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THE ASSEMBLY OF THE 30S RIBOSOMAL SUBUNIT OF ESCHERICHIA COLI

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The present article is concerned with ribosome assembly. However, this theme is really only a device which is introduced to explore the functions of that two-thirds of the prokaryotic ribosome which is RNA. A close look at ribosome assembly turns out to be a convenient way to approach this problem.

A clear distinction between ribosome assembly in vivo and the reconstitution process in vitro must be made at the outset in order to avoid errors in the interpretation of the different sets of data. The data utilized here concern the components of the 30S subunit of the Escherichia coli ribosome and their behavior in the reconstitution system of Traub and Nomura (1). A strong hint that this system may turn out be a subtle but extremely useful artifact is the simple fact that under the same conditions in vitro the reproducible reconstitution of active E. coli 50S subunits has so far eluded a number of expert laboratories.

Potential sources of artifacts in the in vitro system are easy to point out. First, the processing of precursor RNA in the course of assembly in vivo involves the cleavage as well as the methylation of specific RNA sequences (2, 3). Quite possibly a parallel processing of proteins may be operative in vivo (4). Accordingly, the reconstitution from mature RNA and protein in vitro may be distinguished from assembly in vivo by differences in the structures of the ribosomal components involved in these two processes. Second, we must consider the possibility that there are factors functioning in vivo which can increase the rate of assembly but which are absent from the purified ribosomes (5). The paradigm for such factors is seen in a much simpler system – namely, the renaturation of reduced ribonuclease. Here, an enzyme has been described which lowers by orders of magnitude the time required to renature reduced nuclease. It does so merely by catalyzing the random exchange rate of disulfide bonds (6). Given the extraordinary complexity of the ribosome it is quite possible that analogous factors are required to accelerate assembly in vivo. For these reasons we might expect the reconstitution process in vitro to proceed via kinetic pathways which are quite different from those preferred in vivo.

No matter how different the assembly pathways may be in vitro and in vivo, the object recovered in both cases can bind mRNA and tRNA as well as functionally interact with 50S subunits to promote protein synthesis. Since there are no indications to the contrary, we assume that the structures of the 30S subunits assembled in vivo and in vitro are very similar. If this is correct, an analysis of the reconstitution process in vitro will have at least one unquestionable use: to explore the molecular interactions that are responsible for maintaining the functional structure of the ribosome. It is the nature of these interactions with which we will be concerned in the present article.

The prokaryotic ribosome contains more nucleic acid than protein. Furthermore, the third of the 30S subunit which is protein contains 21 different proteins, none of which are present in amounts greater than one copy per particle (7, 8). These structural features immediately suggest that the assembly of 30S subunits is likely to be quite different from that of a virus. In particular, we can not expect the assembly of ribosomes to entail formation of an internal core of RNA enclosed by a shell of repeated proteins whose principal molecular interactions are with each other. Instead, the data suggest that the proteins of the 30S subunit are dispersed through a matrix of RNA. Consequently, the class of molecular interactions which dominate the assembly process seems to be protein–nucleic-acid interactions. The structural constraints on the functional 30S subunit as well as the assembly interactions between 30S components are summarized in what I will refer to as the mixed neighborhood model. Some of the novel features of this system are illustrated in what follows.

ONE VIEW OF THE PROBLEM

The major component of the 30S subunit is the 16S RNA molecule; estimates of its size range from 1,600 to 1,900 nucleotides (9, 10). The length of such an extended chain of nucleotides would be more than 20 times greater than the longest dimension of the 30S subunit. In solution the RNA behaves as a flexible polyelectrolyte coil with a radius of gyration close to 140 Å, while the radius of gyration of the 30S subunit under the same conditions is close to 70 Å (11). Therefore, an important feature of the assembly process is a collapse of the RNA so that it is restricted to a much smaller domain when associated with ribosomal proteins than it occupies in a free state. This collapse or folding of the RNA strikes me as the most significant aspect of the assembly process because it poses a question which is at the very heart of ribosome structure: What sorts of molecular interactions are responsible for constraining the structure of the RNA in that particular ordered array which supports protein synthesis?

A brief survey of the potential constraints on the RNA structure in the ribosome provides the first intimations that the proteins must play a critical role in folding the RNA. This is deducible from the data concerning the alternative molecular interactions available to the RNA. In particular, it seems very unlikely that intramolecular interactions between distant nucleotide sequences of the 16S RNA could generate a specific RNA fold without essential contributions from the 30S proteins. The arguments for this conclusion are the following:

Since the phosphate-ribose backbone is a repeat structure, only base-specific interactions of the sort that stabilize the DNA double helix are likely to provide any sequence-specific intramolecular interactions between distant regions of the 16S RNA. Therefore, were such interactions important, the assembly of the RNA into a 30S subunit should be

attended by dramatic changes in those optical properties of the RNA which reflect nucleotide-nucleotide interactions. Surprisingly this is not observed.

Much of the free RNA is organized as local helical loops (12). Comparisons have been made of the hyperchromicity as well as the optical rotatory activity of ribosomal RNA both in the free state and incorporated into the ribosome (13, 14, 15). The data show that in appropriate buffers close to 60% of the RNA in both states is organized as local hairpin loops with an average size of about 40 nucleotides. In addition, there is evidence that much of the secondary structure of the ribosomal RNA is preserved when the proteins are removed. Thus, degradation of ribosomes with ribonuclease leads to the release of short helical segments which can also be identified in digests of free RNA (16, 17).

In contrast, there are observations which indicate that phosphate-backbone interactions may be quite important to the RNA fold in the ribosome. Thus, the suspension of 30S subunits in media containing EDTA converts the subunits into flexible ribonucleoprotein coils (18, 19, 20). Such results are consistent with the interpretation that the compact configuration of the RNA in the ribosome is stabilized in large measure by ionic interactions, as for example by the apposition of the phosphate groups of distant RNA sequences stabilized by Mg^{++} bridges.

Although ionic interactions of this sort are likely to be quite important for the energetics of the RNA fold, something else is probably needed to provide stereospecificity. Thus, in the absence of evidence to the contrary, it seems unlikely that ionic interactions between phosphates of the RNA backbone could be by themselves site-specific. Consequently, such ionic interactions are likely to be supplemented by protein-RNA interactions which are nucleotide sequence-specific and, therefore, could steer the backbone interactions.

The data discussed so far hint at still another aspect of 30S structure; this is the possibility that most of the 30S proteins have specific binding sites on the 16S RNA. Such an interpretation of the coil-like structure generated by removing divalent cations from the 30S subunits depends on two assumptions. One is that the proteins retained in these complexes are bound in a site-specific manner. The other is that the proteins are distributed more or less uniformly along the coil rather than bunched in clusters stabilized by protein-protein interactions. At the time that these experiments were done it was not possible to check the validity of these assumptions. However, more recent data discussed in the next section suggest that both assumptions are likely to be correct.

RNA-BINDING SITES

There are rather clear indications that a subset of the 30S proteins binds to specific sites on the 16S RNA. Thus, incubation of either S4, S7, S8, S15, or S20 with 16S RNA leads to complex formation (21–24). The complexes so formed are site-specific as indicated by the stoichiometry of the reactions (22–24), as well as by the absence of competitive effects between the different proteins (21–24). The data indicating that two other proteins may also bind in a site-specific manner to the 16S RNA are somewhat more complicated. Here, evidence for both nonspecific and site-specific binding of S13 and S17 has been reported (21–25). Accordingly, there are at least five and possibly seven of the 30S proteins which independently bind in a site-specific manner to the 16S RNA in the absence of other proteins.

The evidence that most of the remaining proteins are bound to the RNA is not so

direct, but it does seem convincing. When 30S subunits are treated with nuclease under a variety of conditions, a population of ribonucleoprotein fragments is released (26–30). These fragments range in size from those with but a few proteins to others with ten or more proteins. Significantly, they all tend to have an RNA/protein ratio which is close to that of the undegraded ribosomes. At first glance such results would seem to indicate that most if not all of the 30S proteins have RNA binding sites and, further, that each protein is associated with a relatively constant mass of RNA in the ribosome.

When they are considered more carefully, however, these results suffer from a serious ambiguity. A critic could argue that the fragments recovered from the degraded ribosomes are artifacts created by the random adsorption of basic proteins onto nucleic fragments released by nuclease. Therefore, independent evidence is required to show that the fragments represent site-specific complexes of components that form neighborhoods in the intact ribosome. Two sorts of experimental results can in fact be used to verify this conclusion. One line of evidence is that the fragments contain clusters of protein which are predictable from assembly data. The other is that the direct analysis of protein neighborhoods in the ribosome yields protein groupings that are also represented in the ribonucleoprotein fragments. We will now consider these corroborative data in somewhat greater detail.

The binding sites on the 16S RNA for five of the 30S proteins have been partially described (31–33); these data are summarized in Fig. 1. S4 and S20 are associated with that quarter of the sequence at the 5′ end of the molecule; S8 and S15 are found near the middle of the molecule, and S7 can be placed at the 3′ end of the 16S RNA. When one or more of these five proteins are bound to the RNA, others will also bind. Thus, S16 will bind to the RNA in the presence of S4 and S20 (34, 25). Similarly, S5 will bind if S8 is present (21, 25). Finally, S9, S13, S14, and S19 will bind if S7 is present (21, 34, 25). Accordingly, each of these clusters of proteins which cooperate during assembly in vitro can be associated with different segments of the 16S RNA molecule. Therefore, we would expect these same groupings of proteins to be reflected in the fragments released by nuclease digestion if the latter are indeed site-specific complexes which reflect the alignment of the proteins along the 16S RNA.

The data summarized in Fig. 1 verify this prediction. Some of the fragments isolated and characterized by Morgan and Brimacombe (29) are displayed along with the three assembly clusters described above. There is a wholly unambiguous correlation between the two independent groupings of the proteins. Furthermore, the analysis of the RNA recovered from a fragment containing S7, S9, S13, S14, and S19 (30) has shown that it indeed corresponds to that part of the 16S RNA to which S7 binds independently (32).

Such correlations indicate that proteins which cooperate with each other during assembly are bound to neighboring stretches of the 16S RNA. Thus, the clusters of cooperative proteins and their associated RNA binding sites represent both physically and functionally defined domains of the ribosome. However, it can not be concluded from this that such clusters of proteins are necessarily near-neighbors. The ambiguity here is that none of the data discussed so far tells us anything about the compactness of these domains. For example, if the RNA segment associated with S7, S9, S10, S13, S14, and S19 were extended through the 30S subunit (instead of being collapsed on itself), the

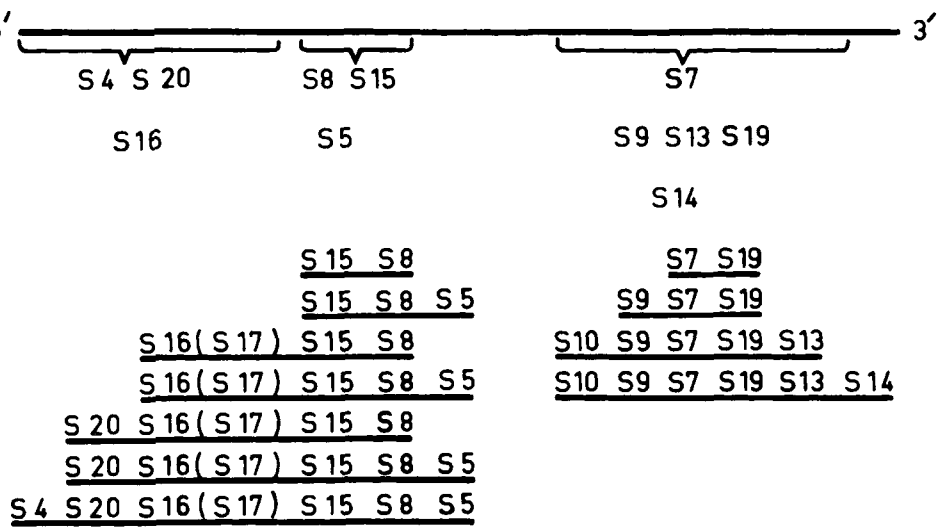

Fig. 1. A diagrammatic representation of the RNA binding sites for several 30S proteins, the cooperative interactions between proteins during assembly, and the composition of the fragments generated by nuclease treatment of 30S subunits is presented here. The experimental sources of these relationships and their interpretation are described in the text.

proteins of this cluster could be positioned at quite some distance from each other. One way of studying the compactness of these domains is provided by bifunctional crosslinking reagents.

We will have occasion to discuss the crosslinking experiments in more detail below. Here, it will suffice to say that it is possible to identify near-neighboring proteins in the intact ribosome by crosslinking them to each other with appropriate bifunctional reagents (35–39). In this way the following relevant neighborhoods have been discovered: S5–S8 (36, 37); S7–S9 and S13–S19 (38); and S14–S19 (39). These four pairs of neighboring proteins correlate remarkably well with the data summarized in Fig. 1. Thus, proteins which cooperate with each other in the course of assembly in vitro are near neighbors in the assembled ribosome. Similarly, those clusters of proteins which are related by their proximity, as well as by interactions during assembly, are recovered in association with well-defined segments of the 16S RNA to which they are bound.

In summary, the fragments recovered from ribosomes degraded with nuclease are clearly not random aggregates. Instead, they represent a series of partial neighborhoods which consist of specific proteins and their associated RNA binding sites. These mixed neighborhoods of RNA and protein may be conveniently viewed as the natural units of 30S subunit assembly. However, before we can more fully explore the implications of this remark it will be necessary to explicitly relate the structure of the intact 30S subunit to the mixed neighborhood concept.

THE MIXED NEIGHBORHOOD MODEL

In the previous section a combination of structure and assembly data was used to introduce the idea that a majority of the 30S proteins have RNA binding sites. Here, a more general structural analysis of the 30S subunit will be presented which indicates that

the immediate neighborhood for any randomly chosen 30S protein is likely to contain both RNA and other proteins.

We begin this analysis by pointing out that less than one-third of the volume of the 30S subunit in solution can be occupied by protein (40). This means that there are two extreme ways that the proteins could be distributed within the particle. One way would be to pack the proteins into an RNA-free domain. Here, the principal molecular interactions of the proteins would be between each other, and only a subset of the proteins would be required to have binding sites on the 16S RNA. An alternative way of distributing the proteins would be to disperse them through a matrix of RNA. Here, the proteins would have principal contacts with RNA, and they might or might not have contacts with each other. It should be stressed that the important distinction between these two models is the different emphasis that they place on the relative contribution of protein-protein and protein-RNA interactions in maintaining the structure of the 30S subunit.

An attempt to determine how the proteins are distributed in the 30S subunit has been made by using bifunctional crosslinking agents (38). This approach takes advantage of the fact that lysines are quite abundant in the ribosomal proteins (there are close to 16 lysines in an average 30S protein). Accordingly, if certain requirements are met, reagents such as diimidoesters which can crosslink lysines in neighboring proteins can be used to study the separations between neighboring proteins.

First, it is necessary to be sure that the lysines are reactive even when the proteins are part of the intact ribosome structure. Second, the crosslinking reagents employed must be able to react with pairs of lysines imbedded in the ribosome. Control experiments have shown that at least two-thirds of the lysines in the 30S subunits are reactive with imidoesters (41, 42). Furthermore, three diimidoesters – DMM, DMA, and DMS (respectively, 5 Å, 9 Å, and 12 Å) – have been shown to form intramolecular crosslinks between lysines of the proteins while they are part of the 30S structure (43).

The two models for the distribution of proteins in the 30S subunit predict different outcomes when the 30S subunits are treated with DMM, DMA, and DMS, because the two models would require the lysines to be separated by different average distances. If the proteins are packed in a single RNA-free domain, the average distance between the ϵ-amino groups of lysines will be close to 11 Å. However, if the proteins are distributed uniformly throughout the 30S subunit, RNA will separate them to some extent and the average distance between the ϵ-amino groups of the lysines will be close to 17 Å. Of course these are just average numbers and the actual separations between lysines are likely to be broadly distributed about these mean values. Furthermore, the trivial calculations used to obtain these figures do not distinguish lysines which are on the same protein from those on different proteins. Nevertheless, the difference between 11 Å and 17 Å average separations provides a rough index to the size of the bifunctional reagents likely to be most effective in crosslinking proteins distributed according to either model.

The effects of the tested reagents give rather straightforward results: no crosslinked complexes are produced by DMM (5 Å); a few are produced by DMA (9 Å); and several complexes along with traces of others are produced by DMS (12 Å). We would expect both DMA and DMS to yield extensive, if not quantitative, crosslinking of the proteins had they been packed in RNA-free domains with average lysine separations of 11 Å. Therefore, we conclude that the proteins are distributed throughout the 30S subunit (38).

There are many ways to distribute the proteins through the 30S subunit, all of which I will refer to as mixed neighborhood models. Although all such models would re-

quire that the proteins have extensive interactions with RNA, some arrangements can be imagined in which none of the proteins interact with each other. For example, if we assume that the proteins are globular they can be uniformly distributed so that they make no contacts with each other; here, RNA would separate all the proteins from each other (44). However, it is also possible to arrange either globular or fibrous forms of the proteins so that they make regular contacts with each other (44). There is no way at present to distinguish these alternatives experimentally.

Finally, there is one other aspect of the crosslinking data obtained to date which deserves comment. At the time of this writing five well-characterized crosslinked pairs of 30S proteins have been reported. These are S5-S8, S7-S9, S13-S19, and S14-S19, which were described above, as well as S18-S21, which is obtained with reagents that crosslink sulfyhydryl groups (36). Thus, four out of five neighborhoods detected with bifunctional reagents contain proteins which the data discussed in the previous section indicate are in intimate association with RNA. If there were clusters of proteins not associated with RNA in the 30S subunit, it seems unlikely that we could have obtained such a result.

The data discussed in this section and in the previous one regretably provide only indirect evidence for the mixed neighborhood model. Fortunately, a completely independent sort of measurement has recently been made which is consistent with the interpretation that the proteins are distributed throughout the 30S subunit. Here, the neutron-scattering properties of deuterated ribosomal subunits have been studied. The preliminary data so obtained suggest that the radii of gyration and the centers of mass for the RNA and for the protein in the 30S subunit are very nearly the same. In other words, the RNA and protein could be distributed nearly uniformly throughout the 30S subunit (46).

At their present level of resolution the neutron-scattering data do not exclude a packing of the 30S proteins in RNA-free domains which are symmetrically distributed around the mass center of the 30S subunit. However, the crosslinking experiments indicate that the proteins are not in general packed in this fashion. Finally, the data discussed in the previous section show that a majority of the proteins are intimately associated with RNA in the intact subunit. Therefore, it seems very likely that the mixed neighborhood model can be used to represent the gross structural features of the 30S subunit.

COOPERATIVITY DURING ASSEMBLY

We have so far used the data obtained from the in vitro reconstitution system to develop a model for the structure of the 30S subunit. Now we can use the model to explicate aspects of the assembly process itself. In particular, the folding of the 16S RNA during the assembly process can be viewed as but one manifestation of the formation of the mixed neighborhoods. Here, cooperative interactions between neighboring proteins which share common regions of the RNA can provide the stereospecificity for the RNA fold.

The interactions between S4 and its RNA binding sites illustrate this idea. The relevant experiments were originally based on the assumption that the RNA binding sites for a given protein could be separated from the rest of the RNA by virtue of the protection from nuclease afforded by the bound protein (31, 47). This assumption turned out to be too naive.

The complex formed between S4 and 16S RNA was incubated with pancreatic ribonuclease, and ribonucleoprotein complex, relatively resistant to nuclease, was recovered. Analysis of the RNA extracted from this complex (referred to as S4aR) revealed that it contains approximately one-fourth of the 16S RNA, but it is not a single continuous nucleotide sequence. Instead S4aR can be fractionated by electrophoresis into five or more components, each with a mass close to 25,000 daltons. More surprising still was the finding that these 4S fragments can reform the original 9S ribonucleoprotein complex when reincubated with S4. None of the other proteins replace S4 in the formation of this complex.

There seems to be no way that a small protein (26,000 daltons) can bind and directly shield from nuclease attack a mass of RNA five times its own size. However, double-stranded RNA is a much poorer substrate for pancreatic ribonuclease than single-stranded RNA. Accordingly it was concluded that the fragments which make up S4aR consist of several discontinuous binding sites of S4 as well as helical loops of RNA that are dispersed between these multiple sites. In other words, the binding sites of S4 are obscured in S4aR by a significant mass of extraneous RNA.

The finding that the binding sites for S4 are multiple and dispersed through approximately one-quarter of the length of the RNA suggests that the binding of this protein could have a dramatic effect on the tertiary structure of the RNA. This is illustrated diagrammatically in Fig. 2. Here, in accordance with the data discussed above, the secondary structure of the RNA is maintained in going from the free state to the complex formed with S4. However, the helical loops are organized into a fixed array once the protein is bound, a feature which will be exploited below. Treatment of this complex with nuclease has the limited effect of cleaving exposed single-stranded regions between the helical loops. As a consequence, the loops are no longer part of a contiguous segment and can be recovered as separate fragments when the complex is disrupted.

Not only is this interpretation of the interaction between S4 and S4aR consistent with the facts, but subsequent experiments have shown independently that something very much like the folding depicted in Fig. 2 must be taking place. Nanninga et al. (48) have studied by electron microscopy the complex formed between S4 and 16S RNA. They found that the binding of S4 causes approximately one-fourth of the RNA to appear as a compact bouquet clearly visible in electron micrographs. Such a collapsed structure is not formed in the absence of S4.

The effect of S4 on the tertiary fold of the RNA can be used to explain another aspect of the assembly process. The data discussed above indicate that there are proteins which can not bind to 16S RNA in the absence of other proteins but which seem to be intimately associated with the RNA in the intact 30S subunit. What sorts of cooperative interactions can account for these apparently contradictory facts?

Part of the answer to this question may be found in Fig. 2. Although this drawing is a much oversimplified representation of the effects of S4 on the RNA fold, it does illustrate an important point: segments of RNA which may be at quite some distance apart in the free RNA can be brought into closer apposition by the binding of a protein to the RNA. For example, the loops A and E in Fig. 2 would not be found in close and stable apposition without some external constraint, such as that provided by the binding of a protein. Once these two loops have been brought near to each other, they could provide a binding site for a second protein. Thus, cooperativity between two proteins during assembly need not require contacts between the proteins. The effect of one protein on the tertiary structure of the RNA could create binding sites for other proteins.

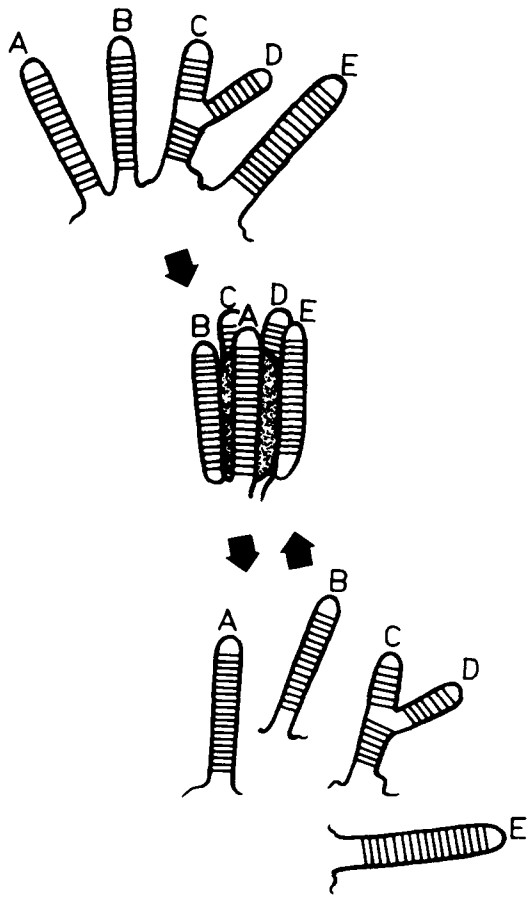

Fig. 2. A diagrammatic representation of the effects of S4 on its RNA binding site and the formation of S4aR with nuclease is presented here. A more detailed description is found in the text.

CONCLUSIONS

Several sorts of experimental results have been discussed in an attempt to account for the orderly folding of RNA during the assembly of the 30S ribosomal subunit.

First, the functional interactions between different proteins during the reconstitution of 30S subunits in vitro operationally defines a set of cooperative interactions. This set of cooperative interactions by itself tells us little about the mechanism of assembly. However, the protein neighborhoods detected in crosslinking experiments are extremely well correlated with the same set of cooperative interactions. This provides grounds for one generalization about the assembly process: proteins which cooperate with each other during assembly are near-neighbors in the assembled subunit.

The interpretation of such a correlation is, however, not straightforward. For example, the attractive inference that such cooperative interactions are a consequence of direct protein-protein interactions has as yet no experimental support. On the other hand,

the finding that clusters of proteins which assemble together are associated with common regions of the 16S RNA provides an alternative way of explaining cooperative interactions between proteins. Here, the effect of one protein on the tertiary fold of the RNA could influence the interaction of a second protein with the RNA. Thus, clusters of neighboring proteins could be functionally associated with each other by their mutual dependence on a specific RNA fold which they in turn create by their interactions with neighboring segments of the RNA.

The important structural question that must be answered next is the extent to which the 30S proteins contact each other. Two extreme forms of the mixed neighborhood model can be imagined. In the one extreme it is possible that the proteins are globular, and in this case they could be distributed so that they make no contacts with each other. Here, the interactions of different regions of the 16S RNA with each other, as well as with proteins, would provide the macromolecular interactions which guide the assembly process.

On the other extreme, predominantly elongated or fibrous proteins could be dispersed through the RNA in the form of a network. Here, protein-protein interactions would supplement the RNA-dependent interactions. Finally, the structure of the 30S subunit could turn out to be a composite of both these alternatives. For example, a central core of proteins associated exclusively with RNA could be surrounded by proteins which have well-developed protein-protein contacts.

We are a long way from being able to experimentally distinguish these structural alternatives. Unfortunately, it will not be possible to describe the physical mechanism of the assembly process until such structural information is available.

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