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Binding of ribosomal protein S1 of *Escherichia coli* to the 3′ end of 16S rRNA

(RNA-protein interaction/initiation of protein synthesis/colicin E3/aurin tricarboxylic acid)

ALBERT E. DAHLBERG* AND JAMES E. DAHLBERG†

* Division of Biological and Medical Sciences, Brown University, Providence, Rhode Island 02912; and † Department of Physiological Chemistry, University of Wisconsin, Madison, Wisc. 53706

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ABSTRACT **Ribosomal protein S1 reversibly binds the 49-nucleotide fragment that is cleaved from the 3′ end of 16S rRNA in ribosomes by colicin E3. The fragment has secondary structure in the form of a hairpin loop. At the base of the stem is a sequence (A-C-C-U-C-C) thought to be involved in the base pairing with complementary sequences in mRNA during the initiation of protein synthesis. The role of S1 may be to stabilize this region of the fragment in an open conformation to allow for base pairing to mRNA. This model is supported by the observation that S1 binds specifically to this region of the fragment. In addition, aurin tricarboxylic acid, an inhibitor of protein synthesis, reverses this effect by disrupting the S1–RNA complex. These results can explain why S1 is an essential component of the ribosome for translation of natural mRNA and why aurin tricarboxylic acid blocks initiation.**

The initiation of protein synthesis in bacteria requires the formation of an initiation complex consisting of the 30S ribosomal subunit, mRNA, fMet-tRNA, GTP, and initiation factors (1). Ribosomal protein S1 is required for maximal binding of the mRNA to the ribosome (2–5). This S1-dependent binding can occur in the absence of fMet-tRNA, GTP, and initiation factors (6, 7). The presence of protein S1 at the mRNA bindng site has been suggested by covalently crosslinking S1 in the ribosome to the synthetic messenger poly(4-thiouridylic acid) (8).

The specificity of initiation of protein synthesis using natural mRNAs is determined, at least in part, by proteins S1 and S12 and 16S rRNA (9–12). Recently it was proposed that 16S rRNA contributes to the specificity by means of the sequence A-C-C-U-C-C near its 3′ end (13, 14). This sequence may base-pair with a purine-rich sequence G-G-A-G-G-U on the 5′ side of the initiator triplet AUG of many natural mRNAs. However, the rRNA may assume a conformation in which a major portion of this pyrimidine-rich sequence is in a base-paired hairpin loop structure (15), thereby making it inaccessible to the mRNA.

The association of ribosomal protein S1 with the 3′ end of 16S rRNA, first suggested by the crosslinking experiments of Kenner (16), was supported by the demonstration that both S1 and the terminal 49 nucleotides of the 3′ end of 16S rRNA (17–19) were removed from the ribosome after colicin E3 treatment (6). We report here that S1 can form a complex with this 3′ RNA fragment, and that the properties of the complex resemble those exhibited by S1 on the intact 30S subunit with respect to stability in the presence of mRNA and certain antibiotics (6). S1 binds specifically to a region within 12 nucleotides from the 3′ end, which includes the sequence A-C-C-U-C-C. We propose that S1 functions by exposing this region, thereby permitting base-pairing to the purine-rich sequence on the mRNA near the initiator triplet.

MATERIALS AND METHODS

Materials. Reagents for cell lysis and gel electrophoresis were as described (6, 20). Reagent grade chemicals were used throughout. Chloramphenicol, tetracycline, streptomycin, aurin tricarboxylic acid, rifampicin, poly(U), RNase T1, and Tris (Sigma 7-9) were purchased from Sigma Chemical Co. Kasugamycin was a gift from Dr. J. Davies, University of Wisconsin; purified ribosomal protein S1 of *Escherichia coli* MRE 600 was a gift from Dr. A. Wahba, University of Sherbrooke, Quebec; purified colicin E3 was a gift from Dr. M. Nomura, University of Wisconsin; Qβ RNA was a gift from Drs. A. Palmenberg and P. Kaesberg, University of Wisconsin.

Methods. *Isolation of colicin E3 RNA fragment: E. coli* MRE 600 growing at 0.5×10^8 cells per ml in 200 ml of low-phosphate L broth (21) at 37° received 10 mCi of carrier-free $^{32}PO_4$ (New England Nuclear Corp.). After two generations, rifampicin (100 μg/ml) was added, and 10 min later the cells were harvested on ice, centrifuged, and lysed by a freeze-thaw lysozyme procedure (22). After a low-speed centrifugation to remove cell debris, the ribosomes were pelleted by centrifugation for 90 min at 60,000 rpm in the 65 rotor in a Beckman L2-65B ultracentrifuge. The pellet was dissolved in 350 μl of 25 mM Tris·HCl pH 8.0 buffer with 60 mM KCl and 10 mM $MgCl_2$, and was reacted with 200 μg of purified colicin E3 at 37° for 30 min. The ribosomes were diluted with an equal volume of water and centrifuged through a 37-ml 5–20% sucrose density gradient, in 25 mM Tris·HCl pH 8.0 buffer with 1 mM $MgCl_2$, for 8⅔ hr at 27,000 rpm, at 3° in the SW27 rotor. Fractions (0.75 ml) were collected and 10-μl aliquots were counted for Cerenkov radiation in 2.5 ml of water to determine the position of the 30S subunits. The 30S particles were pooled, pelleted by centrifugation at 60,000 rpm for 6 hr at 3° in the 65 rotor, and resuspended in 400 μl of 25 mM Tris·HCl pH 8.0 buffer without $MgCl_2$. After 45 min at 0° the sample was centrifuged at 35,000 rpm for 8½ hr through a similar sucrose gradient of 11 ml, but without $MgCl_2$, in the SW 41 rotor. Samples of 0.25 ml were collected and radioactivity was determined (see Fig. 1).

Gel Electrophoresis Conditions. The preparation of the gels and the electrophoretic techniques were as described (6, 20), with the vertical gel cell of E. C. Apparatus Corp., St. Petersburg, Fla. Samples were electrophoresed into slab gels of 10% or 15% polyacrylamide or composite slab gels of 2.5% polyacrylamide, 0.5% agarose, in Tris/EDTA/boric

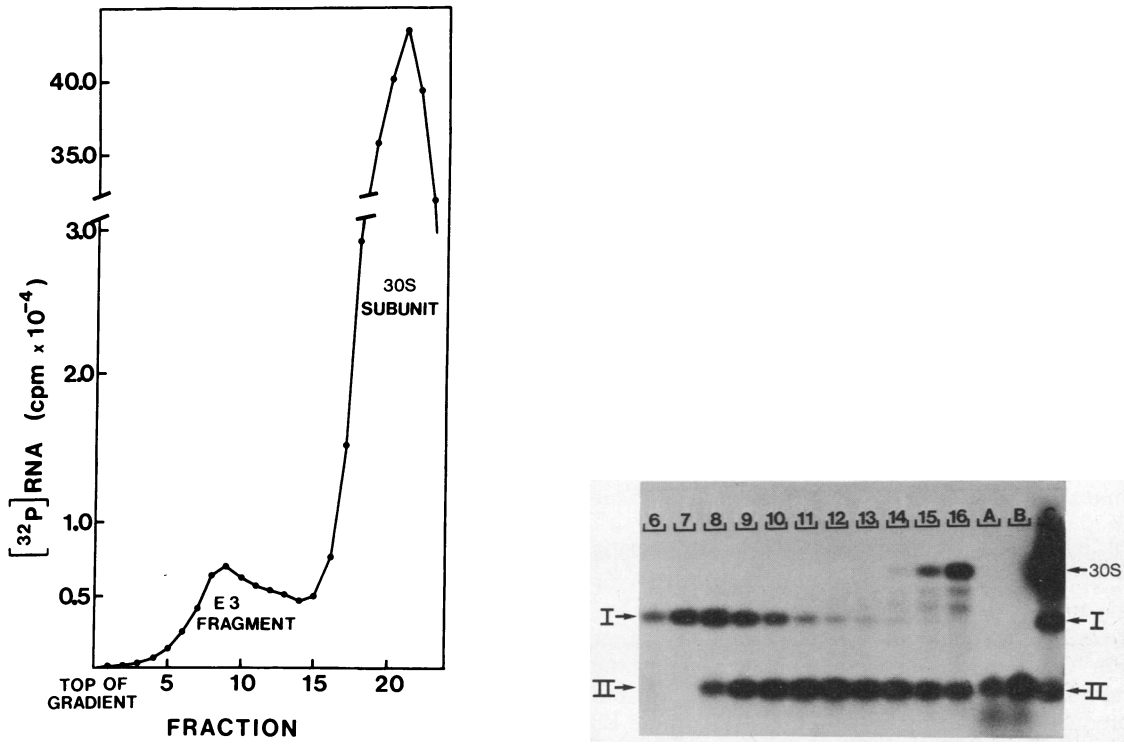

FIG. 1. Isolation of the E3 RNA fragment. ^{32}P-Labeled ribosomes, treated with colicin E3, were fractionated by two sucrose gradient centrifugations, with and without $MgCl_2$ *(Materials and Methods)*. The profile of the [^{32}P]RNA in the second gradient is seen on the *left*. Gradient fractions (20 μl) were electrophoresed into a 2.5% polyacrylamide/0.5% agarose gel for 90 min *(Materials and Methods)*. An autoradiograph of the gel is shown on the *right*. The direction of electrophoresis is from top to bottom. Samples: Gradient fractions 6–16; tRNA of the first gradient (A, B); 30S subunits of the first gradient (C). Two bands from samples near the top of the gradient are labeled I and II.

acid, pH 8.3 buffer at 200 V at 0° for times indicated in legends to figures. The gels were then autoradiographed.

Partial RNase T1 Treatment of E3 RNA. Fifty units of RNase T1 were added to a 400-μl aliquot of E3 RNA (fractions 11 and 12, Fig. 1) from the second sucrose gradient. After incubation at 25° for 10 min, the sample was extracted by shaking with 0.2% sodium dodecyl sulfate and an equal volume of water-saturated phenol at 25° for 10 min. The RNA in the aqueous phase was precipitated with ethanol, dissolved in 100 μl of 25 mM Tris·HCl pH 8.0 buffer, and divided into two aliquots of 50 μl, one of which was mixed with 10 μg of S1 at 0° for 5 min prior to application of both aliquots to the gel.

RNA Fingerprint Analysis. After polyacrylamide gel electrophoresis of RNA fragments, the RNAs were isolated from the gel, precipitated, and digested with RNase T1 or pancreatic RNase as described elsewhere (23, 24). The resulting oligonucleotides were separated by two-dimensional paper electrophoresis (fingerprinting) as described by Sanger *et al.* (23).

RESULTS

Isolation of two fragments produced by colicin E3

The colicin E3 fragment from the 3′ end of 16S rRNA was isolated from ^{32}P-labeled ribosomes by two successive sucrose gradient fractionations as described in *Materials and Methods*. All of the fragments remained attached to 30S subunits in the first gradient with 1 mM $MgCl_2$; only tRNA was found near the top of the gradient. The fragments were dissociated from the subunits in the absence of added $MgCl_2$ in the second gradient and were free of tRNA contamination. Fig. 1 shows the profile of [^{32}P]RNA in the second sucrose gradient and an autoradiograph of a gel in which aliquots from the gradient were subjected to electrophoresis. Two bands were obtained from the samples from near the top of the gradient. The more slowly migrating band (I) also had a slower sedimentation rate, peaking in gradient fraction 8, while the faster migrating band (II) peaked in fractions 11–12. Band II migrated slightly faster than the marker tRNA (slots A and B) in the 2.5% polyacrylamide/0.5% agarose gel, and considerably faster than tRNA in a 10% gel (data not shown). The 30S subunits appeared in lower fractions from the gradient (fractions 15 and 16). The 30S sample from the first sucrose gradient yielded both bands I and II (slot C), which did not remain associated with the subunit in the absence of $MgCl_2$.

The two colicin E3-produced fragments contain the same RNA

The RNAs of bands I and II (Fig. 1) were analyzed by digestion of appropriate fractions with RNase T1, followed by two-dimensional RNA fingerprinting (data not shown). They were found to be identical to each other and to the colicin E3 RNA fragment reported previously (17). The RNA fragment is hereafter referred to as E3 RNA.

Protein S1 binds the E3 RNA reversibly

The difference between bands I and II appears to be the presence of S1 on band I. Addition of purified S1 at 0° converted band II RNA (fraction 12) to the more slowly migrating band I (Fig. 2), just as it converts F-30S subunits to the more slowly migrating S-30S subunits (6). Moreover, phenol extraction did not change the electrophoretic mobility of band II. These results suggest that no additional proteins re-

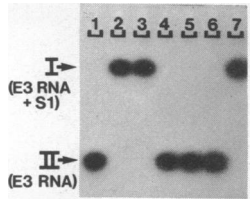

FIG. 2. Binding of S1 to the E3 RNA fragment. Samples containing predominantly band I or band II from the sucrose gradient of Fig. 1, with or without added S1 and subsequently added mRNA or antibiotics, were electrophoresed into a 10% polyacrylamide gel for 4 hr *(Materials and Methods)*. Samples: Gradient fraction 13 (slot 1); fraction 7 (slot 2); fraction 13 plus S1 (approximately 2-fold excess) at 0° (slot 3); aliquots of the sample in slot 3 plus 1 μg of poly(U) at 0° (slot 4); 4 μg of Qβ RNA (slot 5); 1 μg of aurin tricarboxylic acid (slot 6); or 200 μg of kasugamycin (slot 7).

mained bound after isolation of E3 RNA by sucrose gradient centrifugation.

S1 was displaced from the E3 RNA, just as from S-30S subunits (6), by subsequent addition of poly(U), Qβ RNA, or the antibiotic aurin tricarboxylic acid (Fig. 2), but not by kasugamycin, tetracycline, chloramphenicol, streptomycin, or erythromycin. The same results were obtained whether the complex was isolated from the sucrose gradient (Fig. 1, band I) or was produced *in vitro* by the addition of S1 to the E3 RNA.

Formaldehyde treatment of the E3 RNA inhibits S1 binding

When E3 RNA was treated with formaldehyde under mild conditions (25), the RNA lost its ability to bind S1 (Fig. 3). This effect was reversed by heating in the presence of $MgCl_2$. A reversible effect on the electrophoretic mobility of the RNA was also noted (Fig. 3). These results suggested that S1 binding was inhibited either by an alteration in RNA secondary structure or by RNA base modifications (26).

S1 binds to the 3′ end of E3 RNA

The site of S1 binding was examined by partial hydrolysis of E3 RNA by RNase T1 in the presence and absence of S1. The partial digestion products were extracted with phenol and analyzed by gel electrophoresis as done for other ribo-

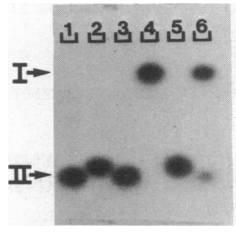

FIG. 3. Effect of formaldehyde treatment of E3 RNA and reversal on electrophoretic mobility and ability to bind S1. E3 RNA (fraction 12, Fig. 1) was extracted with sodium dodecyl sulfate and phenol, precipitated with ethanol, and redissolved in 25 mM triethanolamine buffer, pH 7.6. An aliquot was treated with formaldehyde (25), precipitated in ethanol, and redissolved in the same buffer. Subsequently an aliquot with $MgCl_2$ added (10 mM) was heated for 2 min at 100° and then cooled slowly to 0°. Samples with and without added S1 were electrophoresed into a gel identical to that of Fig. 2. Samples: E3 RNA (slot 1); formaldehyde-treated E3 RNA (slot 2); formaldehyde-treated E3 RNA heated in $MgCl_2$ (slot 3); an aliquot of the sample in slot 1 plus S1 (slot 4); an aliquot of the sample in slot 2 plus S1 (slot 5); an aliquot of the sample in slot 3 plus S1 (slot 6).

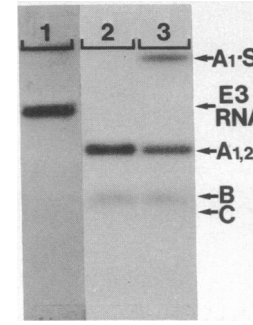

FIG. 4. S1 binding to RNase T1 partial digestion products of E3 RNA. E3 RNA and its RNase T1 partial digestion products, with or without S1, were electrophoresed into a 15% polyacrylamide gel for 4 hr *(Materials and Methods)*. Samples: Fraction 13 of Fig. 1, untreated E3 RNA (slot 1); partial digestion products of E3 RNA without S1 (slot 2), and with added S1 (slot 3). RNA fingerprint analysis of bands labeled A–C is summarized in Fig. 5.

somal proteins (27). The products were identical in both samples and gave the pattern shown in Fig. 4, slot 2. Therefore, S1 did not confer resistance to RNase T1. The "native" S1-E3 RNA complex (fraction 7 of Fig. 1) used in this experiment contained no endogenous RNase, as assayed by incubation at 25° in the absence of RNase T1, prior to phenol extraction and gel electrophoresis (data not shown).

Since S1 conferred no obvious protection of the E3 RNA against RNase T1 digestion, the possibility of S1 binding to a site within one of the digestion products was examined. In this experiment, the partial RNase T1 digestion products (digested in the absence of S1) were added to purified S1 and the mixture was subjected to electrophoresis. As seen in Fig. 4, slot 3, an RNA fragment did bind to the added S1. A pattern identical to Fig. 4, slot 3, was obtained when "native" S1–E3 RNA complex was treated with RNase T1 and applied directly to the gel.

The structures of the partial digestion products in the bands shown in Fig. 4 were identified by total digestion with RNase T1 or pancreatic RNase and fingerprinting. The results of these analyses are summarized in Fig. 5. Band A contained several oligonucleotides 11–12 residues long, whereas bands B and C contained oligonucleotides 7 and 3 residues long, respectively. Analysis of the bands in slot 3 of Fig. 4 showed that one oligonucleotide, the one from the 3′ end of the E3 RNA (partial digestion product A1), was selectively removed from the digest by S1, and migrated as the band denoted A_1·S1 in Fig. 4. Therefore, we conclude that S1 binds specifically to the 3′-terminal oligonucleotides of 16S rRNA.

Protein S1 binds the E3 RNA and the 3′-oligonucleotide in the same way

Since S1 binds the 3′-terminal oligonucleotide (band A_1, Figs. 4 and 5), the possibility was investigated that the binding might account for all of the interactions that we observed between S1 and the E3 RNA. Therefore, experiments similar to those described above for E3 RNA were performed on the 3′-oligonucleotide. As in the case of E3 RNA, binding to S1 was reversed by addition of aurin tricarboxylic acid, Qβ RNA, or poly(U). Interestingly, mild formaldehyde treatment of the 3′-oligonucleotide inhibited its ability to bind S1, and this inhibition was reversed by heating the treated oligonucleotide in the presence of $MgCl_2$. Thus, in all characteristics that we assayed, E3 RNA and its purified 3′-oligonucleotide bound to S1 in the same way. These re-

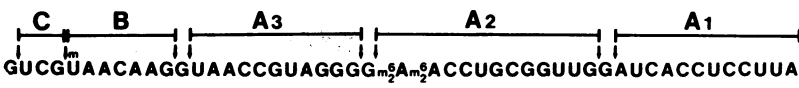

FIG. 5. A summary of the nucleotide sequence data obtained for the RNA fragments A to C from the gel shown in Figure 4. RNAs were eluted from the gel shown in Fig. 4 and identified by digestion with RNase T1 or pancreatic RNase, followed by two-dimensional paper electrophoresis (23). Individual oligonucleotides were identified by their mobilities on the fingerprint pattern and by their compositions as determined by redigestion with pancreatic RNase or RNase T1, respectively. The RNAs in band A in slots 2 and 3 (Fig. 4) were analyzed separately and differed only by the absence of the 3'-oligonucleotide from the preparation in band A of slot 3. That oligonucleotide was found in band A_1·S1 of slot 3. The RNA of band A_1·S1 was isolated after soaking the gel band in 0.1% sodium dodecyl sulfate and re-electrophoresing in a second 15% polyacrylamide gel, to separate it from protein S1. The sequences shown are drawn to match the sequence of this region as determined by others (13, 15).

sults indicate that the S1 binding site is primarily, if not entirely, within twelve nucleotides of the 3' end of the RNA.

DISCUSSION

The present studies demonstrate that S1 associates with the 49-nucleotide fragment of RNA cleaved from the 3' end of 16S rRNA by colicin E3. The interactions of S1 with the E3 RNA resemble in many respects those reported previously for the intact 30S subunit (6): (i) Purified S1 binds at low ionic strength to both E3 RNA and 30S subunits; (ii) S1 binding retards the sedimentation and the electrophoretic mobility of both; (iii) Particles produced by S1 binding *in vitro* to E3 RNA or to 30S subunits have the same electrophoretic mobilities as the respective "native" complexes; (iv) S1 is displaced by aurin tricarboxylic acid and by natural and synthetic mRNAs at 0° from both E3 RNA and 30S subunits; (v) About 50% of the recovered E3 RNA from the second sucrose gradient (Fig. 1) carried S1; loss of S1 from the rest of the E3 RNA may have occurred in a fashion similar to its loss from intact ribosomes during sucrose gradient centrifugation (6).

The site on E3 RNA to which S1 bound was investigated by digestion with RNase T1 in the presence and absence of S1. In both cases, several RNase T1 partial digestion products were obtained, presumably due to secondary structure of the RNA fragment. However, S1 conferred no detectable protection against RNase T1 digestion. S1 did bind to one oligonucleotide in the digest, the 3' dodecamer, indicating that the S1 binding site is within 12 nucleotides of the 3' terminus. The binding of S1 to the pyrimidine-rich 3' oligonucleotide agrees well with the observation (28) that S1 binds to polymers of pyrimidines.

When E3 RNA was incubated at 25° with added purified S1, the RNA was degraded to small products (1 to 3 nucleotides long). This degradation was not observed when the complex was kept cold, nor did it occur when "native" S1-E3 RNA complexes were incubated at 25°. Therefore, the protection experiments were performed on native, rather than reconstituted, complexes. The nuclease in the S1 samples did not appear to affect the binding *per se*, since addition of S1 to the RNase T1 partial digestion products (kept at 0°) and RNase T1 digestion of the "native" complex both led to binding of the 3'-oligonucleotide to S1.

When the 3'-oligonucleotide was treated with formaldehyde, it lost its ability to bind S1. The unusual sequence of this oligonucleotide makes it very unlikely that this inhibition occurs as a result of alterations of secondary structure. It seems more likely that the inhibition results from a direct reaction of the formaldehyde with the bases. Likewise, we propose that a similar modification of the 3' sequence in E3 RNA leads directly to inhibition of S1 binding of that RNA.

The inhibition of S1 binding by poly(U) presumably is a result of direct competition for the S1. The same explanation may be true for Qβ RNA, since it also has a long stretch of pyrimidines at its 3' end (29). Alternatively, the ribosome binding sites in Qβ RNA may bind to the 3'-oligonucleotide sequence and inhibit its binding to S1. The mechanism by which aurin tricarboxylic acid interacts with, and displaces, S1 from E3 RNA or the 3'-oligonucleotide is unknown. However, if binding to the RNA is required for S1 function, this displacement by aurin tricarboxylic acid provides a molecular explanation for the inhibitory effect of the antibiotic on initiation of protein synthesis.

Two structures can be drawn for E3 RNA in which there is considerable pairing of the bases at the 3' end (Fig. 6A). However, since S1 binds the 3' end, we think it unlikely that either structure would exist in the presence of S1. We propose that in the presence of S1 the terminal 3' nucleotides are unpaired and stabilized in the open form of Fig. 6B,

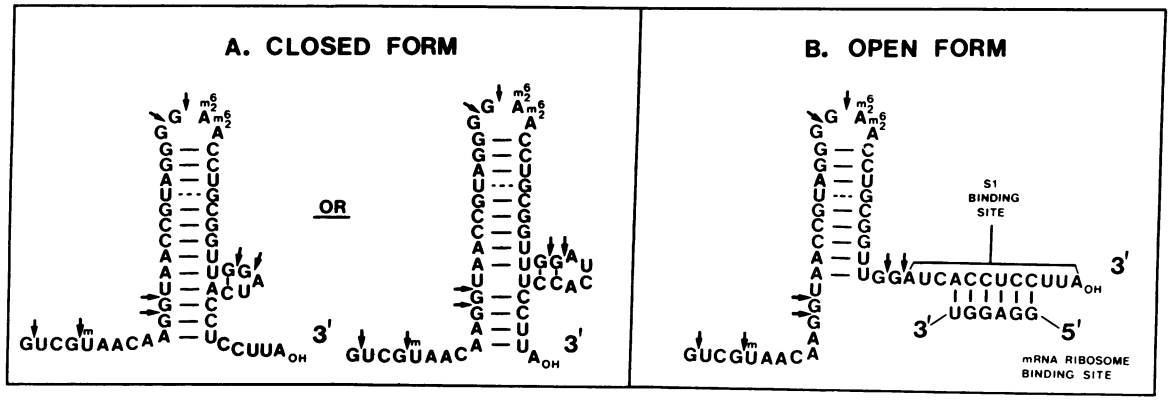

FIG. 6. Proposed structures of E3 RNA in the presence and absence of S1. (A) Structure proposed by Ehresmann *et al.* (15) *(left)* and an alternate structure proposed by us in which there is more pairing of the 3'-terminal bases *(right)*. (B) Structure which has the 3'-terminal bases in an open form. Note that this structure, which may exist with or without S1, unfolds the A-C-C-U-C-C sequence, which is complementary to ribosome binding sites of several mRNAs. Sites susceptible to cleavage by RNase T1 are shown by arrows.

thereby making this region accessible for hydrogen bonding with the ribosome binding sites in mRNA. Although there may be an equilibrium between the open and closed forms in the absence of S1—our data do not rule out this possibility—the absolute requirement for S1 in translation of natural mRNA (9, 10) and the observation that S1 is found on polyribosomes in about one copy per ribosome (30) would suggest that the stabilized, open form is a required structural feature for proper ribosome function.

While this work was in progress J. Steitz and K. Jakes (personal communication) isolated an RNA·RNA hybrid of E3 RNA and the A protein initiator region from R17 RNA following colicin E3 treatment of an initiation complex. This strongly supports the base pairing of 16S rRNA with mRNA proposed by Shine and Dalgarno (13, 14).

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