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Initiator proteins for the assembly of the 50S subunit from *Escherichia coli* ribosomes

(total reconstitution/rRNA-binding proteins/regulation of L protein genes)

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ABSTRACT **An rRNA-binding protein that binds to the rRNA independently of other proteins during the course of ribosomal assembly is termed "assembly initiator protein." In spite of the large number of rRNA-binding proteins (more than 17 out of 32 proteins have been identified in the case of the large ribosomal subunit), only a very small number of proteins should actually initiate ribosomal assembly for theoretical reasons. Here we demonstrate that only two of the L proteins derived from the large subunit (50S) function as assembly initiator proteins. Two different techniques are used to identify these initiator proteins: reconstitution experiments with purified proteins and pulse–chase experiments during *in vitro* assembly. Both methods independently identify L24 and L3 as initiator proteins for the 50S assembly. The existence of two initiator proteins (not just one) resolves an apparent contradiction—namely, that on the one hand, rRNA is synthesized in excess under unfavorable growth conditions, whereas on the other hand, rRNA-binding proteins should be available for translational control.**

The majority of the ribosomal proteins in both the small and the large ribosomal subunit bind *in vitro* to their respective rRNA. In the small subunit, 12 out of 21 proteins have been identified as "RNA-binding proteins" (1, 2), and in the large subunit, 17 out of 32 (3, 4). Proteins derived from the large ribosomal subunit are designated L.

In the case of the small ribosomal subunit, it has been shown that the cooperativity of the assembly process is so high that certainly not all 12 but at most 2 or 3 proteins (or protein complexes) can bind independently to 16S rRNA (5).

In the case of the large (50S) subunit, it is not yet clear whether even the seven most strongly binding proteins (4) bind independently to their rRNA in the course of the assembly. If this were so, then these proteins would be distributed independently of each other over the various individual rRNA molecules. If the rRNA were present in excess, this random distribution would disturb the formation of ribosomal subunits with a constant and stoichiometric protein content and, as a result, dramatically decrease the output of active ribosomes. In fact, a synthesis of rRNA in a molar excess of more than threefold over ribosomal proteins† has been observed under unfavorable growth conditions (6). Therefore, one would expect that, in spite of the large number of 23S rRNA-binding proteins concerned in the assembly of the 50S subunit, only a few of these proteins should be able to bind independently to the rRNA (i.e., without cooperativity), thus selecting the rRNA molecules for the 50S assembly in the presence of an rRNA excess. These proteins we term "assembly initiator proteins".

In this paper we demonstrate that the *in vitro* assembly of the large ribosomal subunit is initiated by only two proteins. Two different methods independently identify these proteins as L24 and L3.

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MATERIAL AND METHODS

Ribosomes and their subunits were isolated from *Escherichia coli* cells as described (7). Isolation of rRNA (7) and that of the total proteins from the 50S subunit (TP50) (8) were as described. TP50 was labeled by means of the reductive methylation method (9) with [^{3}H]- and [^{14}C]formaldehyde. The specific activity of ^{3}H-labeled TP50 (^{3}H-TP50) was 3.00×10^5 cpm per $A_{230\ nm}$ unit, and that of ^{14}C-labeled TP50 (^{14}C-TP50) was 1.55×10^6 cpm per $A_{230\ nm}$ unit. One $A_{230\ nm}$ unit of TP50 is equivalent to 250 μg or to 10 equivalent (equiv.) units (1 equiv. unit of TP50 is that amount of protein present on 1 $A_{260\ nm}$ unit of 50S subunits). Isolation of purified proteins was as described (10). Reconstitution experiments and subsequent activity measurements in the peptidyltransferase assay or the poly(U)-dependent poly(Phe) synthesis system were performed as described (7).

For the pulse–chase experiment, seven samples, each containing 24 $A_{260\ nm}$ units (23S + 5S) of rRNA and 12 equiv. units of ^{14}C-TP50, were incubated in a 1-ml volume under the conditions of the first step of the total reconstitution (7) at 44°C. At different times (as indicated), 36 equiv. units of ^{3}H-TP50 was added (for the 0-min sample the ^{14}C- and ^{3}H-TP50 fractions were mixed before addition to rRNA). After a 20-min incubation at 44°C, a standard second-step incubation followed. Aliquots (40 μl) in duplicate were withdrawn and tested in a modified peptidyltransferase assay (9), and the remainder was subjected to a sucrose gradient run (10–30% sucrose in 20 mM Tris·HCl, pH 7.5/20 mM Mg acetate/400 mM NH_4Cl; SW40 rotor at 20,000 rpm for 16 hr). After fractionation, the peak containing the reconstituted particles was precipitated with trichloroacetic acid (final concentration, 5%), and the proteins were extracted into 66% acetic acid and precipitated with 5 volumes of acetone (2 hr at −20°C). The precipitate was dried *in vacuo*, and the proteins were separated by two-dimensional gel electrophoresis and stained (11). Gel pieces containing stained spots were cut out (diameter, 5 mm), dried at 60°C for 12 hr, and burnt in a Packard Sample Oxidizer. The ^{3}H and ^{14}C isotopes were collected separately and assayed.

RESULTS AND DISCUSSION

The Number of Initiator Proteins of the 50S Assembly. Let us assume that we have a molar excess E of (23S + 5S) rRNA

Abbreviations: TP50, total proteins from the 50S subunit; equiv. unit, equivalent unit (1 equiv. unit is that amount of protein present on 1 $A_{260\ nm}$ unit of 50S subunits).

† For clarity, it should be emphasized that herein a 3-fold molar excess of rRNA is a factor of 3 times the stoichiometric level of ribosomal proteins.

over TP50. Then the probability of finding one distinct initiator protein on a given rRNA molecule would be E^{-1}, and the probability for n initiator proteins to be present on one single rRNA molecule would be E^{-n}. Because the probability is the same for all rRNA molecules, the probability A of finding n initiator proteins on any of the rRNA molecules is

$$A = (E^{-n}) \times E = E^{1-n}.$$

A formula giving the fraction of rRNA molecules used for the 30S assembly as a function of both rRNA excess and the number of initiator proteins was reported by Nomura *et al.* (5). We presume that all initiator proteins must be present on one rRNA molecule in order to allow a successful assembly and the formation of active particles. If so, A gives the fraction of active subunits formed in the presence of a molar excess E of rRNA over TP50 for n initiator proteins. If these assumptions are valid, a double logarithmic plot of A vs. E should yield a straight line of which the slope is $(1 - n)$ and, thus, gives the number n of initiator proteins.

Fig. 1*A* presents such double logarithmic plots, in which the theoretical output of active particles (in percentage of the molar input of TP50) in the presence of an increasing rRNA excess is calculated for 1, 2, 3, and 10 initiator proteins. Accordingly, a reconstitution experiment was performed with a constant amount of TP50 and an increasing excess of rRNA. The activities of the reconstituted particles were determined in a poly(Phe) synthesis system, and the values were corrected for the inhibitory effect of the excess rRNA in the poly(Phe) system. The activity values obtained are incorporated in Fig. 1*A*. The coincidence of the experimental points with the theoretical line for two initiator proteins is striking. We conclude that for the 50S assembly, only two initiator proteins (or protein complexes) exist, which, in the presence of an rRNA excess, select that 23S rRNA molecule used for the assembly of the 50S subunit.

Identification of the Initiator Proteins: Reconstitution with Purified Proteins. Two presumptions can be made as to the nature of the initiator proteins. (*i*) The initiator proteins should belong to those proteins that bind strongly to 23S rRNA under the condition of the first step of the 50S total reconstitution. These proteins are L1, L2, L3, L4, L9, L20, L23, and L24 (ref. 4). (*ii*) The initiator proteins probably belong to the early assembly proteins, which are defined as proteins essential for the formation of an early assembly intermediate, the RI^*_{50} (1) particle. These proteins are L2, L4, L13, L22, L24, and possibly L3 (12). However, because the assembly proteins had been identified in reconstitution experiments with roughly stoichiometric amounts of rRNA and proteins, the necessity of initiator proteins for selecting the rRNA molecule that is used for the assembly could have been overlooked. Therefore, we did not restrict our search to the early assembly proteins but performed a systematic screening.

With 1, 2, and 4 M LiCl, increasing amounts of proteins are split off the 50S subunit. The resulting core particles, designated 1.0c, 2.0c, and 4.0c cores, contain about 25, 15, and <10 L proteins, respectively, in addition to 23S rRNA (13, 14). The proteins in these cores were extracted by the acetic acid method and were used for an rRNA excess experiment as described above for TP50. In the first step of the reconstitution procedure, a constant amount of core proteins was reconstituted with an increasing excess of rRNA. In the second incubation step, TP50 was added to allow only the completion of those assembly intermediates that have achieved a distinct stage of the early assembly [RI^*_{50}(1) conformation; in the second step, active particles cannot be formed *de novo*; ref. 15]. Because L24 is the only early assembly protein not present on the 4.0c cores (16), this protein was added in equimolar amount to the proteins

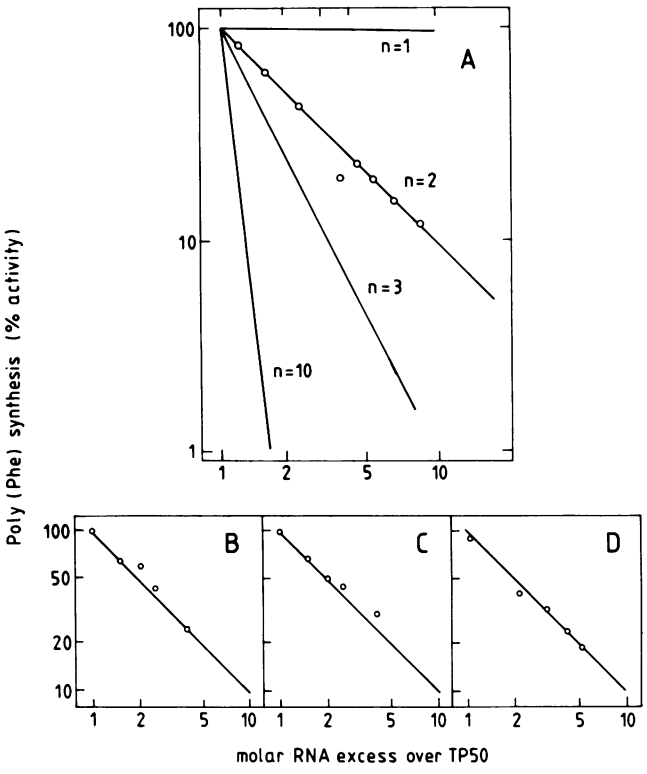

FIG. 1. (*A*) Double logarithmic plot of A vs. E of the formula $A = E^{1-n}$. The theoretical lines for 1, 2, 3, and 10 initiator proteins are shown. The experimental points were obtained after reconstitution of 2.5 equiv. units of TP50, with the molar excess of rRNA as indicated. The activities of the reconstituted particles were determined in the poly(Phe) synthesis system and corrected for the inhibitory effect of the respective excess RNA on poly(Phe) synthesis. The 100% value was 43.230 cpm (obtained with a reconstituted particle of 1 $A_{260\ nm}$ unit), and the correction factors for 2-, 5-, and 10-fold RNA excesses were 1.42, 2.27, and 3.24, respectively. (*B–D*) Proteins of 1 equiv. unit extracted from 1.0c, 2.0c, and 4.0c cores, respectively, were incubated with the rRNA excess indicated under the conditions of the first step. The proteins derived from the 4.0c cores were complemented with equimolar amounts of L24. After raising the Mg^{2+} concentration to 20 mM, 1 equiv. unit of TP50 was added, and a standard second-step incubation followed. After reconstitution, the missing ingredients required for the poly(Phe) synthesis system were added, and the poly(Phe)-synthesizing activity was determined (7) and corrected for rRNA excess as described above.

derived from this core particle. The results obtained with the core proteins are shown in Fig. 1 *B–D* in addition to the theoretical line calculated for two initiator proteins. It is obvious that all protein families tested, including the proteins from the 4.0c core plus L24, must contain both initiator proteins. Therefore, we restricted our analysis in the next experiment to the proteins of the 4.0c core plus L24.

The proteins were divided into four groups (Table 1). Three of these groups were incubated with stoichiometric amounts of rRNA under the conditions of the first step. After 10 min, the rRNA amount was raised to a 3-fold molar excess over proteins, then the missing group was added, and the first-step incubation was continued for another 10 min. In the second-step incubation, TP50 was added.

The rationale of this experiment was the following: if the initiator proteins are both present in the first 10-min incubation, all rRNA molecules (present in stoichiometric amounts) will carry both initiator proteins. Therefore, the rRNA excess given subsequently will not significantly affect the activity. On the other hand, if the initiator proteins are added when the rRNA

is already present in excess, the initiator proteins will be distributed independently over all of the rRNA molecules, thus dramatically reducing the output of active particles. Whenever the four proteins L3, L4, L23, and L24 were present during the first 10 min, then significant activity was obtained (Table 1, experiment 1). In contrast, the addition of the four proteins to the second 10-min incubation (in the presence of excess rRNA) resulted in only poor activity. We conclude that the two initiator proteins are among these four proteins and analyzed these four proteins in the next experiment.

In the following experiment, the strategy was slightly modified. In the first 10 min of the first-step incubation with an equimolar amount of rRNA, either two or only one out of the four proteins were added. In the second 10-min incubation of the first step, the rRNA excess was increased to 7-fold before TP50 was added. A clear result was obtained (Table 1, experiment 2): the most significant activity was found when L3 and L24 were present during the first 10-min incubation. The lowest activity was found when these proteins were both absent. L24 seemed to exert a slightly stronger effect than L3: whenever L24 was present and L3 was not, higher activity was obtained than in the reverse case (compare in Table 1, for example, L3 + L23 with 560 cpm and L24 + L23 with 870 cpm). According to the formula described above, one should expect a 7-fold higher activity in the case where the initiator proteins are given to an equimolar amount of rRNA than in the case where both initiator proteins are mixed with a 7-fold rRNA excess. In good agreement with the expectation, we found that the mixture of L24 and L3 and stoichiometric amounts of rRNA (before establishing the rRNA excess) yielded a 6-fold higher activity than did the mixture of TP50 (which contain both initiator proteins) with a 7-fold molar rRNA excess (compare 1,818 with 302 cpm). We conclude that L24 and L3 are the initiator proteins for the 50S assembly.

Table 1. Start of the 50S assembly with purified proteins

	Incubation conditions: first step					
	Incubation 1, 10 min				Incubation 2, 10 min	Poly(Phe) synthesis, cpm
Exp.	L3, L4 L23, L24	L13 L21	L17 L22	L2 L20		
1	+	+	+	+	—	1,053
	+	+	+	−	L2, L20	1,248
	+	+	−	+	L17, L22	1,828
	+	−	+	+	L13, L21	1,104
	−	+	+	+	L3, L4, L23, L24	28
2	L3	L4	L23	L24		
	+	+	−	−	TP50	662
	+	−	+	−	TP50	560
	+	−	−	+	TP50	1,818
	−	+	+	−	TP50	125
	−	+	−	+	TP50	783
	−	−	+	+	TP50	870
	+	−	−	−	TP50	782
	−	+	−	−	TP50	312
	−	−	+	−	TP50	267
	−	−	−	+	TP50	1,001
	−	−	−	−	TP50	302

The data are mean values of double determinations; the SEM was below 5%. Background values (equimolar amounts of TP50 and rRNA incubated only under the second-step condition) were subtracted. In experiment 1, the first step of the two-step procedure of the total reconstitution was divided into two successive incubations, each 10 min long. In the first incubation, 1 equiv. unit of the indicated proteins was incubated with an equimolar amount of (23S + 5S) rRNA. For the second incubation (first-step condition), the molar rRNA was increased to a 3-fold excess, and 1 equiv. unit of a second set of proteins was added where indicated. After the first-step reconstitution, the Mg^{2+} concentration was raised from 4 to 20 mM, and after addition of 1 equiv. unit of TP50, a second-step incubation followed. The missing ingredients necessary for the poly(Phe) synthesis were added, and the poly(Phe)-synthesizing activity was determined (7). In experiment 2, 1 equiv. unit of the indicated, purified proteins was incubated with an equimolar amount of (23S + 5S) rRNA. Then the RNA was increased to a 7-fold molar excess, 1 equiv. unit of TP50 was added, and the second incubation (first-step condition) followed. Afterward, a standard second-step incubation was performed, and the poly(Phe)-synthesizing activity was determined as described above.

However, although we showed in many experiments that our isolated proteins can be used for the reconstitution of active particles, we do not know whether all of our isolated individual proteins are of comparable activity. Comparable activities are a prerequisite for the quantitative studies we have described above. Therefore, it would be satisfying if we could confirm the identities of the initiator proteins with a different approach, avoiding the use of isolated single proteins. Such an approach is described in the next section.

Identification of the Initiator Proteins: Pulse–Chase Experiment During the 50S Assembly *in Vitro*. Our concept of initiator proteins implies that L24 and L3 are the only ones among the rRNA-binding proteins that bind independently and with high affinity to the 23S rRNA in the course of the assembly. Accordingly, in a pulse–chase experiment performed during the assembly process with various pulse times, the initiator proteins should be chased most poorly among all L proteins.

rRNA was incubated with less than stoichiometric amounts (0.5×) of ^{14}C-TP50. At various times (from 0 to 20 min) a 1.5-molar excess of ^{3}H-TP50 was added to different samples.

After the two-step incubation, the reconstituted particles showed at least 50% of the activity of native 50S subunits in a modified peptidyltransferase assay (9). The reconstituted particles were purified in a sucrose gradient run, and the proteins were extracted and separated by two-dimensional gel electrophoresis. The ^{14}C/^{3}H ratio was determined from each protein spot. A representative example of the cpm values actually derived from one gel (0-min pulse) is shown in Table 2. The cpm ratios usually show a variance from 1.0 to 2.3. Obviously, the proteins had been differently affected during the ^{14}C and ^{3}H labeling. Control reconstitutions performed with the same mixture (^{14}C-TP50/^{3}H-TP50) revealed an excellent reproducibility of the ^{14}C cpm/^{3}H cpm ratio from the assembled proteins (data not shown).

The cpm ratios of a protein derived from different plates were normalized by dividing all values by the smallest one. The results obtained are compiled in Table 3, which together with Fig. 2 strikingly demonstrates that the protein most strongly resisting the chase is protein L24. The second best protein is L3, whereas all the other proteins can be chased to about the same extent. Obviously, most of the proteins with the exception of L24 and L3 are loosely bound in an early assembly particle [RI^{*}_{50} (1)] and, therefore, are exchangeable. The results of this section confirm the findings of the preceding one—namely, that the two initiator proteins of the 50S assembly are L24 and L3.

CONCLUSIONS

In vitro, more than half of the ribosomal proteins can bind directly to their corresponding rRNA. However, the term "rRNA-binding protein" does not mean that such a protein binds independently to rRNA in the course of the assembly. In fact, only a very few out of the large number of rRNA-binding proteins are able to bind independently to the rRNA during the assembly, thus initiating assembly domains. The small number of ini-

Table 2. ^{14}C and ^{3}H label of the assembled proteins at 0-min pulse time

	Label, cpm		^{14}C cpm
	^{14}C	^{3}H	^{3}H cpm
L1	5,415	2,399	2.26
L2	4,951	2,583	1.92
L3	3,588	2,169	1.66
L4	1,277	1,059	1.21
L5	3,313	1,460	2.27
L6	2,185	988	2.21
L7/12	765	3,697	0.21
L9	1,825	1,077	1.70
L10	992	980	1.01
L11	2,605	1,265	2.06
L13	4,747	3,458	1.37
L15	9,338	5,626	1.66
L16	1,784	1,190	1.50
L17	3,181	2,228	1.43
L18	4,389	2,952	1.49
L19	4,164	2,343	1.78
L20	94	85	1.11
L21	1,401	792	1.77
L22	6,474	4,006	1.62
L23	4,741	3,154	1.50
L24	3,748	1,899	1.97
L25	1,880	1,398	1.35
L27	1,246	615	2.03
L28	637	398	1.60
L29	191	1,298	0.15
L30	761	402	1.89
L32	661	345	1.92

For the 0-min pulse–chase during the 50S assembly, the ^{14}C- and ^{3}H-TP50 were mixed before they were added to rRNA. After the standard two-step incubation (7), an aliquot was withdrawn and tested for peptidyltransferase activity; from the remainder, the reconstituted particles were isolated and the ratio of $^{14}C/^{3}H$ of the individual proteins was determined. In contrast to the other gels, this gel showed only a minor spot for L20. In the gel derived from the 0.5-min pulse, for example, the L20 values were 640 and 517 cpm for ^{14}C and ^{3}H, respectively, yielding a quotient of 1.24. The proteins L14, L31, L33, and L34 were not separated satisfyingly on the gels and, therefore, were not processed.

Table 3. Normalized $^{14}C/^{3}H$ quotients of the L proteins

Pulse time, min	0.0	0.5	1.0	1.5	3.0	6.0	20
L1	1.02	1.00	1.084	1.06	1.07	1.03	1.105
L2	1.00	1.01	1.043	1.04	1.03	1.07	1.42
L3	1.00	1.51	1.80	1.90	2.30	2.94	6.80
L4	1.00	1.20	1.31	1.12	1.1	1.04	1.27
L5	1.003	1.00	1.022	1.093	1.041	1.06	1.255
L6	1.13	1.00	1.052	1.013	1.005	1.033	1.112
L7/12	1.014	1.059	1.069	1.039	1.02	1.00	1.02
L9	1.06	1.00	1.095	1.11	1.053	1.019	1.044
L10	1.072	1.00	1.006	1.041	1.02	1.27	1.10
L11	1.00	1.104	1.18	1.18	1.09	1.19	1.223
L13	1.00	1.317	1.42	1.482	1.511	1.592	1.788
L15	1.002	1.047	1.00	1.057	—	1.04	1.10
L16	1.07	1.06	1.02	1.00	1.05	1.05	1.09
L17	1.00	1.064	1.09	1.068	1.124	1.22	1.395
L18	1.00	1.321	1.31	1.3	1.22	1.37	2.09
L19	1.00	1.154	1.12	1.18	1.20	1.323	2.26
L20	1.00	1.21	1.24	1.354	—	1.34	1.491
L21	1.03	1.09	1.03	1.00	1.00	1.22	1.21
L22	1.00	1.349	1.385	1.482	1.59	—	2.65
L23	1.00	1.275	1.368	1.473	1.726	2.043	3.373
L24	1.00	5.04	5.00	6.51	7.24	7.2	10.57
L25	1.01	1.09	1.08	1.012	1.00	1.066	1.108
L27	1.05	1.01	1.06	1.00	1.03	1.09	1.95
L28	1.09	1.00	1.178	—	1.21	1.32	1.67
L29	1.00	1.21	1.37	1.34	1.765	1.721	1.783
L30	1.021	1.04	1.05	1.04	1.03	1.00	1.52
L32	1.06	1.14	1.00	1.09	1.18	1.01	1.36

The pulse time indicates the incubation time of RNA/^{14}C-TP50 after which ^{3}H-TP50 was added. The $^{14}C/^{3}H$ quotients were normalized to 1 by dividing all values of a given protein derived from the various gel plates by the smallest value. —, Sample lost during processing. Because ^{3}H-TP50 was added in a 3-fold molar excess over ^{14}C-TP50, normalized values of <3 are expected. However, control experiments demonstrated that 23S rRNA not complexed with L24 and L3 is inactivated during the first-step incubation, thus decreasing the rRNA amount available for the 50S assembly (data not shown). This kinetic reduction in the apparent amount of rRNA amplifies the $^{14}C/^{3}H$ ratio of the early assembly proteins, which bind tightly to 23S rRNA, thus giving rise to normalized values >3.

tiation proteins for both the 30S (5) and the 50S assembly (this paper) underlines the enormous cooperativity governing the start phase of the assembly process. However, maximal cooperativity for the initiation of the assembly would be achieved with only one initiator protein. Is there any advantage for the ribosome in the fact that the assembly of both subunits is initiated by more than one protein?

In vivo, the rRNA is synthesized in slight excess (1.1:1 over ribosomal proteins up to a generation time of 1.5 hr). At a further slowing down of the generation time, the rRNA excess is increased and can even exceed 3:1 (ref. 6). On the other hand, under these unfavorable growth conditions (long generation time), the translational control of the synthesis of the ribosomal proteins should be effective. The principle of this translational control is that some of the rRNA-binding proteins are able to bind to their corresponding transcript (i.e., to ribosomal initiation sites), thus competing with the ribosomal initiation and, therefore, reducing the frequency of translation of that transcript (for review, see ref. 17). The translational control requires availability of ribosomal proteins for the binding to their corresponding rRNA. This paradox—namely, a significant rRNA excess on the one hand and availability of some rRNA-binding proteins on the other hand—can be explained by the existence of more than one initiator protein.

L24 is an early assembly protein that binds to the 5′-end of 23S rRNA (18), thus selecting an rRNA molecule very soon after the synthesis of this molecule has been started. In contrast, L3 is most probably a late assembly protein for the following reasons: (*i*) it is not found on the first precursor particle of the *in vivo* 50S assembly (19); (*ii*) it is not essential (but stimulatory) for the formation of an early reconstitution intermediate (12); and (*iii*) it binds near the 3′ region of 23S rRNA (18). It follows that the term "initiator proteins" implies that, in the presence of an rRNA excess, these proteins select the rRNA molecules used for 50S assembly. Under balanced synthesis of ribosomal proteins and rRNA, the early assembly protein L24 is the protein initiating the assembly in the real sense, but L3 may not be involved. Therefore, it seems likely that, at an rRNA excess slightly exceeding one, the effect of L24 will dominate that of L3 (see also Fig. 2), with the result that the assembly cooperativity depends mainly on L24. However, if the rRNA excess becomes significantly higher, L3 becomes more important as an initiator protein, leading to a rapid increase of assembly "dead ends." These assembly dead ends have most probably a loose structure similar to assembly intermediates, thus providing ribosomal proteins for the translational control. In support of this idea, a mutant harboring a temperature-sensitive L24 accumulates loose protein–23S rRNA complexes sedi-

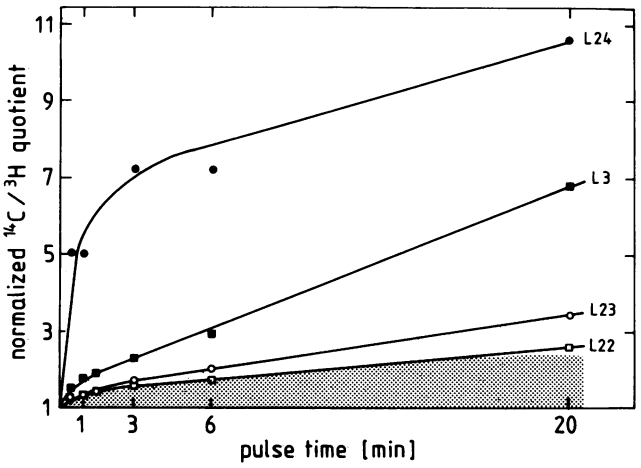

FIG. 2. Plot of the normalized quotients of the proteins (Table 3) vs. time. The larger the quotient, the tighter the binding of the respective protein and the less the chasing effect. ●, L24; ■, L3; ○, L23; □, L22. All other proteins have values below 2.3 and fall in the shaded region. Pulse time indicates the incubation of ^{14}C-TP50/rRNA after which a 1.5 molar excess of ^{3}H-TP50 was added (conditions of the first step). After 20 min, a standard second-step incubation followed.

menting with 30S (20) at nonpermissive temperatures. It appears that the requirement for two initiator proteins is a prerequisite for the formation of loose rRNA–protein complexes in the presence of the rRNA excess; the existence of only one initiator protein would direct all ribosomal proteins toward the assembly process, leaving no proteins available for translational control.

In summary, two initiator proteins could represent an optimum for the assembly of 50S subunits in the presence of rRNA excess. More than two would be deleterious for the efficiency of the assembly process, whereas less than two would impair or even prevent the translational control.

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