides 1535-1540 (5′-CCUCCU-3′) of 16S rRNA (Weiss *et al.*, 1987). Thus it has been proposed that both translation start and stop signals work independently to elevate the amount of frameshifting on strings of repetitive nucleotides.

The hypothesis that the mechanism of high-level frameshifting on this 15-nucleotide sequence entails base pairing between the S-D segment and nucleotides 1535-1540 of 16S rRNA as the ribosome traverses this region thus causing an increase in +1 shifting at the appropriately spaced

pyrimidine string is tested here directly. A defect in the level of frameshifting caused by a single base substitution within the AGGGGG segment is corrected by supplying the compensating single base change in the 1535–1540 region of 16S rRNA. This result implies that the 3′ end of 16S rRNA can base pair with the mRNA close to the ribosome's decoding sites during the elongation phase of protein synthesis.

Results

If base pairing between the S-D sequence in the synthetic *RF2-lacZ* frameshift window mRNA and the 3′ end of 16S rRNA increases the amount of frameshifting by a string-decoding tRNA at the shifty stop, then changes in the core nucleotides of either the S-D sequence or the 1535–1540 region of 16S rRNA should decrease the amount of frameshifting, and conversely, this decrease should be corrected by restoring complementarity between these two regions. This premise is tested in the following experiments. The level of frameshifting is measured *in vivo* by assaying the amount of β-galactosidase activity generated by a hybrid protein A–*lacZ* gene in which translation is required to shift frame at a small defined sequence wedged between two detectable proteins (protein A, 55 kd = unshifted, protein A–β-galactosidase transframe fusion, 170 kd = shifted) in order to yield active β-galactosidase (see Figure 1). The gene is constructed so that the ribosome binding site at the beginning of the gene and the frameshift window located between the 504-codon protein A zero frame segment and the 1023-codon +1 frame *lacZ* segment can be replaced with synthetic sequence. Thus both the ribosome binding site and frameshift window can be altered. The protein A coding subsegment from *Staphylococcus aureus* has been placed at the 5′ end of the construct so that purification for protein A, which tightly binds the F_c part of IgG, by immunoaffinity chromatography recovers both the unshifted 55-kd protein A and the frameshifted 170-kd protein A/β-galactosidase fusion, thus allowing direct estimation of the efficiency of frameshifting.

Table I shows the β-galactosidase activity displayed by three distinct *proA–lacZ* genes when translated by ribosomes with normal 16S rRNA. These constructs (G starts) all have an AGGGGG ribosome binding site S-D sequence. pBW2101 G start is a 15-nucleotide minimal RF2 window containing the AGGGGG 'shifty S-D' element, pBW1604 G start is a similar window with a 'non-shifty' AGCGGG S-D element and pBW901 G start is an in-frame control window. When the efficiency of frameshifting is estimated by calculating the percentage in-frame activity of the frameshift constructs, the relative levels of 21 and 4% (Table I) for the two frameshifts shows both the high efficiency of the 15-nucleotide minimal window and the 5-fold decrease when the shifty AGGGGG element is changed to AGCGGG.

If this decrease in frameshifting is caused by disruption of the postulated base pairing between the mRNA and 16S

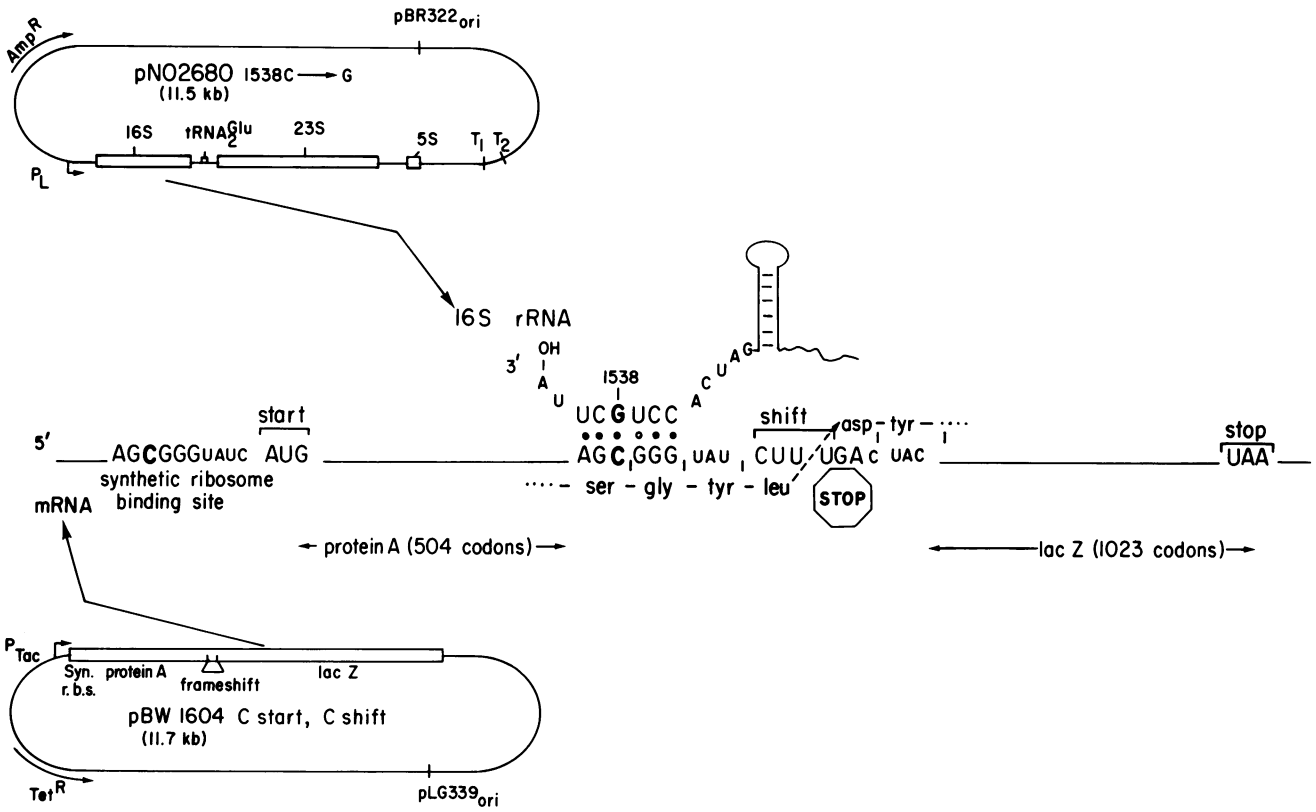

Fig. 1. The structure and expression of the *rrnB* and *proA–lacZ* genes for testing the consequences of base pairing between the 3′ end of 16S RNA and the shifty S-D sequence on frameshifting. The *rrnB* operon is carried on a pBR322 replicon and is expressed from a bacteriophage λP_L promoter (Gourse *et al.*, 1985). The *proA–lacZ* gene is carried on a pSC101 replicon and is expressed from a synthetic Tac promoter (see Materials and methods). The center of the diagram displays the frameshift window located in the interior of the *proA–lacZ* mRNA, with the postulated base pairing occurring between the 3′ end of a mutant 16S rRNA and the shifty S-D sequence in the window. The shift site is indicated by the arrow connecting the protein sequence at the frameshift junction.

rRNA, then a C to G alteration at position 1538 of 16S rRNA should restore pairing between the 1535–1540 region and the AGCGGG window, as well as disrupt pairing with the AGGGGG window. In other words, the relative efficiencies of the two frameshifts should reverse their order when translated by ribosomes carrying 16S rRNA molecules with a 1538 C to G change. The ribosome binding sites on the *proA–lacZ* genes were also changed to match the 1538 C to G change allowing preferential translation of these genes by ribosomes containing mutant 16S rRNA (see Figure 1). With wild-type ribosomes, the AGGGGG to AGCGGG ribosome binding site SD element change causes the level of β-galactosidase production from an in-frame *proA–lacZ* gene to decrease from 7800 to 105 units when translated by wild-type ribosomes (pBW901 G start versus pBW901 C start; Table I), while leaving the relative order of frameshifting efficiencies unchanged.

The change of 1538 C to G in 16S rRNA was constructed by site-directed mutagenesis of a cloned copy of the *rrnB* operon controlled by a bacteriophage λP_L promoter and carried on a high copy number plasmid (Jacob *et al.*, 1987). This plasmid, pNO2680 1538 G, and a compatible plasmid carrying the temperature-sensitive λcI857 repressor, pCI857 (Remaut *et al.*, 1983) were introduced into the *proA–lacZ* plasmid strains. When grown at 30°C, the synthesis of the mutant 16S rRNA is repressed by the cI857 repressor. Upon

Table I. Effects of a 5′-AGGGGG-3′ to 5′-AGCGGG-3′ SD sequence change on the level of translation and frameshifting

Plasmid	mRNA sequences 5′–3′ r.b.s. SD	5′–3′ frameshift window SD	β Galactosidase units
(A) pBW901 G; in-frame	AGGGGG	–	7800 (100%)
pBW2101 G; +1 frame	AGGGGG	AGGGGG	1700 (21%)
pBW1604 G; +1 frame	AGGGGG	AGCGGG	350 (4%)
(B) pBW901 C; in-frame	AGCGGG	–	105 (100%)
pBW2101 C; +1 frame	AGCGGG	AGGGGG	65 (62%)
pBW1604 C; +1 frame	AGCGGG	AGCGGG	10 (10%)

The host is SU1675, grown in M9 + 0.4% glucose + 18 amino acids (0.05 mg/ml, -met, -cys). cultures were grown at 30°C to an OD_{600} of 0.25, temperature-shifted to 42°C, grown for two cell doublings and the β-galactosidase activity determined by whole cell assays; the percentage efficiency equals the percentage frameshift activity versus the in-frame activity.

Table II. Effects of a 3′-UCCUCC-5′ to 3′-UCGUCC-5′ change in the 1540–1535 region of 16S rRNA on the level of translation and frameshifting

Plasmid	mRNA sequences 5′–3′ r.b.s. SD	5′–3′ frameshift window SD	β-Galactosidase units
(A) 30°C, 16S rRNA	1540–1535: UCCUCC (3′–5′)		
pBW901 C; in-frame	AGCGGG	–	110 (100%)
pBW2101 C; +1 frame	AGCGGG	AGGGGG	70 (64%)
pBW1604 C; +1 frame	AGCGGG	AGCGGG	15 (14%)
(B) 42°C, 16S rRNA	1540–1535: UCGUCC (3′–5′)		
pBW901 C; in-frame	AGCGGG	–	1200 (100%)
pBW2101 C; +1 frame	AGCGGG	AGGGGG	160 (13%)
pBW1604 C; +1 frame	AGCGGG	AGCGGG	630 (53%)

The host is SU1675, grown in M9 + 0.4% glucose + 18 amino acids (0.05 mg/ml, -met, -cys). β-Galactosidase units are for whole-cell assays, and the percentage efficiency equals the percentage frameshift activity versus the in-frame activity.

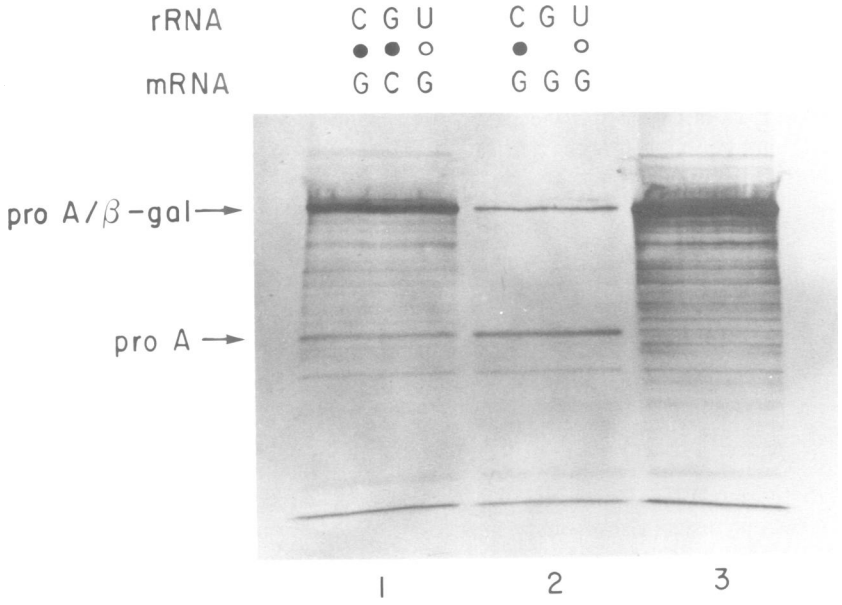

Fig. 2. Direct estimation of the amount of frameshifting by affinity purification of [^{35}S]methionine labelled protein A and protein A–β-galactosidase fusion. Cells carrying the plasmid pNO2680 1538 G and a *proA–lacZ* plasmid were labelled as described in Materials and methods. Eluates from IgG affinity chromatography of labelled cell extracts were electrophoresed on a 10% SDS–polyacrylamide gel, and the arrows indicate the position of the 55-kd protein A (unshifted) and the 170-kd protein A–β-galactosidase (frameshifted) as determined by unlabelled standards. **Lane 1**: pBW1604 C start, C shift; **lane 2**: pBW2101 C start, G shift; **lane 3**: pBW901 C start; in-frame. The potential for base pairing between nucleotides 1539–1537 of the mutant 16S rRNA and the shifty S-D sequence in the frameshift window is shown above the lanes.

temperature shift to 42°C, synthesis of the mutant 16S rRNA is induced leading to accumulation of the mutant 16S rRNA in >50% of the ribosomes present after two doublings of cell mass (Jacob *et al.*, 1987).

The effects of 16S rRNA 1538 G expression upon the levels of *proA−lacZ* translation and frameshifting are shown in Table II. At 30°C, where the synthesis of the mutant rRNA is repressed, the levels of β-galactosidase activity are comparable to temperature-shifted strains without rRNA plasmids (see Table I). After temperature shift to 42°C and two cell doublings, two effects can be observed. First, the ribosome binding site defect is corrected by induction of the 16S rRNA 1538 G as seen by the 11-fold increase in the level of β-galactosidase activity accumulating from the in-frame construct (Table II, pBW901 C start 30°C = 110 units versus 42°C = 1200 units). No significant increase is seen by temperature shifting the pBW901 C start in a host lacking the mutant *rrnB* plasmid (Table IB). Second, the efficiencies of frameshifting for the AGGGGG versus the AGCGGG window are reversed when the mutant 16S rRNA 1538 G is expressed (Table II, 64 versus 14% at 30°C compared with 13 versus 53% at 42°C). In each case, the frameshift window which has the better base-pairing match to the 3′ end of 16S rRNA displays the higher efficiency of frameshifting.

Direct estimation of the level of frameshifting for the AGGGGG versus AGCGGG frameshift window when translated by ribosomes carrying 16S rRNA 1538 G is shown in Figure 2. In this experiment, the 16S rRNA carries a second mutation (1192 C to U) which renders the ribosome resistant to spectinomycin (Sigmund *et al.*, 1984). Addition of spectinomycin allows for preferential translation by ribosomes carrying the 16S rRNA 1192 U (Hui and de Boer, 1987). Figure 2 shows the amount of protein A (55 kd, unshifted) and protein A/β-galactosidase fusion (170 kd, shifted) synthesized during a pulse labelling with [^{35}S]-methionine after temperature induction of the 16S rRNA 1538 G, 1192 U and addition of spectinomycin; the ratio of these two proteins is a measure of the efficiency of frameshifting. In agreement with the β-galactosidase activity measurements, the frameshift window that has the better base-pairing match to the 3′ end of 16S rRNA has a higher efficiency of frameshifting.

Discussion

Defective S-D sequences in a ribosome binding site and a high-level frameshift window caused by a single nucleotide substitution in the mRNA sequence 5′-AGGGGG-3′ are corrected by restoring the base-pairing potential with a complementary change in the 3′ end of 16S rRNA. The correction of the initiation defect confirms the well-documented role of the S-D sequence in ribosome binding sites (Steitz and Jakes, 1975; Stormo, 1986; Hui and de Boer, 1987; Jacob *et al.*, 1987), but our results also strongly support the idea that the mRNA and the 3′ end of 16S rRNA can base pair during elongation, with this interaction occurring close to the decoding sites. This finding raises the question of whether there are other functional roles for interactions between the 3′ end of 16S rRNA and mRNA close to the decoding sites during elongation, besides this specific frameshift event.

The details of the programmed ribosomal frameshift within the *RF2* gene of *E.coli* are apparent from these experiments and the saturation mutagenesis of the minimally required sequence (Weiss *et al.*, 1987). The central component is a tRNA decoding in the zero frame on a string of repetitive nucleotides; such sequences, which allow the decoding tRNA to base pair effectively in both the zero and +1 and −1 frame, can be termed 'slippery'. This requirement for slippery codons at high-level frameshift sites is also seen at retroviral frameshift sites (Jacks *et al.*, 1987; Moore *et al.*, 1987). The amount of shifting at these sites can be increased by flanking sequence elements, in this case, a 3′ stop codon and a precisely spaced 5′ S-D sequence. Either of these elements can work independently to elevate the level of shifting on the string of repetitive nucleotides (Weiss *et al.*, 1987), which implies the effect of base pairing observed here is not dependent on the stop codon but can occur within coding sequences. The results presented here confirm the idea that the 5′ S-D sequence elevates the level of frameshifting by the string-decoding tRNA via base pairing to the 3′ end of 16S rRNA. In the earlier *lacZ* constructs, the frameshift occurs between codons 11 and 12 of the gene, and in the *proA−lacZ* constructs described here, the shift occurs between codons 509 and 510. This indicates that the ability to form base pairs between the 3′ end of 16S rRNA and the mRNA is not confined to the beginning of coding sequences and occurs with equal efficiency at two arbitrarily chosen locations from the AUG start codon. This implies that the 3′ end of 16S rRNA scans the mRNA during elongation in order to efficiently detect and base pair with these internal S-D sequences. Since single nucleotide insertions between the S-D element and the shift site disrupt the high level of frameshifting (Weiss *et al.*, 1987), our results also suggest that the elevation of the level of +1 shifting on the string of reptitive nucleotides is caused by the precisely spaced rRNA−mRNA helix, whose terminal base pair would form three mRNA nucleotides 5′ of the codon−anticodon helix at the zero frame CUU leucine shifty codon. The occurrence of this rRNA−mRNA helix within elongating ribosomes should also provide a new constraint upon models of the structure of 16S rRNA in the decoding region (Noller *et al.*, 1986).

One possible functional role for continuous base pairing between the 3′ end of 16S rRNA and the mRNA during elongation may be to shepherd the mRNA away from the decoding sites through transitory base pairing with the mRNA. It has recently been suggested (Trifonov, 1987) that base pairing between various polypyrimidine segments of 16S rRNA and mRNA are the primary determinants of the reading frame. Our results show that while base pairing can indeed occur, the effect is quite the opposite: in the appropriate context such base pairing severely disrupts the reading frame. However, this disruption only occurs with a tRNA decoding a string of repetitive nucleotides, emphasizing that the primary determinant of the reading frame is the codon−anticodon pair.

After formation of an extensive hybrid between the AGGGGG sequence and the 3′ end of 16S rRNA, this hybrid must melt out as the ribosome continues to translate the message; the inability to melt this hybrid would preclude continued translation by upstream ribosomes. When a second S-D sequence is placed nearby the S-D sequence used for frameshifting, a decrease in the amount of shifting is observed, indicating interference between the two S-D sequences (Weiss *et al.*, 1987). One interpretation of this interference is that formation of an mRNA−rRNA hybrid at

the first 'interfering' S-D sequence prevents formation at the second 'shifty' S-D sequence. If the mRNA−rRNA hybrid is melted at a discrete distance from the decoding sites, then interference between the two S-D sequences should diminish when the length between them is greater than this average distance; experiments are in progress to test this idea.

The role of the S-D interaction in promoting frameshifting may set precedents for other hypothetical mRNA−rRNA hybrids within elongating ribosomes, such as stop codon recognition. Base pairs between the S-D element and 16S rRNA in translationally coupled genes may also form before the termination codon of the upstream gene is decoded. The ability to preferentially translate *lacZ* by matching the ribosome binding site to a mutant 1535−1540 region of 16S rRNA was critical in obtaining the results described here, and should be a useful tool for analyzing the functional effects of 16S rRNA changes *in vivo*, although it must now be taken into account that 1535−1540 changes may themselves have functional consequences, in addition to those seen in ribosomal initiation and frameshifting.

Materials and methods

Strains and plasmids

E.coli K-12 SU1675 is a *recA56* derivative of CSH26 (Miller, 1972). pNO2860 1538 C to G is derived from pNO2860 (Gourse *et al.*, 1985) by site-directed mutagenesis, it has a C to G transversion at the position corresponding to position 1538 in 16S rRNA. The C to U transition at 1192 conferring spc^R was also constructed by site-directed mutagenesis. Both the 1538 and 1192 regions were verified by sequencing the plasmid DNA of the final rRNA plasmid constructs. The *proA−lacZ* genes were constructed as follows. The *lacZ* frameshift windows 1604 and 2101 have been previously described (Weiss *et al.*, 1987) and were initially modified by addition of a *SalI* site just inside of the *Hin*dIII site located between codons 2 and 4 of a modified *lacZ* gene; this *lacZ* vector has *lac* operon sequence from codon 5 of *lacZ* through codon 66 of *lacY*, replacing the *Eco*RI−*Ava*I section of pBR322. A 1.5-kb fragment containing a *proA−lacZ* fusion segment was then cloned between the *Hin*dIII and *Sal*I sites at the beginning of *lacZ*. This 1.5-kb *Hin*dIII−*Sal*I fragment contains a staphylococcal protein A coding segment on 370 bp. *Hin*dIII−*Eco*RI *proA* fragment from pRIT2T (Nilsson *et al.*, 1985) fused to 1.1-kb *lacZ* segment spanning a region from a synthetic *Eco*RI site at *lacZ* codon 6 to a synthetic *Sal*I inserted into the *Eco*RV site at codon 376. A synthetic ribosome binding site was then cloned into the *Hin*dIII site [sequence = 5′-GAATTCGTTCT-TAG(G or C)GGGTATAATGAAAAGCTT-3′], along with a synthetic Tac promoter bounded 5′ by a *Xho*I site. The 4.9-kb *Xho*I−*Dra*I fragment containing the *proA−lacZ* fusion gene was then inserted between the *Xho*I and *Pvu*II sites of pLG339 (Stokes *et al.*, 1982). All synthetic inserts and cloning junctions were verified by dideoxy sequencing of the plasmid DNA (Chen and Seeburg, 1985). Oligonucleotides were synthesized by an ABI 380A or B DNA synthesizer and the crude material was cloned directly.

β-Galactosidase activity measurements

Whole-cell assays were based on the procedure according to Miller (1972). Whole-cell assays were carried out in a final volume of 1.0 ml Z-buffer, the assays were started with the addition of 0.2 ml of 4 mg/ml *O*-nitrophenyl-β-D galactopyranoside and stopped with the addition of 0.5 ml of 1 M Na_2CO_3, pH 11. All reactions were run at 28°C. The assay tubes were centrifuged before reading the A_{420}. The whole cell unit definition is β-gal units = $[1000(A_{420})]/[OD_{600} \times \text{time(min)} \times \text{vol.(ml)}]$.

Labelling and purification of protein A/β-galactosidase fusions

Cells were grown in M9 minimal = 0.2% glucose + 18 amino acids (0.05 mg/ml, no met or cys) + 1 μg/ml thiamine, heat-shocked at 42°C and grown for two doublings with rapid aeration. Spectinomycin (Sigma) was added to a final concentration of 0.5 mg/ml; and after 5 min 0.1 mCi of [^{35}S]methionine (>1000 Ci/mmol, NEN) was added per culture (10-ml cultures, OD_{600} = 0.8). The 10-min labelling period was followed by a 10-min chase with excess cold methionine, and the cells were harvested by centrifugation. The cell pellet was resuspended in 1.0 ml of 50 mM KPO_4 (pH 7.4), 150 mM NaCl, 0.1% Tween-20, 10 mM β-mercaptoethanol and 1 mg/ml lysozyme; the cells were lysed by rapid freeze-thawing. Protein A and the protein A/β-galactosidase transframe fusion were purified by passage through a human IgG agarose (Sigma) affinity column, eluted with 0.5 M HAc, pH 3.4, and the eluate was dried in a speed-vac. Quantitative retention of the protein A−β-galactosidase fusion was monitored by assaying the flow-through for β-galactosidase activity. The samples were analyzed by electrophoresis on 10% SDS−polyacrylamide gels, fixed, stained, dried and autoradiographed on Kodak XAR-5 film for 48 h at room temperature.

Acknowedgements

Our thanks to Paul Makogky for constructing pNO2860 1538 C to G. This research was supported by the Howard Hughes Medical Institute, NSF grant DMB-8408649 to R.G., NIH grant GM19756 to A.D. and a fellowship from the Helen Hay Whitney Foundation to R.W.

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Received on November 1987; revised on February 2, 1988