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The 3′-Terminal Sequence of *Escherichia coli* 16S Ribosomal RNA: Complementarity to Nonsense Triplets and Ribosome Binding Sites

(terminal labeling/stepwise degradation/protein synthesis/suppression)

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ABSTRACT **With a stepwise degradation and terminal labeling procedure the 3′-terminal sequence of *E. coli* 16S ribosomal RNA is shown to be Pyd-A-C-C-U-C-C-U-U-A_{OH}. It is suggested that this region of the RNA is able to interact with mRNA and that the 3′-terminal U-U-A_{OH} is involved in the termination of protein synthesis through base-pairing with terminator codons. The sequence A-C-C-U-C-C could recognize a conserved sequence found in the ribosome binding sites of various coliphage mRNAs; it may thus be involved in the formation of the mRNA·30S subunit complex.**

In a previous communication we reported that the 3′-terminal hexanucleotide of 18S ribosomal RNA from eukaryotes is complementary to known eukaryotic terminator codons (1). We suggested that this may indicate a direct role for the 3′-end of the small ribosomal RNA in the termination of eukaryotic protein synthesis. In this report we provide sequence data which could imply a similar role for the 16S rRNA from *Escherichia coli*. The 3′-terminus of 16S RNA also bears a stretch of nucleotides which is complementary to ribosome-binding sequences previously found in the intercistronic regions of a number of coliphage mRNA species.

We discuss these results in the light of recent observations on the role and structure of the 3′-terminus of *E. coli* 16S RNA. Considered together the data are consistent with the view that this region of 16S RNA may play a direct part in the termination and initiation of protein synthesis in *E. coli*.

MATERIALS AND METHODS

Isolation of 16S RNA. *E. coli* B were grown to mid-log phase in M9 medium (2) and converted to protoplasts by treatment with lysozyme–EDTA (3). RNA was extracted directly from the protoplasts by homogenisation in 20 volumes of phenol/cresol–aminosalicylate (2). DNA and low-molecular-weight RNA species were removed by extraction with 3 M sodium acetate (pH 6). 16S RNA was isolated by centrifugation in 5–20% (w/v) sucrose gradients in 0.1 M NaCl, 0.01 M sodium acetate (pH 5) for 16 hr in a Spinco SW27 rotor at 25,000 rpm, and stored at −20° after the addition of 2 volumes of ethanol.

Labeling of 3′-Termini. 16S RNA (1 mg/ml) in 0.1 M NaCl, 0.01 M sodium acetate (pH 5) was oxidized with a 100-fold molar excess of sodium periodate at 20° for 45 min (2, 4) and precipitated with 2 volumes of ethanol at −20°. Excess periodate was removed by resuspension of RNA in the same buffer and reprecipitation with ethanol. The oxidized RNA was resuspended at 1 mg/ml in 0.1 M NaCl, 0.01 M sodium acetate (pH 5), and incubated for 18 hr at 20° with a 50-fold molar excess of [^{3}H]isonicotinic-acid hydrazide (iNzd) (1 Ci/mmol). The labeled RNA was absorbed to a 1 × 3-cm column of DEAE-cellulose (Serva, DEAE-SS) in 0.1 M NaCl, 0.01 M sodium acetate (pH 5), and free iNzd removed by washing the bound RNA with 50 ml of the same buffer. The RNA was eluted with 10 ml of 1.5 M NaCl, 0.01 M sodium acetate (pH 5), and precipitated with 2 volumes of ethanol at −20°. This procedure also removes the low levels of labeled contaminating polysaccharide.

Stepwise Degradation. The sequential removal of the 3′-terminal nucleotides from periodate-oxidized 16S RNA was carried out by incubation in 0.33 M aniline (pH 5) at 20° for

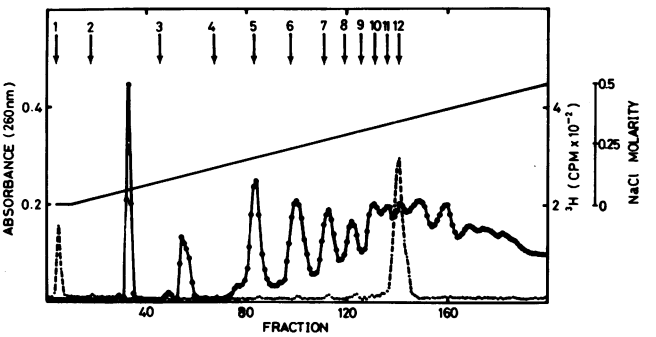

FIG. 1. DEAE-Sephadex chromatography of a T_1-ribonuclease digest of *E. coli* 16S RNA terminally labeled with [^{3}H]iNzd. Terminally labeled RNA (10,000 cpm) was resuspended in 1 ml of 0.01 M Na/K phosphate buffer (pH 7.4) and digested with 10μg of T_1-ribonuclease at 20° for 4 hr. The sample was mixed with 5 mg of a T_1-digest of carrier RNA, made 7 M in urea and applied to a 1 × 25-cm column of DEAE-Sephadex A-25. Elution was with a 1-liter linear gradient of 0–0.5 M NaCl in 7 M urea, 0.01 M Na/K phosphate buffer (pH 7.4) at a flow-rate of 30 ml/hr. Absorbance of the effluent was recorded with an ISCO UV monitor. Five-milliliter fractions were collected and 1 ml of each assayed for radioactivity. No further radioactivity was eluted when the column was washed with 1 M NaCl. Peaks of oligonucleotide hydrazones are designated (*arrows*) according to the number of constituent nucleoside residues (10). Above eight nucleosides the numbering is somewhat arbitrary. The small amount of radioactivity which elutes in the void volume of the column is free [^{3}H]iNzd. (●——●) Absorbance at 260 nm; (· – – ·) (^{3}H); (——) NaCl gradient. The numbers on the ordinate indicating ^{3}H counts are the product of the factor, 10^{-2}, and the experimental values.

Abbreviation: iNzd, isonicotinic acid hydrazide.

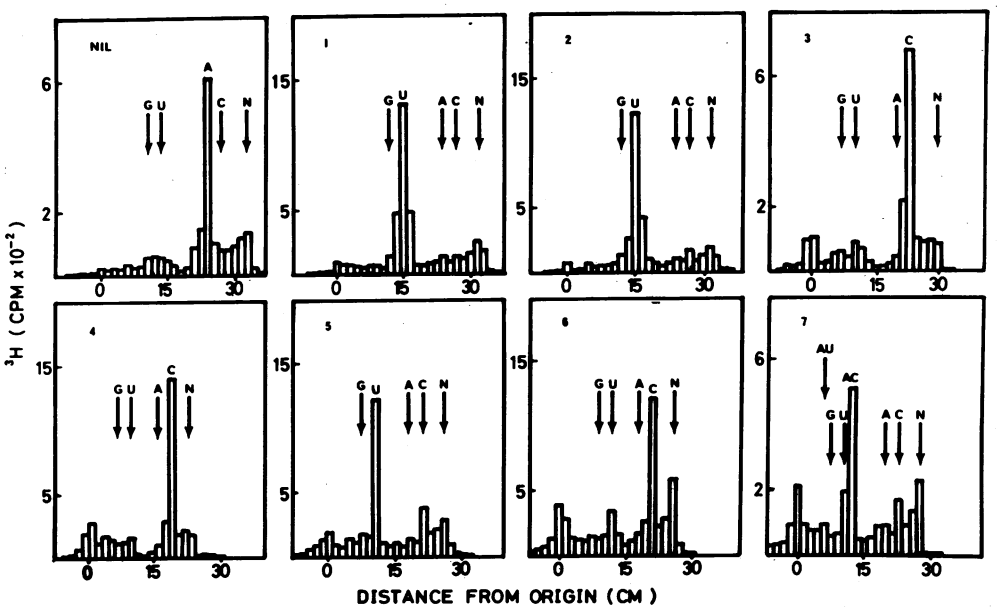

FIG. 2. Paper electrophoresis of pancreatic ribonuclease digests of stepwise degraded, terminally labeled 16S RNA. 16S RNA was subjected to seven cycles of stepwise degradation. After each cycle a sample was removed, labeled with [^{3}H]iNzd and digested with pancreatic ribonuclease (100 μg/mg RNA) at 20° for 4 hr in 0.01 M Na/K phosphate buffer (pH 7.4). A sample of the digest (10 or 20 μl) was mixed with 20 μl of unlabeled mononucleoside hydrazones (4) and electrophoresed on Whatman 3MM paper at 40 V/cm for 2 hr in 0.1 M sodium formate buffer (pH 3). Under these conditions the two hydrazone derivatives differing least in mobility (U-iNzd and G-iNzd) are separated by about 1 cm. After drying, the positions of marker nucleoside hydrazones and free iNzd were detected under ultraviolet light, the electropherogram cut into strips and each strip assayed for radioactivity (4). The *arrows* labeled G, U, A, C, and N indicate the positions of the respective marker hydrazones and free iNzd. Nil–7 refers to the number of stepwise degradations before labeling with [^{3}H]iNzd. A dinucleoside monophosphate hydrazone was released by pancreatic ribonuclease digestion of RNA after 7 stepwise degradations; it was identified as ApC-iNzd by co-electrophoresis with the two possible dinucleoside monophosphate derivatives, ApU-iNzd and ApC-iNzd.

4 hr, followed by digestion with alkaline phosphatase (5). This was repeated six times after periodate oxidation of the resultant 2',3'-hydroxyl groups. Following the removal of successive nucleotides, a sample of the RNA was labeled with [^{3}H]iNzd.

RESULTS

The composition of the 3'-terminal oligonucleotide of *E. coli* 16S RNA has been determined by Fellner *et al.* (6) as GAU-[(AC)(C)$_4$(U)$_3$]A$_{OH}$. Since such pyrimidine-rich oligonucleotides are difficult to sequence by the standard methods of Sanger's group (7–9) for ^{32}P-labeled RNA, we have used the methods developed by Hunt (5) for terminal sequence analysis of unlabeled RNA. This involves the stepwise degradation of periodate-oxidized RNA and 3'-terminal labeling with [^{3}H]iNzd. The combination of these procedures with the use of pancreatic and T_1-ribonuclease has allowed double checks at each step and an unambiguous sequence determination.

T_1-ribonuclease digestion of *E. coli* 16S RNA labeled with [^{3}H]iNzd releases a single 3'-terminal oligonucleotide containing about 12 nucleoside residues (Fig. 1). Since [^{3}H]iNzd reacts specifically with the 3'-terminus of periodate-oxidized ribosomal RNA in a 1:1 molar ratio (2), this shows that virtually all 16S RNA molecules possess the 3'-terminal sequence G-(X-)$_{\sim 11}$N$_{OH}$ where N is any nucleoside and X any nucleoside other than G.

Isolated 16S RNA was then subjected to seven stepwise degradations. After the removal of successive 3'-terminal nucleotides, a sample of the oxidized RNA was condensed with [^{3}H]iNzd and digested with pancreatic ribonuclease. Paper electrophoresis of the digest (4) was used to identify the mononucleoside hydrazones or dinucleoside monophosphate hydrazones released from the 3'-terminus (Fig. 2). The completeness of each stepwise degradation can be confirmed by chromatography of the corresponding T_1-ribonuclease digest on DEAE-Sephadex which shows a reduction in chain length of the T_1 oligonucleotide hydrazone (Fig. 1) by one nucleotide at each step. By this method we demonstrate that the 3'-terminal sequence of 16S RNA is G-(X-)$_{\sim 2}$Pyd-A-C-C-U-C-C-U-U-A$_{OH}$ (Fig. 2; Table 1). From Fig. 2 it can be seen that, within the limitations of the methods used, there is no evidence for any sequence heterogeneity at the 3'-terminus. The

TABLE 1. *Determination of the 3'-terminal sequence of E. coli 16S RNA.*

Number of stepwise degradations before labeling RNA with [^{3}H]iNzd	Labeled hydrazone released by pancreatic ribonuclease*	Sequence
None	A-iNzd	Pyd-A$_{OH}$
1	U-iNzd	Pyd-U-A$_{OH}$
2	U-iNzd	Pyd-U-U-A$_{OH}$
3	C-iNzd	Pyd-C-U-U-A$_{OH}$
4	C-iNzd	Pyd-C-C-U-U-A$_{OH}$
5	U-iNzd	Pyd-U-C-C-U-U-A$_{OH}$
6	C-iNzd	Pyd-C-U-C-C-U-U-A$_{OH}$
7	ApC-iNzd	Pyd-A-C-C-U-C-C-U-U-A$_{OH}$

* From Fig. 2.

TABLE 2. *Ribosome binding sites and possible pairing with 3'-terminus of 16S rRNA**

RNA	Cistron	Possible pairing arrangement	Ref.
R17, MS2†	A-protein	(5') U-A-G-G-A-G-G-U-U (3')	
		$_{OH}$A-U-U-C-C-U-C-C-A-C-U-A-G (5')	29, 30
Qβ	A_2-protein	(5') A-A-G-A-G-G-A-C-A (3')	
		$_{OH}$A-U-U-C-C-U-C-C-A-C-U-A-G (5')	31
R17	Coat protein	G‡ ↕ (5') C-C-G-G-A-G-U-U-U (3')	
		$_{OH}$A-U-U-C-C-U-C-C-A-C-U-A-G (5')	29, 32
MS2†	Coat protein	(5') C-C-G-G-A-G-U-U (3')	33
		$_{OH}$A-U-U-C-C-U-C-C-A-C-U-A-G (5')	
f2	Coat protein	(5') C-C-G-(G,A)-G-U-U (3')	
		$_{OH}$A-U-U-C-C-U-C-C-A-C-U-A-G (5')	34
R17, MS2†	Replicase	(5') A-U-G-A-G-G-A-U (3')	
		$_{OH}$A-U-U-C-C-U-C-C-A-C-U-A-G (5')	29, 35
Qβ	Replicase	(5') A-A-G-G-A-U-G-A-A (3')	
		$_{OH}$A-U-U-C-C-U-C-C-A-C-U-A-G (5')	36
"Early" T7 mRNA	—	(5') A-U-G-A-G-G-U-A (3')	
		$_{OH}$A-U-U-C-C-U-C-C-A-C-U-A-G (5')	37

* The sequence of 11 nucleotides preceding the initiation codon for the *trpE* polypeptide in *E. coli* has recently been determined (38). Although limited pairing of this region is possible with the 3'-end of 16S rRNA (G-A-G with C-U-C), it is not known whether the sequence reported represents the complete ribosome binding region. Pairing with nucleotides located on the 5'-side of this sequence cannot be excluded.

† The sequences determined for MS2 RNA are assumed to represent ribosome binding sites by their identity to R17 ribosome-binding sites and their positions relative to the known initiator triplet.

‡ Two sequences have been reported for this region (29, 32); the G ↔ A transition probably represents a spontaneous mutation which occurred after the two stocks used in these studies were first separated (32).

sequence obtained is consistent with the composition of the 3'-oligonucleotide determined by Fellner *et al.* (6) and the 3'-sequence is therefore probably G-A-U-C-A-C-C-U-C-C-U-U-A_{OH}. This is in disagreement with the sequence recently proposed by Santer and Santer (11) who find G-A-U-C-C-U-C-A-C-U-U-C-A_{OH} (an isomer of the above sequence) at the 3'-terminus of 16S RNA from *E. coli* MRE600. It seems unlikely that this reflects a real difference in the 3'-sequence between *E. coli* B and *E. coli* MRE600, and we feel that the sequence proposed by these authors is incorrect. The difficulties mentioned earlier in the sequence analysis of pyrimidine-rich oligonucleotides have previously resulted in the publication of sequence isomers for particular regions of *E. coli* 16S RNA (Santer and Santer, quoted in ref. 6).

DISCUSSION

Termination. In view of previous reports that the 3'-terminal trinucleotide of 18S rRNA from widely divergent eukaryotes is U-U-A_{OH} (1, 5), it is noteworthy that the same trinucleotide is found at the 3'-end of 16S rRNA from *E. coli* and *Salmonella hirschfeldii* (J. Shine, unpublished results). We have previously suggested that the conserved terminal hexanucleotide, U-C-A-U-U-A_{OH}, of eukaryotic 18S rRNA may be directly involved in the termination of protein synthesis (1). We proposed that U-U-A_{OH} could recognize the terminator codons UAA and UAG and that U-C-A could pair with UGA. If the 3'-terminal U-U-A_{OH} of 16S rRNA is involved in interaction with terminator codons, recognition of UAA on mRNA could occur by standard base-pairing mechanisms with 'wobble' (12) in the 3rd position for recognition of UAG as was proposed for eukaryotes. Interaction of the 3'-terminal U-U-A_{OH} with UGA may also occur as a result of pairing in the 1st and 3rd positions. The apposition of U and G residues in the 2nd position should not seriously interfere with the stability of such an interaction (13, 14).

However, the nature of the proposed interaction of U-U-A_{OH} with the three terminator triplets may be different in certain respects from that expected in tRNA–mRNA interactions. It would be expected, for example, that the absence of a 3'-terminal phosphate from 16S rRNA could strengthen base-pairing interactions involving the two terminal bases (15), and that any flexibility associated with the 3'-terminus of 16S rRNA (see below) would also modify pairing strengths and may increase the possibility of interactions between G and U.

It has previously been found that the extent of suppression of a nonsense triplet reflects a competition between suppressor tRNA and release factors for the termination signal (16). However, attempts to demonstrate that release factors *per se* can discriminate between individual nonsense triplets have proved inconclusive (17). On the other hand, if we assume base-pairing between the 3′-terminal U-U-A_{OH} of 16S rRNA and nonsense triplets, the likely stability of interaction of the three nonsense codons with U-U-A_{OH} can be graded in a manner which relates to their general levels of suppressibility and which is therefore consistent with a role for U-U-A_{OH} in chain termination. Thus UAA, which is only weakly suppressed by *ochre* suppressors (1–15%; refs. 18 and 19) could form three A·U pairs with U-U-A_{OH}. The *amber* triplet UAG forms A·U pairs only in the 1st and 2nd positions with 'wobble' pairing in the third; *amber* suppressors have an efficiency of suppression of 30–63% (20, 21). The triplet UGA can pair with U-U-A_{OH} in only the 1st and 3rd positions with no net contribution to the stability of interaction from G·U in the 2nd position (13, 14). This is consistent with the observation that UGA is a 'leaky' terminator codon in enterobacteria since even suppressor$^-$ strains read UGA as sense with low efficiency (22–28). These considerations are therefore consistent with suppression primarily representing an initial competition between suppressor tRNA and the 3′-terminal U-U-A_{OH} for the nonsense triplet. Thus interaction of U-U-A_{OH} with terminator codons may in fact represent the signal for the binding or activation of release factors.

Ribosome Binding Sites. Recent studies (29–37) have shown that although the ribosome-binding sites of different coliphage mRNA species do not bear regions of extensive sequence homology, all contain some substantial part of the sequence (5′)G-G-A-G-G-U(3′). In each species this sequence is found at a similar relative position on the 5′-side of the initiator triplet. The 3′-terminus of 16S rRNA contains, adjacent to U-U-A_{OH}, the sequence (5′)A-C-C-U-C-C(3′) which has not been found elsewhere in the molecule (6). Since recognition of messenger initiation signals in prokaryotes appears to be primarily a function of the 30S subunit (39, 40), it is significant that this sequence is complementary to (5′)G-G-A-G-G-U(3′). It may therefore represent a stretch of nucleotides which promotes interaction of the small subunit with ribosome-binding sites on mRNA. The extent of interaction possible would clearly vary with the ribosome-binding site concerned. Since the degree of continuous base-pairing possible is greatest with the R17 and MS2 A-protein binding site (Table 2), it would be expected that this site would be a more efficient initiator than the coat protein or replicase initiator regions. This is confirmed by the observation that *E. coli* ribosomes interact with the isolated A-protein initiator fragment of R17 RNA some 40 and 11 times more efficiently than with the coat and replicase fragments, respectively (41). The highly efficient binding of A-protein initiator fragments contrasts with the translation of intact coliphage RNA *in vitro* where 20 mol of coat protein and 5 mol of replicase are synthesized for each mol of A-protein (42). During translation of intact phage RNA, the secondary structure of the molecule presumably impedes binding of the 30S subunit to the A-protein initiation region (41).

Special Features of the 3′-Terminus of 16S rRNA. If the 3′-terminus of 16S rRNA is involved in recognition of ribosome-binding sites and terminator codons on mRNA, it seems likely that other properties of the 3′-terminus would reflect an involvement in these important functions. The activity of two antibiotics, colicin E3 and kasugamycin, supports the view that an intact and unaltered 3′-end of the 16S RNA is indispensable for protein synthesis.

Colicin E3 induces a rapid shutdown of protein synthesis in susceptible *E. coli* (43) although the particular stage involved is not known (44). Colicin E3 treatment of susceptible cells, or of ribosomes *in vitro*, results in the removal of about 50 nucleotides from the 3′-terminus of 16S RNA. This appears to be the sole lesion induced and is due to the introduction of a single endonucleolytic cleavage (45–47). Kasugamycin binds to the 30S ribosomal subunit (48) specifically inhibiting initiation (49, 50). Kasugamycin-resistant mutants lack the ability to methylate two adjacent adenosine residues (51) 28 nucleotides from the 3′-terminus of the 16S rRNA (6), implying that the interaction of kasugamycin with the ribosome involves the 3′-end of the 16S RNA. Our results support the view that the role of this region in the initiation of protein synthesis may be related in part to the recognition of ribosome binding sites on mRNA.

From sequence studies a model for the secondary structure of the 3′-terminus of *E. coli* 16S RNA has been proposed (6) in which the 13 3′-terminal nucleotides are free rather than involved in any base-paired structure. This supports the view that the 3′-end may be available for interactions with other polynucleotides as proposed in this communication.

If the 3′-terminus of 16S rRNA is involved in both ribosome binding to mRNA and recognition of terminator codons, it would presumably be in a different orientation (relative to the ribosomal P and A sites) during formation of the 30S initiation complex and during termination on the 70S ribosome. Thus, during binding of mRNA to the 30S subunit, the 3′-terminal adenosine of 16S rRNA would be some 12–18 nucleotides from the initiator AUG in the P site. At termination the 3′-adenosine might be expected to lie near the A site in order to interact with the incoming terminator codon. It is therefore significant that substantial changes in ribosome conformation occur during a number of steps in protein synthesis (52–54) and that an appreciable change in conformation of the 30S subunit occurs during association with the 50S subunit (55). Further, colicin E3 will only inactivate 30S subunits when they are associated with the 50S subunit (56). This suggests a conformational change in the 3′-terminal region of 16S rRNA as a consequence of 70S ribosome formation.

The presently available data are therefore consistent with the view that a short terminal section of the 3′-end of the small ribosomal RNA of *E. coli* may have a direct base-pairing role in the termination and initiation of protein synthesis on mRNA.

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