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Assembly map of the large subunit (50S) of *Escherichia coli* ribosomes

(reconstitution/23S-5S RNA–protein complex/organization of L-protein genes)

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ABSTRACT **Stoichiometric amounts of ribosomal proteins and RNA derived from the 50S subunit reconstitute to fully active particles under the conditions of a two-step incubation procedure. After the first incubation, all components are found in a particle that is activated in the second incubation [Dohme, F. & Nierhaus, K. H. (1976) *J. Mol. Biol.* 107, 585–599]. Here we describe the assembly dependences of the ribosomal components in the first incubation. Assembly dependence is the requirement of one protein that, before it binds, another must be first built into the ribosome. After incubation of 23S RNA and the proteins under observation, the mixture was subjected to sucrose gradient analysis. The RNA–protein complex was precipitated with trichloroacetic acid and the proteins were identified by $NaDodSO_4$ gel electrophoresis. The assembly dependences of 26 proteins could be elucidated. In a second series of experiments, the incorporation of ^{3}H-labeled 5S RNA in the 23S–protein complex was analyzed. It was found that L5, L15, and L18 are absolutely required for 5S RNA incorporation. In addition, two of the three proteins L2, L3, and L4 are needed, in excellent agreement with the protein dependences. The data are summarized in an assembly map. Comparison with other data shows a structural domain at the 5′ end of 23S RNA around protein L20 combining all proteins essential in the early assembly. All the proteins essential for the reconstitution of the peptidyltransferase form a skeleton of strong assembly dependences. Finally, L proteins whose genes are present in large transcriptional units on the chromosome depend on each other during assembly.**

The assembly of the small subunit (30S) of *Escherichia coli* ribosomes has been studied in detail by the reconstitution technique, and much information has accumulated about the precise assembly dependences of the 30S components (30S assembly map; for review, see ref. 1).

In assembly, both *in vivo* and *in vitro*, of the 50S subunit, three intermediate particles occur that have similar *S* values (for review, see ref. 2). The reconstitution intermediates formed subsequently are designated as RI_{50} [1] → RI^*_{50} [1] → RI_{50} [2] → reconstituted 50S subunit. The first two particles contain the same complement of components (≈20 proteins and 5S and 23S RNAs) but differ markedly in their *S* values (33S and 41S, respectively). Similarly, the latter two particles (RI_{50} [2] and 50S) differ only in their conformation, since the RI_{50} [2] particle consists of the full complement of 50S components but is totally inactive. The two conformational changes (RI_{50} [1] → RI^*_{50} [1] and RI_{50} [2] → 50S) require different ionic conditions and temperatures; a two-step incubation is therefore needed for the reconstitution of *E. coli* 50S subunits starting from totally separated rRNA and protein fractions (3, 4).

In the first incubation of the two-step procedure, all reconstitution intermediates are formed, including the RI_{50} [2] particle. Therefore, the assembly of all 50S components can be studied in the first incubation. An assembly map that includes most of the components of the RI_{50} [1] particle—i.e., 17 proteins and 23S RNA—has been described recently (5). Here we present an assembly map comprising both rRNAs (23S and 5S) and 26 proteins. Structural and functional domains are evident, and a relationship is found between assembly dependences and the gene organization of 50S proteins.

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MATERIALS AND METHODS

70S ribosomes and 23S and 5S RNAs were prepared from *E. coli* cells as described (6). For the isolation of 5S [^{3}H]RNA, *E. coli* cells (MRE600) were grown in 2 liters of minimal medium (7). When the culture reached an $OD_{650\,nm}$ of 0.06 units/ml, 300 nmol of [5,6-^{3}H]uridine (specific activity, 40 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) was added, and the cells (5 g) were harvested one generation later. The isolation procedure of 5S [^{3}H]RNA followed ref. 6. However, to reduce radiolysis, the material was processed as quickly as possible and not frozen until the labeled RNA was isolated. The specific activity was ≈1,000,000 cpm/$A_{260\,nm}$ unit. The isolation of highly purified ribosomal proteins followed ref. 8.

Standard Assembly Mapping Experiments. These experiments were as reported (5) with slight modifications. Five $A_{260\,nm}$ units of 23S RNA and 10 equivalent units (where 1 equivalent unit is the amount of protein on 1 $A_{260\,nm}$ unit of 50S subunits) of proteins were incubated for 20 min at 44°C in 200 μl of 20 mM Tris·HCl, pH 7.5/4 mM $Mg(OAc)_2$/0.2 mM EDTA/400 mM NH_4Cl/4 mM 2-mercaptoethanol (i.e., the conditions of the first reconstitution step). After incubation, the sample was subjected to sucrose gradient centrifugation (10–30%, 2 hr 45 min at 50,000 rpm, Beckman SW60). Fractions containing 23S RNA–protein complex were pooled and 0.1 vol of 50% trichloroacetic acid was added. After storage overnight at 4°C, the samples were centrifuged at low speed and the pellet was resuspended in 25 μl of 1.5% $NaDodSO_4$/15% glycerol/0.1 M Tris (unbuffered)/1 M 2-mercaptoethanol/0.001% bromphenol blue and incubated for 5 min at 90°C. The sample was subjected to one-dimensional $NaDodSO_4$ gel electrophoresis and the gel was stained according to ref. 9 with the modification that the thickness of the gel was reduced to 1.2 mm.

5S RNA Assembly Experiments. These experiments were carried out with 5S [^{3}H]RNA. One $A_{260\,nm}$ unit of unfractionated (23S/5S) RNA was mixed with ≈100,000 cpm (≈0.1 $A_{260\,nm}$ units) of 5S [^{3}H]RNA and 2 equivalent units of proteins. Incubation and sucrose gradient analyses were as described above. The integration of 5S [^{3}H]RNA was detected by monitoring the radioactivity in the gradient fractions.

Protein Binding to 5S RNA. Binding was tested by incubation of 0.3 $A_{260\,nm}$ units of 5S RNA and 10 equivalent units of proteins under standard conditions as described above. After sucrose gradient centrifugation (4–15%, 18 hr at 40,000 rpm,

Beckman SW60) and trichloroacetic acid precipitation of those fractions containing 5S RNA, the bound proteins were analyzed by $NaDodSO_4$ slab gel electrophoresis (9).

RESULTS

The technique used for assembly-mapping experiments was a modification of that used by Mizushima and Nomura (10) for 30S mapping experiments and essentially as described (5). 23S RNA was incubated with the proteins to be tested, the RNA–protein complex was separated from unbound proteins by sucrose gradient centrifugation, and the bound proteins were identified by gel electrophoresis. The example of a mapping experiment shown in Fig. 1 demonstrates the assembly dependences of L5. In the presence of L4, significant but low L5 binding is observed (compare lanes 1 and 2), whereas L2 mediates full binding of L5 (compare lanes 1 and 4). We designate the L5 binding promoted by L4 as "weak binding" and that promoted by L2 as "strong binding."

If an experiment suggested an assembly dependence, the dependence was analyzed in detail, and the decisive experiment was repeated two to five times and performed again with a second protein set derived from different protein preparations. The assembly dependences are classified as follows. Controls of the proteins in question were included in the gel runs in that 3 equivalent units of these proteins were applied directly to the gel. If the binding of protein Lx (which otherwise showed no or weak binding) could be stimulated by protein Ly so that the Lx bound did not significantly differ from the Lx control, then the binding of Lx is considered to be strongly dependent on Ly. If Ly significantly enhanced Lx binding but the Lx band was clearly less intense than that of the Lx control, the binding of Lx is considered to be weakly dependent on Ly. About 1500 assembly experiments led to the establishment of the 50S assembly map, including 26 of 32 proteins (see Fig. 3). Detailed documentation will be given elsewhere (13).

In a second series of experiments, the binding dependences of 5S RNA were investigated. In these assays, [^{3}H]uridine-labeled 5S RNA was used that had been shown to be active in the total reconstitution of 50S subunits (data not shown). ^{3}H-Labeled 5S RNA was mixed with unlabeled (23S/5S) RNA and incubated with various proteins. The integration of ^{3}H-labeled 5S RNA into the 23S RNA–protein complex was analyzed by sucrose gradient centrifugation.

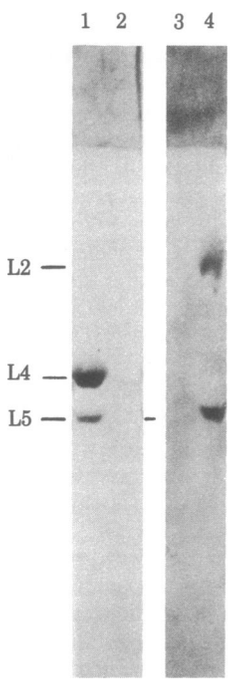

FIG. 1. $NaDodSO_4$ gel analysis of assembly mapping experiments. The reconstitution assay used 23S RNA and proteins L4 and L5 (lane 1), L5 (lanes 2 and 3), or L2 and L5 (lane 4).

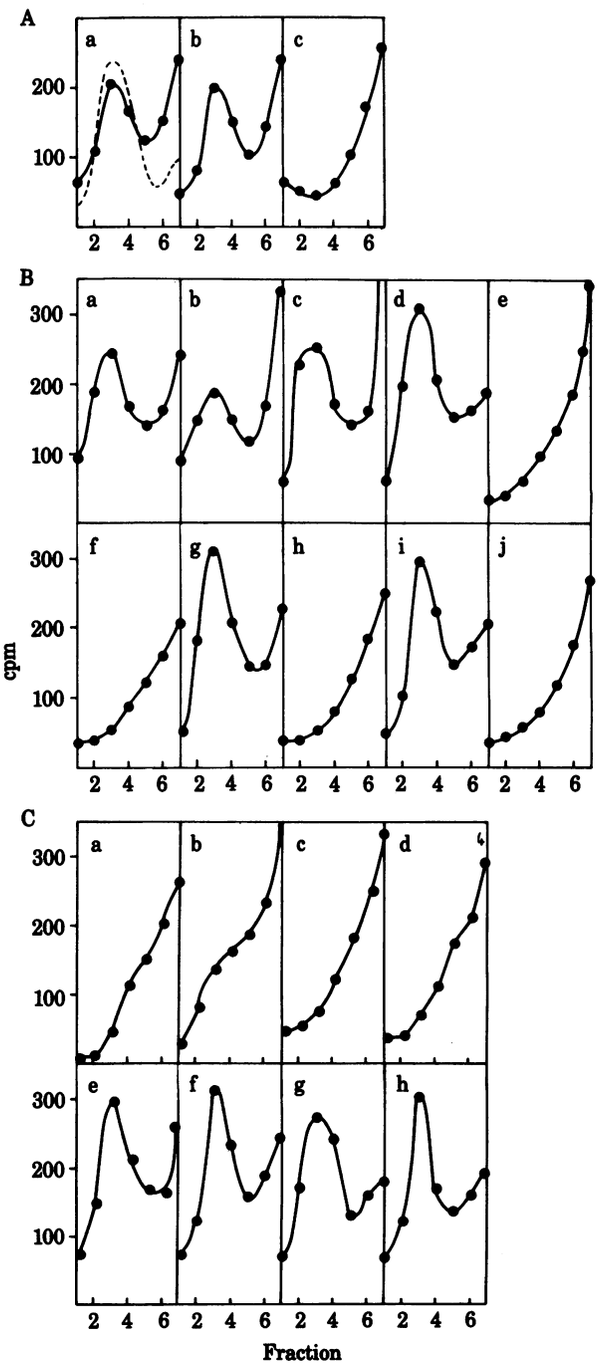

FIG. 2. Radioactivity profiles after sucrose gradient centrifugation of binding experiments with 5S [^{3}H]RNA and 23S RNA in the presence of various proteins. (*A*) Binding with total protein (a); L2, L3, L4, L5, L15, L16, L18, and L25 (*b*); no protein (c). ----, 23S RNA monitored at 290 nm. (*B*) Binding with protein mixture I (L2 , L3, L4, L5, L15, L16, L18, and L25). (a); with mixture I lacking L2 (b), L3 (c), L4 (d), L5 (e), L15 (f), L16 (g), L18 (h), and L25 (i); with no protein (j). (*C*) Binding with protein mixture II (L5, L15, and L18) (a) and with protein mixture II containing L2 (b), L3 (c), L4 (d), L2 and L3 (e), L2 and L4 (f), L3 and L4 (g), L2, L3, and L4 (h).

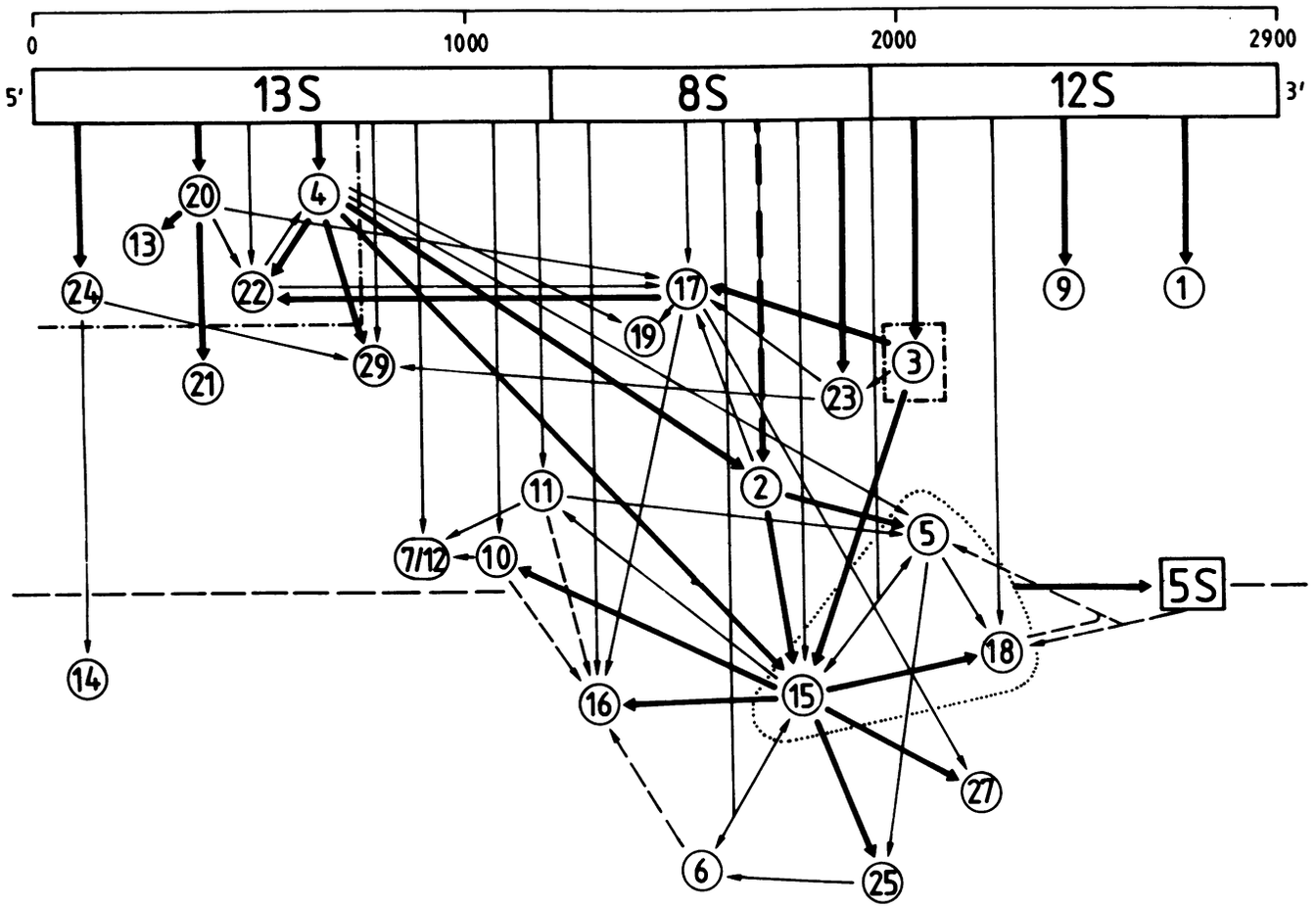

FIG. 3. Assembly map of the 50S subunit. The main fragments of 23S RNA (13S, 8S, and 12S) are indicated. The proteins are arranged according to their binding regions on 23S RNA (11). ➞, Binding of Lx is strongly dependent on Ly; → binding of Lx is weakly dependent on Ly; RNA → Lx, Lx is a RNA binding protein; broken arrow to L2, some L2 preparations bind strongly to 23S RNA while others bind weakly; → from L6, L10, and L11, respectively, to L16, the effects of these proteins on the binding of ^{14}C-labeled L16 have been shown recently (12); → from 5S RNA to L5 and L18, 5S RNA binds L18 and L18 supports L5 binding to 5S RNA; ····· around L5, L15, and L18, these proteins are essential for mediating the binding of 5S RNA to 23S RNA. Proteins enclosed by –·–·– are important or essential for the conformational change RI_{50} [1] → RI^*_{50} [1]. Components below – – – are not present on the RI^*_{50} [1] particle. The results of RNA binding studies (9) and those of a first set of assembly mapping experiments (5) have been integrated.

The first experiment (Fig. 2*A*) shows that 5S RNA binding to 23S RNA is protein dependent and that this binding can be mediated by a set of eight proteins—L2, L3, L4, L5, L15, L16, L18, and L25—as well as by the total protein (TP50) of 50S subunits. The importance of each of the eight proteins was investigated by a single-omission test in the next experiment (Fig. 2*B*). The essential role of L5, L15, and L18 for the binding of 5S RNA to 23S RNA is conspicuous. L16 and L25 have no significant effect and were omitted in the next experiment. L2, L3, and L4 support the binding of L15 (Fig. 3). Therefore, the effects of L2, L3, and L4 were tested in a reaction mixture containing in addition to the RNAs the essential proteins L5, L15, and L18. Fig. 2*C* shows that the three essential proteins alone are not sufficient for mediating 5S RNA binding, and even a fourth protein, L2 or L3 or L4, does not significantly improve 5S RNA binding. In contrast, any two of proteins L2, L3, and L4 added to the essential proteins confer full binding of 5S RNA comparable with that induced by L2, L3, and L4 and the essential proteins or even by the total protein. These results were integrated into the assembly map (Fig. 3).

Finally, proteins L2, L3, L4, L5, L15, L16, L18, and L25 were tested for their ability to bind directly to 5S RNA under the first-step incubation conditions. L18 was the only protein that could bind to 5S RNA; L5 binding was observed only in the presence of L18 in agreement with ref. 14. In contrast, all other proteins, including L25, did not show any binding under our conditions, whether they were added as single entities or in combination with other proteins (data not shown). These results are also incorporated in Fig. 3.

DISCUSSION

(5S·23S) RNA Complex Formation. In addition to the extension of the 50S assembly map, which now comprises 26 L proteins and the RNA molecules, we demonstrate that for the binding of 5S RNA to 23S RNA proteins L5, L15, and L18 play an essential role and, in addition, any two of the three proteins L2, L3, and L4 are required. The experiments were carried out under conditions that allow the assembly of active 50S subunits. These results are in agreement with the protein-assembly dependences derived from a different series of experiments: the assembly of L5 and L18 depends on L15 and that of L15 depends on L2, L3, and L4 (Fig. 3).

It was repeatedly reported that L5 and L18 are involved in (15, 16) or even sufficient for (17, 18) mediating the binding of 5S RNA to 23S RNA. The notion that L5 and L18 are sufficient disagrees with our findings. Two reasons for this discrepancy can be put forth. (*i*) The incubation and ionic conditions differed

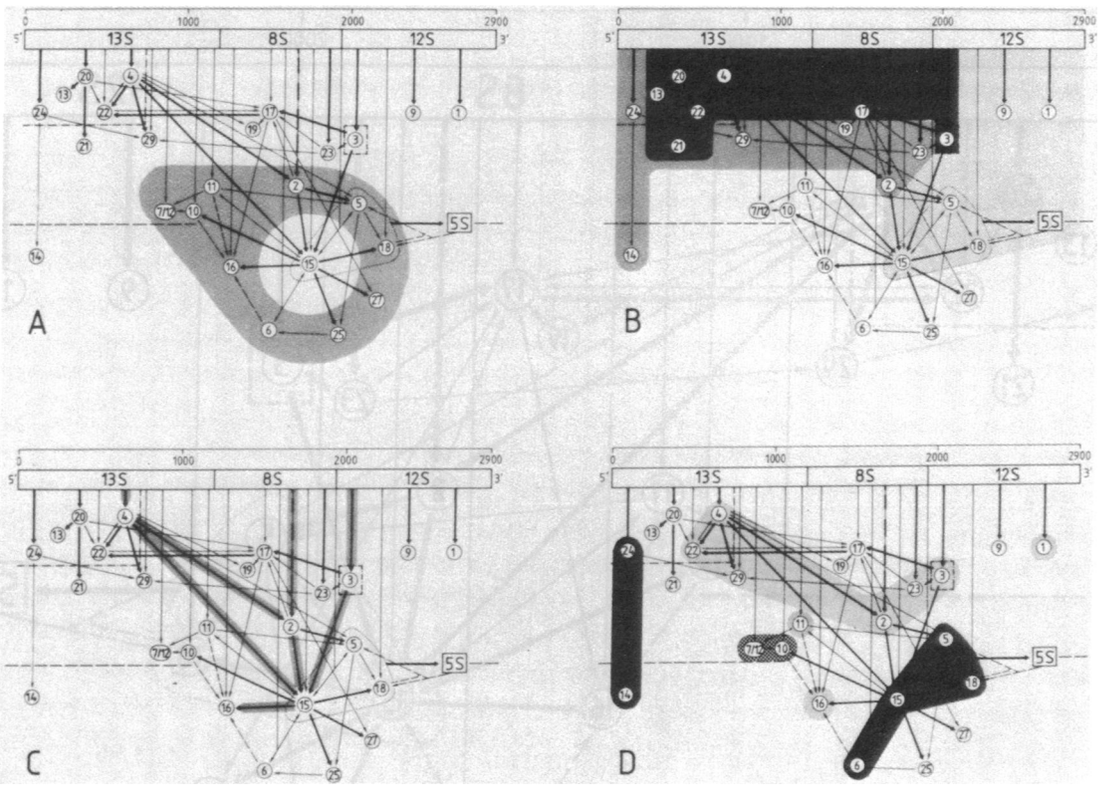

FIG. 4. Comparison of the 50S assembly map with other data. (*A*) ■, proteins exposed to trypsin attack or modification reactions within the 50S subunit. The various reactions (19–22) allow classification of exposure into high, medium, and low degree and not exposed. Only those proteins are indicated that show at least medium exposure on average. The protein nomenclature used is that of ref. 22. (*B*) Proteins split off the 50S subunit as the concentration of LiCl is increased. ▭, Proteins split off by 1.3 M LiCl; ▬, proteins split off in addition by 3 M LiCl; ■, proteins split off in addition by 4 M LiCl; ■, proteins present on the complementary 4.0c core particle (ref. 23 and unpublished results). (*C*) Proteins essential for the reconstitution of peptidyltransferase activity form a skeleton of strong assembly dependences. (*D*) Groups of L-protein genes present in various transcriptional units. ▩, *S10* operon; ■, *spc* operon; ▩, *L7/L12* operon; ▨, *L11* operon.

from those we have used. (*ii*) The proteins had been contaminated, and the contaminants did support 5S·23S RNA complex formation. In fact, efficient complex formation was reported in ref. 15 with fractions containing at least 12 proteins including L2, L3, and L15. Also, the protein preparation used in ref. 18 contained at least L3 and L15. The protein purity in ref. 17 was not documented.

In contrast to our incubation conditions, those used by the other groups (20 mM Mg^{2+}/≈300 mM K^+, 15–45 min at 30–37°C) do not yield active particles. However, it could well be that a partial assembly step is obtained directly under conditions far from the standard ones that allow totally correct assembly, whereas the standard ones lead to that partial assembly step only after a defined sequence of assembly reactions. We tested this possibility in a control experiment in which the binding of L5 and L18 to 23S RNA dependent on the presence of 5S RNA was analyzed under the conditions reported in ref. 17 (20 mM Mg^{2+}/300 mM K^+, 15 min at 30°C). In fact, we found significant binding of L5 and L18 that could be improved significantly by the addition of L15 and L2, L3, and L4 (data not shown). It appears that interactions taking place during the assembly can be studied in model systems far from the conditions of total reconstitution, as has been done for the analysis of 5S·23S RNA interaction in the presence of L5 and L18 (15–18). However, in this paper, we show that, under standard conditions, L5 and L18 are not sufficient and at least three other proteins are required. Therefore, if in a model system component A binds to component B the involved binding sites might well reflect regions interacting in the course of assembly. However, in the course of the assembly the binding of A and B could be mediated by additional components. Thus, it is misleading to derive a sequence of assembly reactions from experimental conditions that do not allow the assembly of active particles. This might be the main reason why the partial assembly map reported in ref. 17 is not compatible with the results reported here (Fig. 3).

Comparison of the 50S Assembly Map with Other Data. Various approaches have been used to determine the "inside-/outside-" location of ribosomal proteins—e.g., trypsin digestion of 50S subunits and subsequent determination of nondigested proteins (19), modification with *N*-ethylmaleimide (20) and glutaraldehyde (21), and the comparison of chemical and enzymatic (lactoperoxidase) iodination (22). Concerning the assembly map, it is strikingly evident that those proteins whose outside location was shown by various techniques are late assembly proteins, most of which show a strong assembly dependence to L15 (Fig. 4*A*).

Another structural hint concerning inside/outside is provided by the order in which the proteins can split off the mature 50S subunit by incubation with LiCl at increasing concentration (23). Fig. 4*B* shows that this order reflects in reverse the assembly sequence.

Recently, we have identified in single-omission experiments the proteins essential for reconstitution of the peptidyltransferase activity—i.e., namely L2, L3, L4, L15, L16, and L18 (ref. 12). With a different strategy—i.e., determination of the minimal set of ribosomal components required for reconstitution of the peptidyltransferase activity—we could further

reduce the number of essential components and show that L18 is not essential to peptide bond formation (unpublished results). Interestingly, all the essential components form a network of strong assembly dependences (Fig. 4C). Furthermore, there is a striking coincidence of components essential for peptidyltransferase activity on the one hand and those essential for the binding of 5S RNA on the other (L2, L3, L4, L15, and L16 vs. L2, L3, L4, L15, and L18). One may speculate whether this "skeleton" of assembly dependences, comprising both structural (5S RNA plays at least an important role in the late assembly of 50S subunit; ref. 24) and functional (peptidyltransferase) components, reflects elements of an "ur-ribosome."

Finally, we compare the 50S assembly map with the gene organization of the L proteins. When we project the known operon structures of the L-protein genes (for review, see ref. 25) onto the assembly map, we find a conspicuous coincidence with assembly domains (Fig. 4D). The *S10* operon comprises the genes for seven 50S proteins, six of them belong to one assembly cluster. The *S10* operon contains all the peptidyltransferase candidates except L15. A relationship of gene organization and regulation on the one hand and assembly dependences on the other is strikingly indicated by the *spc* operon. This operon contains two groups of *L* genes, the first one (genes for L14 and L24) is not regulated by the mechanism found with the second group (genes for L5, L6, L18, L30, and L15 in addition to those of some S proteins; see ref. 26). The separation of this transcriptional unit into two differently regulated groups coincides with two assembly clusters; the second one comprises those proteins (L5, L15, and L18) that are essential for the binding of 5S to 23S RNA.

The striking coincidence of assembly dependences and gene clusters of the respective proteins shows that assembly dependences play an important and possibly decisive role for the gene organization of the L-protein genes. A similar coincidence of assembly dependences of the 30S subunit components (1) and the organization of the respective genes is not apparent. An exception concerns proteins S6 and S18, which stimulate the incorporation of each other during reconstitution (10) and can form a protein complex (26) and whose genes are located in a single operon (27).

The relationship described above between assembly dependences and the organization of L-protein genes, as indicated by the common transcription of assembly groups of ribosomal proteins, corresponds to an overlapping of 23S rRNA transcription and 50S assembly: clearly, 50S assembly already starts at the 5′ end of 23S RNA before synthesis of this RNA has been completed (assembly gradient: for review, see ref. 2). Thus, 50S assembly is related to both the transcription process of 23S RNA and the transcription order of the L proteins.

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