

How ribosomes select initiator regions in mRNA: Base pair formation between the 3' terminus of 16S rRNA and the mRNA during initiation of protein synthesis in *Escherichia coli*

(specificity in polypeptide chain initiation/RNA secondary structure)

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ABSTRACT Initiation complexes formed by *E. coli* ribosomes in the presence of ³²P-labeled A protein initiator region from R17 bacteriophage RNA have been treated with colicin E3 and disassembled by exposure to 1% sodium dodecyl sulfate. Electrophoresis on 9% polyacrylamide gels reveals a dissociable complex containing the 30-nucleotide-long messenger fragment and the 50-nucleotide-long colicin fragment, which arises from the 3' terminus of the 16S RNA. The complex is a pure RNA-RNA hybrid; it is apparently maintained by a seven-base complementarity between the two RNA fragments.

Detection of this mRNA-rRNA complex strongly supports the hypothesis that during the initiation step of protein biosynthesis the 3' end of 16S RNA base pairs with the polypurine stretch common to initiator regions in *E. coli* and bacteriophage mRNAs. The implications of our findings with respect to the molecular mechanism of initiation site selection and mRNA binding to ribosomes, the role of rRNA in ribosome function, and species specificity in translation are explored.

Shine and Dalgarno (1) originally suggested that a sequence near the 3' terminus of *Escherichia coli* 16S ribosomal RNA participates directly in the initiation of protein biosynthesis by forming several Watson-Crick base pairs with the messenger RNA. Indeed, one of the few common features of all ribosome-protected initiator regions analyzed so far is a polypurine stretch of 3 to 8 nucleotides located about 10 bases 5' to the initiator codon (Table 1). From 3 to 7 contiguous bases within this region of each mRNA can potentially pair with some portion of the polypyrimidine sequence found in the 3'-terminal T1 oligonucleotide of 16S RNA. Although the relevant 16S RNA sequence as determined by Shine and Dalgarno (1) conflicted with previous reports (2), its validity has now been confirmed in three additional laboratories (3-5).

The suggestion of Shine and Dalgarno seemed attractive for several reasons. First, crosslinking (6-8) and other chemical (9) data suggest that the 3' end of 16S RNA, the binding sites for initiation factors, and certain ribosomal proteins implicated in initiation (10-13) may all be neighbors in the 30S ribosome. Second, studies of several antibiotic inhibitors of initiation (14, 15) indicate that their sites of action likewise lie in the vicinity of the 3' end of 16S RNA. Third, random copolymers rich in A and G are the best competitive inhibitors of initiation on natural mRNAs (16), underscoring the importance of polypurines in ribosome binding to initiator regions. Finally, in the single well-documented instance of ribosome recognition of a noninitiating RNA sequence (17), ribosomes from *Bacillus stearothermophilus* bind to Q β phage RNA at a site containing no initiator triplet, but possessing a long polypurine stretch which is potentially complementary to the 3' terminus of *B. stearothermophilus* 16S RNA (18).

To test the idea of Shine and Dalgarno, we formed ribo-

somal initiation complexes with a messenger fragment of known sequence, cleaved the 16S RNA at a specific site about 50 nucleotides from its 3' terminus with colicin E3 (20, 21), and fractionated the components of the disassembled ribosome on a polyacrylamide gel. An mRNA-rRNA hybrid, which contains approximately equimolar amounts of the 30 nucleotide-long mRNA fragment and of the colicin fragment, is detected. We thus provide direct evidence that base-pairing between the *E. coli* 16S ribosomal RNA and messenger RNA does occur during the initiation of protein biosynthesis.

METHODS AND RESULTS

Choice of the R17 A protein initiator region for study

Of the prokaryotic initiator regions whose sequences have been determined so far, the beginning of the A cistron from R17 bacteriophage RNA is ideally suited for the experiment outlined above. First, because its polypurine stretch exhibits the longest known complementarity to the 16S RNA 3' end (seven base pairs), the bimolecular complex should be sufficiently stable for analysis. (D. Crothers has calculated a lifetime of approximately 30 hr in 1 M NaCl at 0° for the structure pictured in Fig. 1.) Second, of the three R17 protein synthesis initiator regions, only the A site is efficiently recognized as an isolated fragment by ribosomes (19). Finally, radiochemically pure preparations of the A protein initiator fragment can be prepared relatively easily (22).

A noncovalent complex including the R17 A site

Fig. 2 illustrates the requirements for the appearance of a complex between the ³²P-labeled R17 A protein initiator fragment and nonradioactive rRNA. Slot 1 shows that the isolated A site (19) migrates on a 9% polyacrylamide gel as a diffuse band in the region expected for a 30- to 40-nucleotide RNA molecule. Its mobility is unchanged after treatment with colicin E3 alone (not shown).

Incubation of all the components required for 70S initiation complex formation (8 min at 38°), followed by colicin treatment (10 min at 38°), disassembly in 1% sodium dodecyl sulfate (15 min at 0°), and gel electrophoresis (5 hr at 6°) significantly alters the mobility of approximately 2/3 of the labeled A site fragments (slots 2 and 6). They migrate just ahead of the fastest tRNAs, behavior consistent with their being involved in the RNA-RNA complex illustrated in Fig. 1.

Slots 3 and 4 are controls showing that the appearance of the new gel band is dependent upon involvement of the labeled A site in a 70S initiation complex before ribosome disassembly. These reactions are important since any two RNA molecules with a seven-base complementarity would be expected to hybridize given sufficient time under the appro-

Table 1. Initiation sequences recognized by *E. coli* ribosomes

mRNA	Ribosome binding site	Ref.
R17 A	GAU UCC <u>UAG GAG GUU</u> UGA CCU AUG CGA GCU UUU AGU G	22
Qβ A	UCA CUG AGU <u>AUA AGA GGA</u> CAU AUG CCU AAA UUA CCG CGU	38
R17 coat	CC UCA ACC <u>GGG GUU</u> UGA AGC AUG GCU UCU AAC UUU	22
Qβ coat	AAA CUU <u>UGG GUC</u> AAU UUG AUC AUG GCA AAA UUA GAG ACU	39
f2 coat	CC UCA ACC <u>G(A)GGUU</u> UGA AGC AUG GCU UCC AAC UUU ACU	40
R17 replicase	AA ACA <u>UGA GGA</u> UUA CCC AUG UCG AAG ACA ACA AAG	22
Qβ replicase	AG UAA <u>CUA AGG AUG</u> AAA UGC AUG UCU AAG ACA G	41, 42
f1 coat	UUU AAU GGA AAC UUC CUC AUG AAA AAG UCU UU	43
f1 gene 5	<u>A AGG</u> UAA UUC ACA AUG AUU AAA GUU GAA AU	43
f1 gene ?	A AAA <u>AAG GUA</u> AUU CAA AUG AAA UU	43
T7 <i>in vitro</i>	AAC AUG <u>AGG UAA</u> CAC CAA AUG AUU UUC ACU AAA GAG	44
φX174 spike (DNA)	TTT CTG CTT <u>AGG AGT</u> TTA ATC ATG TTT CAG ACT TTT ATT	45
<i>trp</i> leader	CAC GUA AAA <u>AGG GUA</u> UCG ACA AUG AAA GCA AUU UUC GUG	46
<i>trpE</i>	GAA CAA AAU <u>UAG AGA</u> AUA ACA AUG CAA ACA CAA AAA CCG	46
<i>trpA</i>	GAA AGC ACG <u>AGG GGA</u> AAU CUG AUG GAA CGC UAC GAA UCU	47
<i>lacZ</i>	AAU UUC ACA <u>CAG GAA</u> ACA GCU AUG ACC AUG AUU ACG GAU	48
<i>galE</i>	AUA AGC CUA <u>AUG GAG</u> CGA AUU AUG AGA GUU CUG GUU ACC	49
16S RNA 3' end:	HOA U U C C U C C A C U A G _{5'}	

Underlining indicates contiguous bases complementary to the 3'-oligonucleotide of *E. coli* 16S RNA. Dots indicate G-U base pairs. Gaps appear at positions where a one-base bulge in the rRNA strand is required to provide the indicated complementary.

appropriate conditions (23). In fact, our mRNA-rRNA complex can be formed in the complete absence of ribosomes (see below). However, slot 3 shows that 2×10^{-5} M aurintricarboxylic acid, a potent inhibitor of messenger binding to ribosomes (24), lowers the amount of complex about 4-fold, as judged by microdensitometry of the autoradiograph shown. Likewise, we observe only minor amounts of complex when labeled initiator fragments are not added until just after the dissociation period in the presence of 1% sodium dodecyl sulfate has begun (slot 4). Thus, in a complete initiation re-

action, the complex appears only if conditions are such that the mRNA fragment can bind to ribosomes.

Slot 4 also shows that some of the labeled A site fragment becomes degraded during incubation at 38°. However, slot 7 and experiments described below indicate that the intact initiator region, rather than small degradation products, is bound to the colicin fragment.

Slot 5 provides more concrete evidence that the labeled A site is in fact complexed with the colicin fragment on the gel. The new band does not appear if colicin treatment is

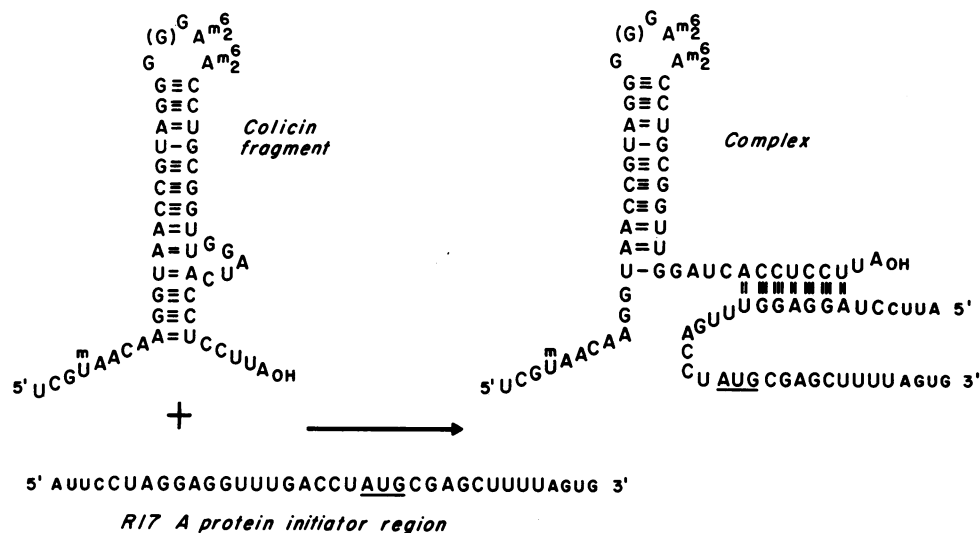


FIG. 1. Postulated hydrogen bonding between the colicin fragment of 16S RNA and the R17 A protein initiator region. The sequence of the colicin fragment is from ref. 25 and M. Santer, personal communication. The secondary structure drawn is predicted to be stable under physiological conditions (23). Not shown is an alternative hydrogen bonding scheme, of comparable predicted stability (23), which would enlarge the bulge loop to 9 bases and pair the CCUU sequence directly adjacent to the 3' end with the AAGG on the 5' side of the lower portion of the stem. Either structure involves the Shine-Dalgarno nucleotides in internal base pairing. The isolated A protein initiator fragment is predicted to assume no stable secondary structure under physiological conditions (23, 19). Ragged ends on the messenger fragment are denoted by small capital letters. Approximately 1/2 of the A site fragments in the preparations used in Figs. 2 and 3 were the length indicated by the larger capital letters (28 nucleotides); the remaining fragments included the entire sequence shown.

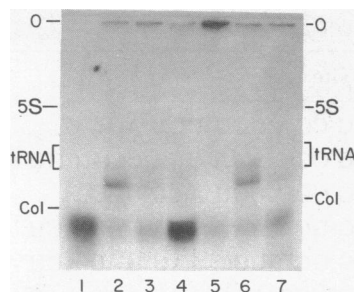


FIG. 2. Polyacrylamide gel analysis of reactions including ^{32}P -labeled A protein initiator fragment. A complete reaction mixture contained in $10\ \mu\text{l}$: $0.1\ \text{M}$ Tris-HCl (pH 7.4), $0.05\ \text{M}$ NH_4Cl , $0.01\ \text{M}$ magnesium acetate, $0.2\ \text{mM}$ GTP, $0.5\ A_{260}$ unit of high salt-washed ribosomes from *E. coli* MRE600 (17), $0.3\ A_{260}$ unit of charged, formylated mixed *E. coli* tRNA (22), and $3000\ \text{cpm}$ of ^{32}P -labeled R17 A site fragment (specific activity = $1 \times 10^6\ \text{cpm}/\mu\text{g}$). A site fragments (see Fig. 1) were isolated by binding ^{32}P -labeled R17 RNA to *B. stearothermophilus* ribosomes as described (17, 22) and further purifying (to reduce the level of contamination by unlabeled rRNA fragments) by preparative electrophoresis on 9% polyacrylamide gels (see below) after heating for 5 min at 55° in $8\ \text{M}$ urea; initiator fragments were electrophoretically eluted (34) and, after phenol extraction and ethanol precipitation, stored at -20° in distilled H_2O . The molar ratio of ribosomes to initiator fragment in the reactions was approximately 30/1; identical results were obtained when initiation complexes were formed at $5\ \text{mM}$ Mg^{++} , or when low salt-washed ribosomes (17) were used (here degradation problems were more severe). After incubation of the reaction mixtures for 8 min at 38° , $5\ \mu\text{l}$ of colicin E3 (purified as described in ref. 39) at $2.2\ \text{mg}/\text{ml}$ in Buffer A (39) were added and incubation continued for 10 min. Then, $3\ \mu\text{l}$ of 5% sodium dodecyl sulfate (Fisher) were added, and the reaction mixture was held on ice for 15 min with occasional shaking. Samples were loaded onto a 9% polyacrylamide 0.3% bisacrylamide slab gel ($20 \times 20 \times 0.15\ \text{cm}$) in $40\ \text{mM}$ Tris-acetate, pH 8.0, $5\ \text{mM}$ magnesium acetate. Electrophoresis was performed at 6° and $200\ \text{V}$, until the xylene cyanol marker dye migrated $\frac{3}{4}$ the length of the plate (4–6 hr). Gels were stained with Stains-all as described in ref. 36, dried (37), and contact autoradiographed. Slot 1, reaction omitting ribosomes and colicin. Slot 2, complete reaction. Slot 3, complete reaction plus $2 \times 10^{-5}\ \text{M}$ aurintricarboxylic acid (Eastman). Slot 4, reaction omitting A site fragment during 38° incubation; fragment added immediately after sodium dodecyl sulfate. Slot 5, no colicin added. Slot 6, complete reaction. Slot 7, complete reaction to which $15\ \mu\text{l}$ of $8\ \text{M}$ urea were added followed by incubation for 5 min at 55° just before electrophoresis. The positions of RNAs visualized in the stained gel are indicated.

omitted (slot 5). Instead the radioactive messenger fragments are trapped at the top of the gel, apparently bound to the intact 16S RNA. Fractionation of the reaction mixture on a sucrose gradient (not shown) rather than on a gel directly supports this conclusion; the mRNA fragments cosediment with the 16S peak.

Finally, the complex between the A site initiator region and the colicin fragment is dissociable, as expected. If the sample is heated either in the presence or absence of $4\ \text{M}$ urea to 55° for 5 min just before electrophoresis (slot 7), the A initiator fragment is released and reappears in the position of unbound fragment. This melting behavior is consistent with a T_m of 50° – 60° calculated for the mRNA-rRNA complex in the buffer used here (D. Crothers, personal communication).

Colicin fragment is the other component of the complex

Above we demonstrated that under conditions of polypeptide chain initiation, the R17 A protein initiator fragment becomes noncovalently complexed with some ribosomal product that is released by colicin E3 action. To prove that

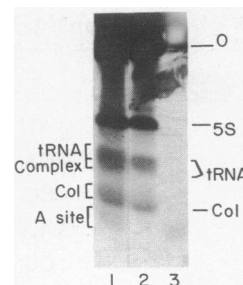


FIG. 3. Polyacrylamide gel analyses of reactions including ^{32}P -labeled ribosomes ($2 \times 10^5\ \text{cpm}/\mu\text{g}$) and ^{32}P -labeled A protein initiator fragment ($1 \times 10^6\ \text{cpm}/\mu\text{g}$). The constituents and processing of reaction mixtures were as described in Fig. 2, except that the gel was autoradiographed wet. ^{32}P -Labeled high salt-washed ribosomes were prepared (17) from *E. coli* MRE600 grown to late logarithmic phase in PGM (5) containing $50\ \mu\text{Ci}/\text{ml}$ of $^{32}\text{PO}_4$ (New England Nuclear). Slot 1, complete reaction including ^{32}P -labeled ribosomes and ^{32}P -labeled A site. Slot 2, ^{32}P -labeled A site omitted. Slot 3, ^{32}P -labeled ribosomes omitted. Regions of the gels that were subjected to fingerprint analysis are indicated on the side of slot 1; the positions of RNAs visualized by staining another portion of the same gel are indicated next to slot 3. The reaction mixture for slot 1 was scaled up 7-fold and fractionated in parallel on the same gel to provide sufficient material for analysis (Fig. 4).

this other component is in fact the colicin fragment, we repeated our experiment using ^{32}P -labeled ribosomes in addition to ^{32}P -labeled initiator region. Thus in the autoradiogram of Fig. 3 we visualize 5S RNA, ribosome-bound tRNA, and the free colicin fragment as well as A site. RNA was eluted from each band indicated in slot 1 and subjected to RNase T1 fingerprint analysis.

The map of Fig. 4a reveals that the region of the gel marked "Complex" in Fig. 3 indeed contains only oligonucleotides previously assigned to the colicin E3 fragment (refs. 21 and 25; M. Santer, personal communication) of 16S RNA and to the R17 A protein initiator region (22). Their molar ratio is 1.5/1. (Note that this slightly high yield of colicin fragment is expected if only 20% of the radioactivity is contributed by background levels of colicin fragment in the gel.) The region of the gel just above the complex (marked "tRNA" in Fig. 3) produces a more complicated fingerprint (Fig. 4b) typical of unfractionated tRNA. Minor amounts of the colicin fragment and the A site are detected in a molar ratio of 1.1/1. By contrast, the fingerprint (not shown) of the uncomplexed colicin fragment (marked "Col" in Fig. 3) contained a 30/1 ratio of colicin fragment to A site, whereas the initiator fragment region ("A site" in Fig. 3, fingerprint also not shown) gave a ratio of 0.16/1. We conclude that the complex contains approximately one mole each of the colicin fragment and the R17 A protein initiator region; no other RNA is present in significant amounts.

We also eliminated the possibility that ribosomal protein is required to maintain the rRNA-mRNA hybrid. Incubation of ^{32}P -labeled A protein initiator fragments with small RNAs purified from colicin-treated ribosomes (the mixture thus includes 5S, tRNA, and colicin fragment) under conditions identical to those used in Fig. 2 yields detectable amounts of the same complex (not shown). In this case, complex formation is not inhibited by as much as $10^{-4}\ \text{M}$ aurintricarboxylic acid. Moreover, complexing is not observed upon incubation of the messenger fragment with tRNA and 5S RNA alone, with 23S RNA, or with 16S RNA lacking the colicin fragment. Thus, the mRNA-rRNA hybrid we visualize contains only RNA molecules and is formed specifically with the 3'-terminal region of 16S RNA.

Finally, we probed whether the mRNA-rRNA interaction

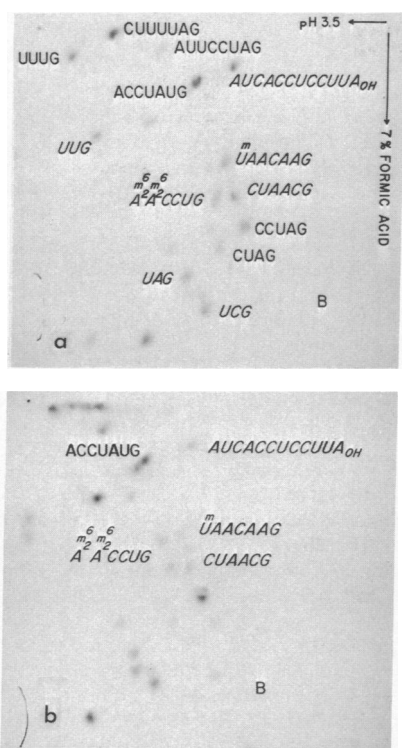


FIG. 4. RNase T1 fingerprints of the A site-colicin fragment complex. RNA was eluted electrophoretically (34) from the regions indicated in Fig. 3, slot 1, and subjected to RNase T1 fingerprint analysis (19). Oligonucleotide spots from each map were quantitated by scintillation counting (19), and their identity was confirmed by analysis of their pancreatic RNase digestion products. (a) Complex region; (b) tRNA region. *Italics* designates rRNA sequences.

depends on specific hydrogen bonding or represents some form of nonspecific aggregation between the colicin fragment and the A site. If base pairing is required, then other initiator regions with comparatively less complementarity would be expected to form less stable complexes. We tested this prediction by using unfractionated ^{32}P -labeled R17 RNA to form initiation complexes at the beginning of all three R17 cistrons, cleaving with colicin, and then trimming the unprotected portions of the mRNA with pancreatic RNase. When the resulting 70S initiation complexes were disassembled in 1% sodium dodecyl sulfate and subjected to gel electrophoresis as in Fig. 2, we found by fingerprint analysis (not shown) that the A site preferentially appears in the complex region of the gel. In one experiment, the molar ratio of the three R17 initiation sites in the original 70S peak was 1:1.4:1.2 (A:coat protein:replicase); a ratio of 1:0.5:0.25 was found in the complex, while 1:3.8:1.4 was observed in the unbound fragments. Thus as predicted (23), the A site, which can potentially form seven base pairs with the colicin fragment, remains most stably bound during the isolation procedure.

DISCUSSION

In a 70S ribosomal initiation complex including the R17 A protein initiator region, the messenger fragment is positioned such that upon gentle dissociation of the ribosome, it is recovered noncovalently complexed with the 16S rRNA. The interaction occurs specifically with that portion of the 16S species which is released by colicin E3 treatment. The residues involved in Watson-Crick base-pairing are presumably those depicted in Fig. 1 since all other potential interac-

tions between these two RNA fragments would not be expected (23) to exhibit the stability and melting behavior we observe for the complex.

mRNA-rRNA complementarity as a determinant in initiation

Our data therefore strongly support the idea that base pairing between the mRNA and the 3' end of 16S rRNA occurs during the formation of a functional initiation complex in *E. coli* (1, 18). If so, the interaction is probably an important component of both initiation site selection and the physical process of mRNA binding to ribosomes.

During ribosome recognition of true initiator triplets, the extent of mRNA-rRNA complementarity may be used to achieve fine control over the relative efficiency of initiation at different cistrons. Although only three base pairs can be formed by some initiator regions (Table 1), recall that this number insures specificity in codon-anticodon interaction; thus ribosomal design may allow three base pairs to provide partial specificity for initiation as well. In addition, the variable distance between the polypurine tract and the initiator triplet is likely to influence the formation and stability of the mRNA-ribosome complex. Here, the flexibility of the 3' terminus of the 16S RNA molecule may be essential for providing the required fit.

On the other hand, rRNA-mRNA complementarity is not the only component of messenger selection and binding by ribosomes. Since any AUG triplet can direct initiation of a polypeptide chain (26), the interaction of the fMet-tRNA with the initiator codon is obviously of prime importance. Possible roles for 30S ribosomal proteins S12 and S1 have been discussed previously (18). Initiation factors also facilitate the reaction, perhaps in part by compensating for or stabilizing weak complementarity between the mRNA and rRNA (18). In the case of the three R17 initiator regions, ribosome binding and protection of the coat and replicase sites are at least 7- to 10-fold dependent upon the presence of initiation factors and fMet-tRNA (17), whereas binding to the A protein initiator region shows only a 2- to 5-fold dependence (17, 19). Thus the high degree of complementarity between the A site and the 16S 3' end may substitute for the usual factor and tRNA requirements in initiation complex formation at this particular initiator region. Conversely, base pairing between the rRNA and initiation sites with weak complementarity may function more to align the mRNA on the ribosome than to provide the primary driving force in messenger binding.

Finally, RNA secondary and tertiary structure are known to negatively influence ribosome recognition of initiation sites in mRNA (19, 27). Specifically, if the polypurine tract and/or the AUG of a potential initiator region is sequestered by intramolecular base pairing, the initiator region may not be utilizable (see ref. 19). On the other hand, the exposure of appropriately situated polypurine sequences could account for ribosome recognition of selected noninitiator AUGs in artificially unfolded mRNAs (27).

RNA-RNA interactions in ribosome function

The diagram of Fig. 1 suggests that upon mRNA binding, one set of RNA base pairs (formed *intramolecularly* within the 16S RNA) is exchanged for another (formed *intermolecularly* between the message and the ribosome). Thus, when the ribosome moves beyond the initiation site during the elongation process, the rRNA base pairs would be expected to reform in the reverse reaction. Although we can only speculate that the illustrated secondary structures exist in the

functioning ribosome, an exchange of base pairs is an attractive mechanism since it would significantly lower the energy required to disrupt the mRNA-rRNA interaction.

The reaction of Fig. 1 is not the first RNA-RNA interaction to be identified as important to ribosome function. In addition to codon-anticodon recognition, considerable evidence suggests that hydrogen-bonding between a specific sequence in 5S RNA and the TΨCG region common to prokaryotic noninitiator tRNAs serves to fix the entering tRNA in the amino-acid site on the ribosome (28, 29). Such examples support the idea that the primeval translational machinery may have consisted almost entirely of RNA molecules (30), many of the proteins being a later addition to the evolving ribosome.

A comparable mechanism in eukaryotic initiation?

Since the basic features of almost all fundamental processes prove to be similar in prokaryotes and eukaryotes, it is tempting to assume that the 3' terminus of 18S RNA in the eukaryotic ribosome likewise interacts with messenger RNA during polypeptide chain initiation. Indeed, all species of 18S RNA that have been analyzed (ranging from yeast to mammals) possess an identical 3'-terminal sequence-GAUCAUUA_{OH} (31). The first ribosome binding site whose sequence was determined from a eukaryotic mRNA (32) does exhibit a four-base complementarity with this terminus. Moreover, the observed compatibility between ribosomes and mRNAs from various eukaryotic species is consistent with this hypothesis (33). By contrast, since prokaryotic messages have presumably been tailored to interact optimally with their own particular 16S ribosomal RNAs [here the 3'-terminal sequences do differ (18)], species specific interactions are observed in bacterial systems (18). Comparably, the expression of most bacterial genes in eukaryotic cells (or the reverse) would be predicted to encounter at least some difficulty at the point of polypeptide chain initiation.

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