Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 7884–7888, August 1994
Biochemistry

Independent *in vitro* assembly of a ribonucleoprotein particle containing the 3′ domain of 16S rRNA

(ribosomes/*in vitro* reconstitution/ribosomal proteins/spectinomycin)

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Contributed by Harry F. Noller, April 26, 1994

ABSTRACT **Small (30S) subunits of *Escherichia coli* ribosomes are composed of 21 proteins and a 1542-nucleotide 16S rRNA, whose secondary structure is divided into three domains. An *in vitro* transcript of the 3′ domain of 16S rRNA (residues 923–1542), assembles efficiently with 30S ribosomal proteins to form a compact ribonucleoprotein (RNP) particle. Isolated particles examined under the electron microscope have a globular appearance, similar in size and shape to the head of the 30S ribosomal subunit. Two-dimensional gel analysis of the particles indicates the presence of proteins S3, S7, S9, S10, S13, S14, and S19 and smaller amounts of S2, all of which have been localized to the head of the 30S subunit by immunoelectron microscopy and neutron diffraction and belong to the S7 assembly family. Interestingly, protein S4, which is believed to interact exclusively with the 5′ domain, is also reproducibly found associated with the particles in significant amounts. Chemical probing of the RNA in the assembled particle reveals characteristic cleavage protection patterns, showing that the proteins assemble with the 3′-domain RNA similarly to the way in which they assemble with 16S rRNA, although some of the later steps of assembly appear to be incomplete. These results show that the 3′ domain of 16S rRNA can indeed assemble independently of the rest of the 30S subunit into a particle that resembles its structure in the ribosome. In addition, the assembled particles are able to bind spectinomycin with an affinity comparable to that of 30S subunits.**

Small (30S) subunits of *Escherichia coli* ribosomes are composed of 21 ribosomal proteins complexed with a 1542-nucleotide 16S rRNA. For very large RNP structures, by analogy with the structures of large proteins, it is reasonable to expect a structural organization based on autonomously assembling structural subdivisions, or domains. With the emergence of its secondary structure, it became evident that 16S rRNA is itself organized into three major domains and one minor domain (1). An important question is whether these RNA secondary structure domains correspond to true three-dimensional structural domains, as defined by structural biologists (2). An early indication of domain organization in ribosomes was the isolation of an RNP complex by Brimacombe and co-workers containing S7, S9, S19, either S13 or S14, and fragments derived from the 3′ half of 16S rRNA after partial ribonuclease digestion of 30S small ribosomal subunits (3, 4). This was extended by Zimmermann (5) to isolation of specific RNP complexes corresponding to the 5′ and central domains and demonstration that the deproteinized RNA fragments from each of the three domains were able to reassemble with specific subsets of ribosomal proteins. Nomura and co-workers showed that there were two to three independent nucleation events for *in vitro* assembly of 30S ribosomal subunits, suggesting independent assembly of different domains (6). Assembly mapping results suggest that one such nucleation event could be the binding of protein S7 to 16S rRNA, upon which the assembly of S2, S3, S9, S10, S13, S14, and S19 depends (7). More recently, studies on 16S rRNA–protein interactions using chemical footprinting have shown that this same group of eight 30S proteins interacts exclusively with the 3′ domain of the RNA (8, 9). These proteins have all been mapped to the head of the 30S subunit by immunoelectron microscopy (reviewed in refs. 10 and 11) and neutron diffraction studies (12). These results all support the idea that the head of the 30S subunit is composed of a RNP domain that might be capable of autonomous assembly.

The experiments presented here show that a subfragment of 16S rRNA, corresponding to the 3′ domain of 16S rRNA, assembles with this same group of eight ribosomal proteins—the S7 family—into a compact, globular RNP particle that resembles the head of the 30S ribosomal subunit. At least some 3′-domain particles are able to bind the antibiotic spectinomycin with an affinity and specificity comparable to that of complete 30S subunits.

MATERIALS AND METHODS

T7 Transcription of rRNAs. Full-length 16S rRNA was transcribed from *Bsu*36I-linearized pBS16S.RS, a derivative of pBS16S.10B (13) in which 21 extra nucleotides preceding the 5′ end of 16S rRNA were deleted by loop-out mutagenesis (14) using the oligonucleotide CTATAGGGCAAATTGAAG. RNA corresponding to the 3′ domain (nucleotides 923–1542) was transcribed from linearized pBS3D.1, which is derived from pBS16S.10B by digestion with *Sph* I and *Apa* I, followed by religation of the large fragment with a synthetic linker comprised of the two partially complementary oligonucleotides 5′-CACGGGGGCC and 5′-CCCGTGCATG. For spectinomycin binding, 3′-domain RNA was transcribed from pFD3 (15), which lacks the spectinomycin-resistance mutation.

After transcription with T7 RNA polymerase (16, 17), transcripts were extracted with phenol and chloroform at 4°C, purified over a Sephadex G-50 column in water, and recovered by precipitation with ethanol from 0.3 M NH_4Cl. RNA was redissolved in 20 mM Hepes, pH 7.5/1 mM EDTA and examined by denaturing 2.2 M formaldehyde/1% agarose gel electrophoresis (18).

***In Vitro* Reconstitution.** Total 30S ribosomal proteins (TP30) were dialyzed overnight at 4°C vs. Rec20 buffer (20 mM Hepes, pH 7.5/400 mM NH_4Cl/20 mM $MgCl_2$/6 mM

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Abbreviations: RNP, ribonucleoprotein; TP30, total 30S ribosomal proteins; DMS, dimethyl sulfate.

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2-mercaptoethanol) in 6 M urea and then against two changes of Rec20 without urea for 1 hr each. Prior to reconstitution, the RNA (dissolved in Rec20) was heated for 15 min at 37°C. Reconstitution (19) was carried out at a final RNA concentration of 0.4 μM by using a 3-fold molar excess of TP30 in Rec20 buffer containing 500 mM NH_4Cl and 0.01% Nikkol detergent (Nikko Chemicals, Tokyo) in 15-min heating steps at 40°C, 43°C, 46°C, 48°C, and 50°C. Reconstitution volumes varied between 0.2 and 1.0 ml.

Electron Microscopy. Reconstituted 30S subunits or 3′-domain RNP particles were purified by sucrose gradient centrifugation and concentrated by ultrafiltration with a Microcon 3000 (Amicon). Samples for electron microscopy were spread on single carbon films with uranyl acetate as negative stain (20).

Chemical Probing. Chemical probing of 16S rRNA, 30S subunits, and 3′-domain rRNA and RNP was performed as described by Stern *et al.* (21). Ten picomoles of RNA, 30S subunits, or RNP particles in 40 μl of buffer A (20 mM Hepes, pH 7.5/100 mM NH_4Cl/20 mM $MgCl_2$/6 mM 2-mercaptoethanol) was heated to 40°C for 30 min prior to addition of dimethyl sulfate (DMS) or Kethoxal. Modification of guanosine residues with DMS at N-7 was detected after borohydride/aniline cleavage (22).

RESULTS

***In Vitro* Reconstitution.** T7 transcripts corresponding to 3′-domain RNA (nucleotides 923–1542) (Fig. 1 *Left*) were reconstituted with a 3-fold molar excess of TP30 (19) and were isolated by sucrose gradient centrifugation (Fig. 2). The 3′-domain rRNA fragment, which sediments at about 8S (Fig. 2*A*), was converted to a species sedimenting as a sharp peak of about 13S following *in vitro* reconstitution with TP30 (Fig. 2*B*), suggesting formation of a compact RNP particle. The overall yield of reconstituted RNP particles was about 30–40% for 3′-domain RNA or full-length 16S rRNA transcripts compared with 80–90% for natural 16S rRNA (data not shown).

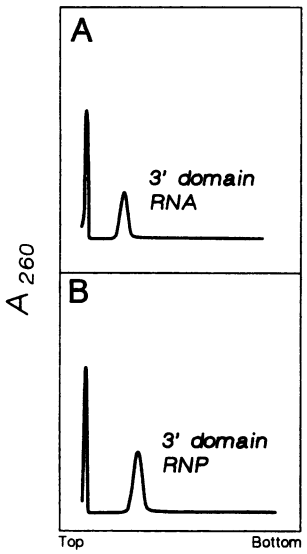

FIG. 2. Sucrose gradient analysis of *in vitro* transcribed 3′-domain rRNA (*A*) and 3′-domain RNP (*B*) particles reconstituted from *in vitro* transcribed 3′-domain rRNA and TP30. Complexes were loaded onto 10-ml 10–40% sucrose gradients in 20 mM Hepes, pH 7.5/100 mM NH_4Cl/20 mM $MgCl_2$/6 mM 2-mercaptoethanol; centrifuged for 13 hr in a SW 41 rotor (Beckman) at 35,000 rpm at 4°C; and scanned with an ISCO density gradient fractionator model 183.

Electron Microscopy. Reconstituted particles were examined by electron microscopy using single-carbon negative-stain preparations. Fig. 3 *Upper* shows reconstituted 30S subunits, and Fig. 3 *Lower* shows mixed fields of 30S subunits and reconstituted 3′-domain RNPs. The 3′-domain RNPs appear as compact globular particles, bearing a close resemblance to the heads of 30S subunits. Some images of the RNP domain show angular projections that are similar in appearance to those seen in the 30S subunit head region when viewed in certain orientations (Fig. 3 *Lower*). Some of the RNP particles appear slightly larger than the heads of 30S subunits, which could be ascribed to incomplete assembly,

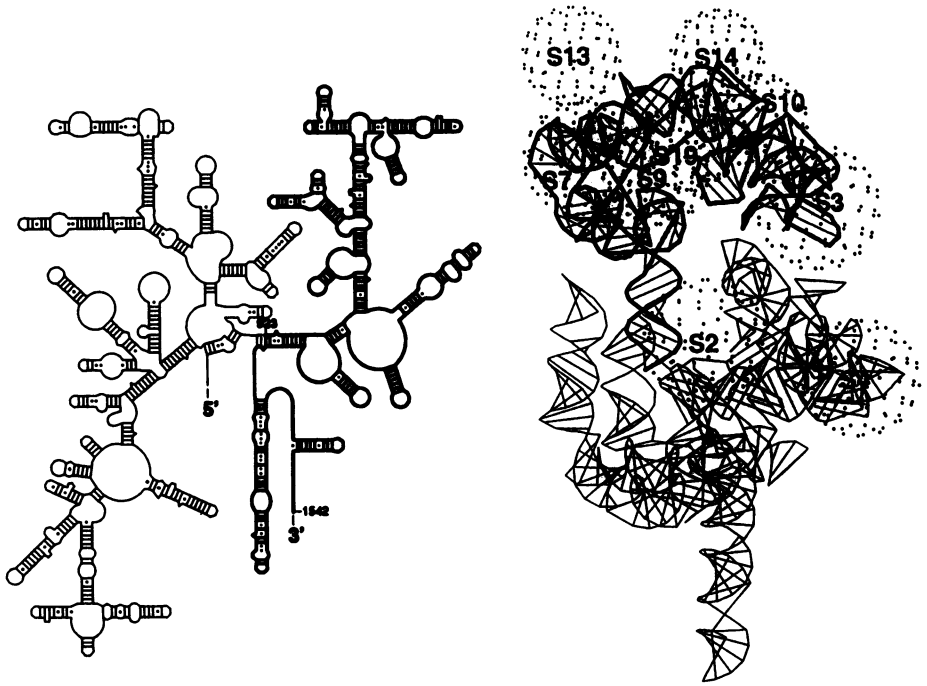

FIG. 1. Secondary structure (*Left*) and a three-dimensional model (*Right*) for folding of 16S rRNA (23). The 3′-domain RNA is emphasized by thicker lines.

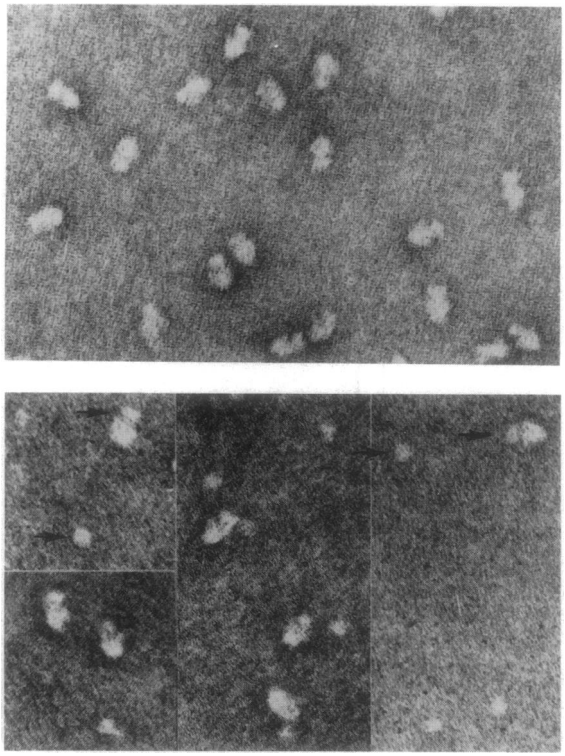

FIG. 3. Electron micrographs of reconstituted 30S subunits (*Upper*) and mixed fields of 30S subunits and 3′-domain RNP particles (*Lower*).

flattening during spreading, and adsorption to the carbon films or to the ability of the particles to lie in a different orientation from that assumed in the context of the whole 30S subunit.

Protein Composition of the 3′-Domain RNP. Fig. 4 shows two-dimensional gels of proteins extracted from isolated 30S subunits (Fig. 4*A*) and from reconstituted 3′-domain particles (Fig. 4*B*). All of the small subunit proteins except S1 and S6 are evident in isolated 30S subunits. In contrast, the 3′-domain RNP contains only proteins S2, S3, S4, S7, S9, S10, S13, S14, and S19; thus, the 3′-domain RNA selectively binds a subset of the total 30S proteins (Table 1). Protein S4, previously observed to associate exclusively with the 5′ domain (26, 27), was reproducibly found to be present in the 3′-domain RNP in reduced but significant amounts. Among the proteins previously localized to the 3′ domain (8, 9), only S2 is present at clearly reduced levels, compared with 30S subunits.

Assembly of proteins was also assessed by chemical footprinting (28) of the 3′-domain RNP (Fig. 5 and data not shown). According to this criterion, assembly of proteins S7, S9, and S19 appeared normal and complete. Although it is more difficult to assess the quality of assembly for the later binding proteins by the footprinting approach, some of the reactivity changes associated with assembly of S2, S3, S13, and S14 appear to be incomplete (assembly of protein S10 cannot be monitored by this method). This suggests that some of the mature RNA–protein or RNA–RNA contacts are not formed in all of the 3′-domain particles.

We conclude that the 3′-domain RNA assembles selectively with a subgroup of 30S proteins containing S2, S3, S4, S7, S9, S10, S13, S14, and S19, of which all but S2 and S4 are present in amounts comparable to those observed for 30S subunits (Table 1). As judged by chemical probing, the regions of the RNA that interact with proteins S7, S9, and S19 appear to be structured in a way that is indistinguishable in detail from that seen in mature 30S subunits. However, folding of the RNA in regions that are usually associated with

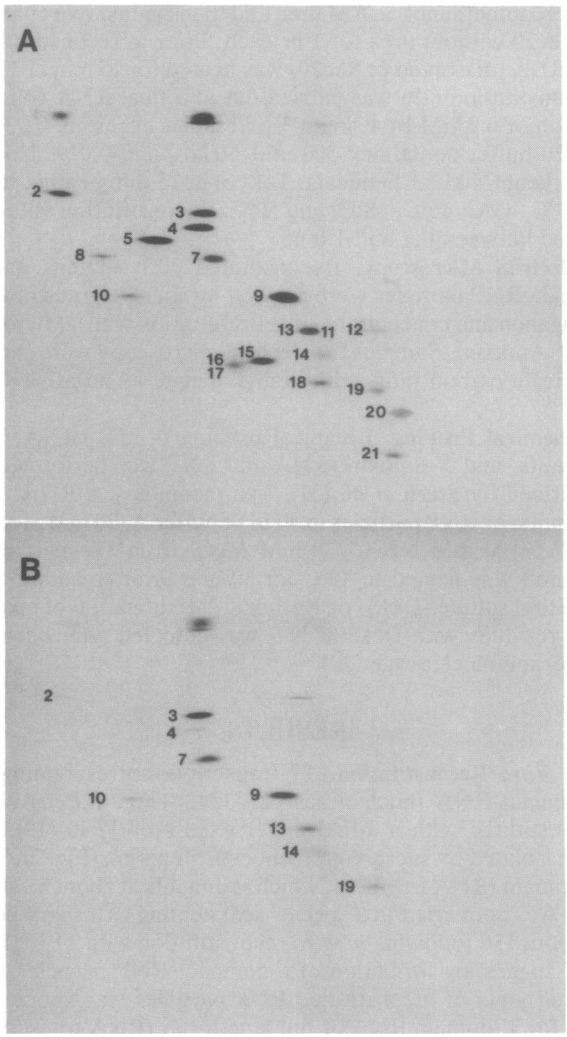

FIG. 4. Protein compositions of 0.1 nmol of natural 30S subunits (*A*) or 0.1 nmol of isolated 3′-domain particles (*B*), extracted as described by Siegmann and Thomas (24) and analyzed by two-dimensional gel electrophoresis as described by Geyl *et al.* (25).

assembly of proteins S3, S13, and S14 appears to be incomplete, even though these proteins are present in the 3′-domain particles in normal amounts.

Table 1. Assembly of ribosomal proteins in the 3′-domain RNP particle

Protein	Analysis of 3′-domain RNP assembly	
	Chemical footprinting	2-D gel analysis
S2	±	±
S3	±	+
S4	−	±
S7	+	+
S9	+	+
S10	ND*	+
S13	±	+
S14	±	+
S19	+	+

+, present; −, absent; ±, incomplete assembly; 2-D, two-dimensional.

*Protein S10 has no effects on the RNA that are observable in the fully assembled particle; therefore, its presence could not be determined by chemical footprinting.

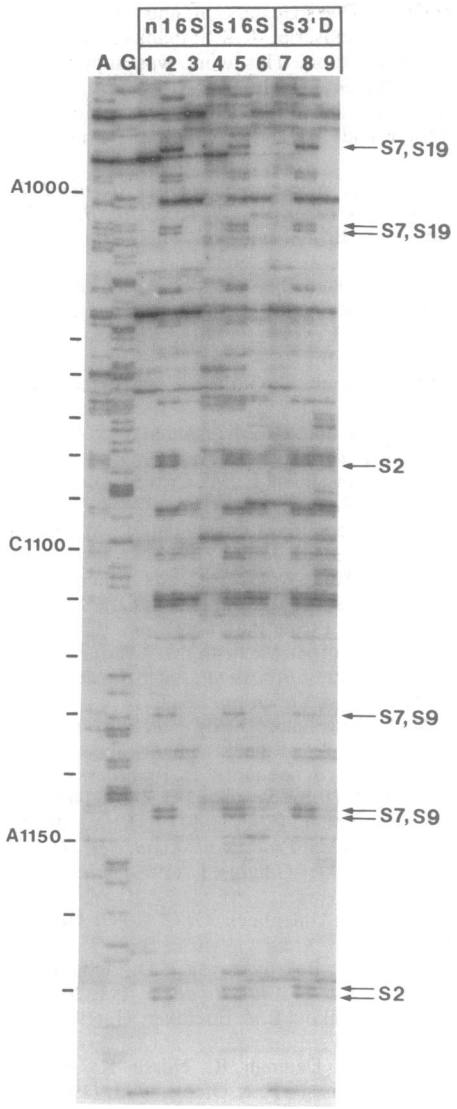

FIG. 5. Primer extension analysis of DMS-modified RNA from 30S subunits or RNP particles reconstituted from TP30 and natural 16S rRNA (lanes n16S), *in vitro* transcribed 16S rRNA (lanes s16S), and 3′-domain rRNA (lanes s3′D). The transcripts were reconstituted with a 3-fold excess of TP30 (lanes 3, 6, and 9) or without TP30 (lanes 2, 5, and 8). Lanes 1, 4, and 7 are control samples in which DMS was omitted. Arrows identify bases whose protections have been shown to be caused by assembly of specific ribosomal proteins (8, 9).

Functional Properties. Because of the many structural similarities between the 3′-domain particles and the corresponding region of 30S subunits, we asked whether they also have functional capability. Chemical footprinting was used to test the interaction of the 3′-domain particles with three ligands that are known to bind specifically to 30S ribosomal subunits: $tRNA^{Phe}$, spectinomycin, and neomycin (29, 30). Although we were unable to detect binding of $tRNA^{Phe}$ or neomycin, we found clear evidence for binding of spectinomycin (Fig. 6). Previously, it was shown that spectinomycin specifically protects the N-7 position of the guanosine residue at position 1064 from attack by dimethyl sulfate in 30S subunits (ref. 30; Fig. 6, lanes 2–5). Although the N-7 position of G-1064 is highly reactive in 30S subunits, its reactivity is less pronounced in 3′-domain particles, possibly because of incomplete assembly. Nevertheless, G-1064 is clearly protected by spectinomycin in the 3′-domain particles (Fig. 6, lanes 7–10). Moreover, the degree of protection follows the same dependence on drug concentration as seen for 30S subunits, indicating that spectinomycin binds to the 3′-domain particles with a binding affinity that is similar to that of 30S subunits.

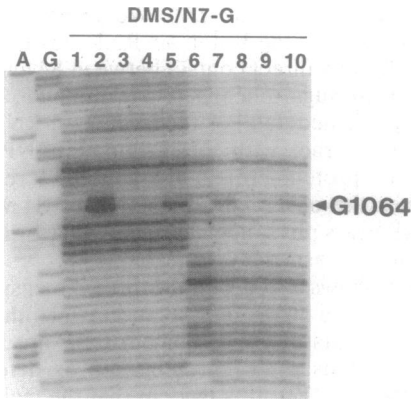

FIG. 6. Protection of G-1064 at N-7 by binding of spectinomycin to 0.2 μM 30S subunits (lanes 1–5) or 3′-domain particles (lanes 6–10) in buffer A. In lanes 1 and 6 there was no DMS modification. Spectinomycin concentrations are 0 (lanes 2 and 7), 50 μM (lanes 3 and 8), 10 μM (lanes 4 and 9), and 1 μM (lanes 5 and 10).

DISCUSSION

These studies show that the 3′ domain of 16S rRNA is capable of independent *in vitro* assembly into a specific RNP particle. Several lines of evidence argue that this RNP particle corresponds to the head of the 30S subunit. First, the proteins found associated with the isolated 3′-domain RNP—S2, S3, S4, S7, S9, S10, S13, S14, and S19—have been found, with the exception of protein S4, to associate exclusively with the 3′ domain of 16S rRNA (Table 1; refs. 4, 5, 8, 9, and 28). All of these proteins (again, with the exception of S4) have been mapped to the head of the 30S subunit either by immunoelectron microscopy (10, 11) or by neutron diffraction (12). Second, they belong to a well-defined pathway in the 30S assembly map that is dependent on the primary binding protein S7 (7), consistent with their independent self-assembly into a structural domain. Finally, the 3′-domain RNP is similar in size and shape to the head of the 30S particle, as shown by electron microscopy (Fig. 3).

These results further show that the main events leading to the three-dimensional folding of the 3′ domain can occur independently of assembly of the 5′ and central domains—i.e., that it is a true structural domain. Nevertheless, there are indications that the structure of the 3′-domain RNP differs in detail from that of the 3′ domain in mature 30S subunits. First, protein S2 is present at low levels, compared with particles reconstituted from full-length 16S rRNA (Fig. 4). This is reflected in the weakness or absence of chemical probing signals for bases whose protection was previously found to depend on S2 or on the combined presence of S2 and S3 (Fig. 5; data not shown). A localized region of the 3′ domain, bounded by nucleotides 1060–1110 and 1160–1210, is structurally immature in a significant fraction of the 3′-domain particles. Although these differences correlate with the observed low levels of protein S2, they could also be due to the absence of possible unidentified interactions involving missing RNA domains.

An unexpected finding was the reproducible presence of low amounts of protein S4 associated with the isolated 3′-domain particles. S4 has been shown to assemble with the 5′ domain of 16S rRNA; indeed, an RNP complex containing near-stoichiometric amounts of proteins S4, S16, S17, and S20 has been assembled *in vitro* from transcripts of the 5′ domain of 16S rRNA (31). The sole indication of interaction of S4 with the 3′ domain is a significant enhancement of the reactivity of G-1221 and G-1222 toward kethoxal upon bind-

ing S4 to 16S rRNA *in vitro* (27). We do not observe these effects in assembly of the 3′-domain RNP, however. The lack of evidence for any direct contact between S4 and any of the bases (27) or the sugar-phosphate backbone (T. Powers and H.F.N., unpublished data) of the 3′-domain RNA, suggests that S4 may interact with the 3′-domain RNP mainly or exclusively via protein–protein interactions. It remains to be seen whether the observed association of S4 with the 3′-domain RNP bears physiological significance or is merely an artifact of *in vitro* assembly.

Fig. 1 *Right* shows a model for the three-dimensional folding of 16S rRNA in the 30S subunit (23), in which the RNA and protein components of the 3′-domain RNP are highlighted. Here, the positions of the proteins are as determined by neutron diffraction (12). It is evident that the majority of the molecular components of the RNP form a compact, self-contained domain in the model, clearly corresponding to the head of the 30S subunit, as defined by electron microscopy. It can also be seen that the location of protein S2 is peripheral to this compact domain. Efficient assembly of S2, incompletely defined in the original assembly mapping studies (7), may depend on interactions involving the other domains of the subunit. One possible candidate is protein S5, which is located just below S2 according to the neutron diffraction map (12); such an interaction could have important functional implications, including clues to the mode of action of spectinomycin.

Resistance to spectinomycin was originally demonstrated to be conferred by mutations in protein S5 (32). The possibility that the target of this drug might include 16S rRNA was raised when it was shown that a C → U mutation at position 1192 can confer spectinomycin resistance (33). The latter interpretation was strengthened when it was found that the N-7 position of G-1064, the Watson–Crick pairing partner of C-1192, was strongly protected from DMS attack by spectinomycin (30). In the present studies, spectinomycin was also found to protect G-1064 in the 3′-domain RNP (but not in the naked 3′-domain RNA; R.R.S. and H.F.N., unpublished results) with an affinity similar to that observed for 30S subunits. Since no detectable S5 is found in the 3′-domain RNP, we conclude that spectinomycin does not bind to protein S5, nor is S5 required for spectinomycin binding. Most likely, mutations in S5 cause spectinomycin resistance by some indirect mechanism. Since proteins S2 and S3 are known to interact with 16S rRNA near the site of the spectinomycin-resistance mutation and spectinomycin footprint, one possible model is that mutations in S5 could indirectly influence the conformation of this region of 16S rRNA via interaction with S2 and/or S3. Alternatively, mutations in S5 could confer spectinomycin resistance by directly perturbing functional targets of spectinomycin, such as translational events involving elongation factor EF-G (34).

Our findings suggest that the 3′ domain of 16S rRNA forms a structure that is, to some extent, an independent structural unit, and the studies of Ofengand and co-workers (31) provide evidence for independent assembly of the 5′ domain. To better understand the mechanism of action of the 30S ribosomal subunit, it will be important to find out whether domain–domain interactions play an important role or whether domains such as the one studied here function independently. If domain–domain communication turns out to be an important facet of the translational mechanism, it may be possible to study the details of such interactions via approaches based on the use of separately assembled domains.

This paper is dedicated to the memory of R. S. Samaha. We thank Ted Powers and Alexander Hüttenhoffer for advice; Bryn Weiser for preparing Fig. 1; Alexander Mankin for encouragement and help with the two-dimensional gels; Léa Brakier-Gingras for providing pFD3; and Rachel Green, Claes Gustafsson, and Simpson Joseph for their comments on the manuscript. R.R.S. thanks Nada Duna for encouragement and inspiration. This work was supported by National Institutes of Health Grants GM-17129 (to H.F.N.) and GM-15438 (to T.W.O.) and by a grant to the Center for the Molecular Biology of RNA from the Lucille P. Markey Charitable Trust.

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