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Ribosomal Protein L20 Can Replace the Assembly-Initiator Protein L24 at Low Temperatures

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ABSTRACT: The assembly of the 50S subunit from *Escherichia coli* ribosomes is initiated by two ribosomal proteins, L24 and L3. A mutant lacking the assembly-initiator protein L24 shows distinct phenotypic features (temperature sensitivity, growth rate reduced by a factor of 6 at permissive temperatures below 34 °C, underproduction of 50S subunits), which could be traced back to assembly effects caused by lack of L24 [Herold, M., Nowotny, V., Dabbs, E. R., & Nierhaus, K. H. (1986) *Mol. Gen. Genet. 203*, 281–287]. As expected, only one assembly protein was effective during in vitro assembly at nonpermissive temperatures, whereas surprisingly the restoration of active particle formation at permissive temperatures was paralleled by the reappearance of two initiator proteins. Here we analyze the initiation of assembly at permissive temperatures in the absence of L24. We demonstrate in a series of reconstitution experiments with purified proteins that the two initiator proteins are L20 and L3. Thus, L20 can replace L24 for the initiation of assembly at permissive temperatures.

L24 and L3 are the two assembly-initiator proteins for the in vitro assembly of the ribosomal 50S subunit (Nowotny & Nierhaus, 1982). Since L24 binds to the 5′-end of 23S rRNA (Kühlbrandt & Garrett, 1978) in contrast to L3 (Zimmermann, 1980), L24 should initiate the assembly in vivo, where the assembly is probably coupled to the rRNA transcription ["assembly gradient"; for discussion, see Herold and Nierhaus (1987)]. L3 would become effective as a second assembly-initiator protein only under unfavorable growth conditions, when the synthesis of rRNA no longer matches that of the r proteins, and can exceed the latter by a factor of 3 (Gausing, 1977). The appearance of a second initiator protein under these conditions leads to "assembly dead ends", loose r protein–rRNA comlexes, which might be important for maintaining translational control of the r protein synthesis [for discussion, see Nowotny and Nierhaus (1982)].

A detailed analysis of the rRNA binding site of L24 has revealed that this protein is very well suited for initiating the assembly. The RNA binding site consists of two rRNA regions; one serves as the "primary attachment site", whereas binding to the second region ("organizer") may induce an rRNA folding which probably facilitates subsequent assembly steps (Egebjerg et al., 1987).

L24 is a mere assembly protein, engaged exclusively in the early assembly but partaking neither in the late assembly nor in the ribosomal functions (Spillmann & Nierhaus, 1978). Under the standard conditions of the two-step reconstitution, L24 is absolutely required for the formation of the $RI^{*}_{50}(1)$ particle, the essential reconstitution product of the first-step incubation (Spillmann et al., 1977). However, when the temperature of the first-step incubation was reduced from 44 to 36 °C, an active peptidyltransferase center could be reconstituted in the absence of L24 (Schulze & Nierhaus, 1982).

Interestingly, a temperature-sensitive, spontaneous mutant could be isolated which lacks L24 (Dabbs, 1982). The phenotypic features of this mutant, including temperature sensitivity and severe growth defects, could be explained by serious impairments in the 50S assembly generated by the lack of L24 (Herold et al., 1986). One surprising finding was that at permissive temperatures two initiator proteins were again effective. Clearly, L24 could be replaced as assembly-initiator protein under these conditions.

Here we show that in the absence of L24 the protein L20 takes over the L24 function at low temperatures, whereas L3 remains the second assembly-initiator protein.

Experimental Procedures

The 70S ribosomes, ribosomal subunits, and 23S + 5S rRNA were prepared and the total reconstitution was performed as described (Nierhaus & Dohme, 1979). The preparation of the LiCl core particles followed the procedure of Homann and Nierhaus (1971), and the preparation of total proteins (TP50)[1] from the 50S subunits and the 3.6 M LiCl core (CP3.6) followed that of Schulze and Nierhaus (1982). The proteins of the core particles derived from 50S subunits by incubation with 3.6 M LiCl were isolated by conventional techniques (Wystup et al., 1979). The purity of the isolated proteins was assessed by two-dimensional gel electrophoresis (Geyl et al., 1981). The poly(U)-dependent poly(Phe) synthesis of the reconstituted particles (Nierhaus & Dohme, 1979) and the determination of the number of the assembly-initiator proteins (Nowotny & Nierhaus, 1982) were carried out as described earlier.

Results

Initiation of Assembly with and without L24. In this section we analyze whether or not a 50S core particle, consisting of 23S rRNA and approximately 10 proteins, also contains the two initiator proteins found at low temperatures in the absence of L24. The core particle is obtained by washing 50S subunits with ca. 4 M LiCl under defined conditions (Homann & Nierhaus, 1971), and all proteins essential or important for the formation of the reconstitution intermediate $RI^{*}_{50}(1)$ (namely, L3, L4, L13, L20, L22, and L24) are found among the 10 core proteins, with the exception of L24 (Spillmann & Nierhaus, 1978).

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

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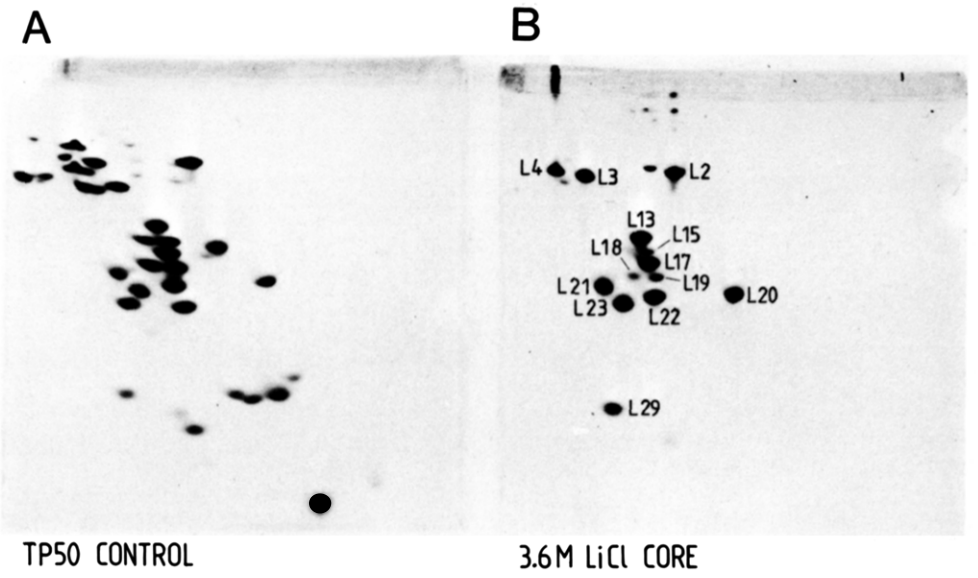

FIGURE 1: Two-dimensional gel electrophoresis of the total proteins (TP50) from the 50S subunit (A) and from the 3.6 M LiCl core particles, i.e., the CP3.6 proteins (B).

Table I: Importance of L24 for the Total Reconstitution at Standard (44 °C) and Low Temperatures (36 °C) during the First Step Incubation[a]

	first step		second	poly([^{14}C]Phe)	
expt	temp (°C)	proteins	step proteins	% relative activity	cpm
1		CP3.6 + L24	TP50	100	3.930
	44	CP3.6	TP50	9	
		control (TP50)			12.126
		CP3.6 + L24	TP50	51	
	36	CP3.6	TP50	64	
		control (TP50)			6.270
2		$\sum$(A+B) + L24	TP50	100	5.057
	44	$\sum$(A+B)	TP50	0	
		control (TP50)			35.766
	36	$\sum$(A+B)	TP50	108	
		control (TP50)			16.193

[a] The reconstitution with no protein additions to the first step, and TP50 added to the second step, is taken as background and has been subtracted. A total of 2.5 A_{260} units of 23S+5S rRNA was present per 100 μL of aliquot. In experiment 1, CP3.6 means the proteins extracted from the 3.6 M LiCl core; the protein:rRNA ratio was 2.3 eu of core proteins plus L24 and 1.5 eu of TP50 per 1 A_{260} unit of rRNA. In experiment 2, $\sum$(A+B) means the proteins L3, L4, L13, L20, L21, L22, L23, and L29. The protein:rRNA ratio was 1.5 eu of $\sum$(A+B), 2.2 eu of L24, and 1.5 eu of TP50 per 1 A_{260} unit of rRNA. Controls with the various protein fractions at the first step but with no TP50 at either step showed no activity at all.

By washing 50S subunits with 3.6 M LiCl we obtain the corresponding 3.6 M LiCl core, which contains a full complement of the ten proteins L2, L3, L4, L13, L17, L20, L21, L22, L23, and L29 and in trace amounts the proteins L15, L18, L19, and L30 (Figure 1B). The core proteins (CP3.6) were extracted and tested for their ability to form the $RI^{*}_{50}(1)$ particle in the first step of the two-step reconstitution procedure. This particle is the essential product of the first-step incubation, and only if this particle is formed does the addition of TP50 to the second step yield active 50S particles (Dohme & Nierhaus, 1976; Spillmann et al., 1978). Table I, experiment 1, shows that at 44 °C significant activity can only be found if L24 is added to the CP3.6 fraction during the first step, in agreement with the previous observation (Spillmann & Nierhaus, 1978). The TP50 control at 36 °C yields only half the amount of active particles as compared to that at 44 °C as expected [see Herold et al. (1986) and Sieber and Nierhaus (1978)]. The fact that we find only one-third of the activity seen with TP50 (3.930 vs 12.126 cpm) could be due to the lower stability of the $RI^{*}_{50}(1)$ particle formed in the presence of the reduced number of proteins in the CP3.6 fraction.

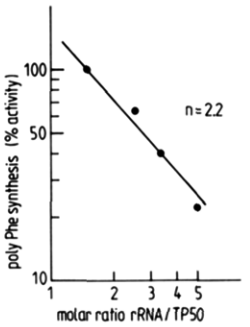

FIGURE 2: Determination of the number of initiatior proteins within the CP3.6 protein fraction. A constant amount of CP3.6 proteins was incubated with increasing amounts of 23S + 5S rRNA at 36 °C in the first step of the two-step reconstitution procedure; TP50 was added to the second step. After reconstitution the rRNA amount in each assay was adjusted to the highest amount present in the experiment, native 30S subunits were added, and the poly(Phe) synthesis was assessed.

However, at lower temperature (36 °C; experiment 1 in Table I) the extent of formation of the $RI^{*}_{50}(1)$ particles is no longer dependent on the presence of L24; i.e., the assembly of active 50S subunits can be initiated in the absence of L24 by the small number of proteins in the CP3.6 fraction. Accordingly, the number of initiation proteins effective at 36 °C within the CP3.6 fraction was tested, following the method described earlier [see the preceding paper (Nowotny & Nierhaus, 1988) and, for detailed discussion, Nowotny and Nierhaus (1982)].

For this purpose, a constant amount of CP3.6 is added to an increasing excess of rRNA during the first step, TP50 is added to the second step, and the activity is assessed in the poly(Phe) synthesis system. Corrections have to be made for the increasing rRNA content; i.e., the RNA content in all the samples is raised to that of the highest amount of rRNA used during reconstitution just before measuring the poly(Phe) synthesis. In this way, a double logarithmic plot of activity versus input excess of rRNA should give a straight line. The slope of this line is $1 - n$, where n is the number of initiator

Table II: Identification of the Initiator Proteins in the Absence of L24 at Low Temperatures (36 °C)[a]

	first step				second step	
	1.1 (20 min/36 °C)		1.2 (20 min/36 °C)			
expt	rRNA	proteins	rRNA	proteins	TP50	% activity of poly([^{14}C]Phe)
1	1×	∑A + ∑B	5×	–	+	100 (2.748)
		∑A		∑B		0
		∑B		∑A		0
				∑A + ∑B		0
2	1×	∑A + L13 + L20 + L21 + L22	7×	–	+	100 (2.620)
		∑A + L20 + L21 + L22		L13		99
		∑A + L13 + L21 + L22		L20		0
		∑A + L13 + L20 + L22		L21		77
		∑A + L13 + L20 + L21		L22		96
				∑A + ∑B		0
3	1×	∑B + L3 + L4 + L23 + L29	7×	–	+	100 (2.218)
		∑B + L4 + L23 + L29		L3		30
		∑B + L3 + L23 + L29		L4		64
		∑B + L3 + L4 + L29		L23		62
		∑B + L3 + L4 + L23		L29		116
				∑A + ∑B		0
4	1×	∑C + L3 + L20	8×	–	+	100 (1.551)
		∑C		L3 + L20		0
		L3 + L20		∑C		21
		L3 + L20 + ∑C[b]		–		52
		∑C + (L3 + L20)[b]		–		3

[a] ∑A contains L3, L4, L23, and L29. ∑B contains L13, L20, L21, and L22. ∑C = ∑A + ∑B − L3 − L20 − L29. In the 1.1 step, the molar ratio of proteins to rRNA was 2:1; 1×, standard amount of 23S+5S rRNA (2.5 A_{260} units in 100 μL). In the 1.2 step, 5×rRNA means, for example, that the amount of total rRNA was increased 5-fold. [b] For 5 min at 0 °C, before establishing the rRNA excess.

proteins. Figure 2 demonstrates that the slope is −1.2, showing that two initiator proteins (n = 2.2) are present in the CP3.6 fraction.

Next, a protein mixture equivalent to the CP3.6 proteins was composed from individual purified proteins, including those present in trace amounts in CP3.6. Several subfractions were prepared: subfraction A consisted of L3, L4, L9, and L23, subfraction B consisted of L13, L20, L21, and L22, and further subfractions contained the proteins L2, L15, L17, L18, L19, and L30. A series of experiments revealed that the proteins outside the subfractions A plus B [∑(A+B)] are not required for $RI^*_{50}(1)$ formation at 36 °C (data not shown). Table I, experiment 2, demonstrates that in fact the combined ∑(A+B) fraction behaves qualitatively like the CP3.6 fraction; viz., L24 is absolutely required at 44 °C in striking contrast to the 36 °C requirement. Here the 44 °C condition (in the presence of L24) yields the same activity as the 36 °C conditions (in the absence of L24, 100 and 108%, respectively), whereas the corresponding data in experiment 1 are different. A probable explanation is that the L24 preparation used in experiment 2 is of lower activity than the one used in experiment 1. We conclude that the two initiator proteins effective at 36 °C are present in the ∑(A+B) fraction.

Identification of the Initiator Proteins under the Low-Temperature Condition as L20 and L3. For these experiments the experimental strategy for the identification of the initiator proteins within the ∑(A+B) fraction is modified. The first step of the two-step reconstitution procedure is split in two incubations (1.1 and 1.2), each of 20 min at 36 °C. In the 1.1 incubation selected proteins are incubated with a standard amount (1×) of rRNA. For the 1.2 incubation the rRNA amount is increased [e.g., in Table II, experiment 1, it is 5-fold (5×)], and a second set of proteins is added. The rationale is that only if both initiator proteins are present in the first set of proteins (added to the 1.1 incubation) will a large amount of complete initiation complexes be formed, consisting of rRNA and both initiator proteins. These initiation complexes will hardly be affected by the rRNA excess added in the 1.2 incubation but will rather form $RI^*_{50}(1)$ intermediates with the help of the second set of proteins. These intermediates result in the formation of active 50S subunits during the second step where TP50 is added. However, if one or both initiator proteins are added only with the second set of proteins (during the 1.2 incubation), the two initiator proteins will be independently distributed among the excess rRNA molecules, yielding very low amounts of complete initiation complexes and thus very low amounts of $RI^*_{50}(1)$ particles and active 50S subunits.

Table II, experiment 1, demonstrates that significant activity is only found if both protein groups ∑A and ∑B are present during the 1.1 incubation, whereas ∑A or ∑B alone shows no activity. The conclusion is that each group contains one initiator protein.

Group ∑B, consisting of L13, L20, L21, and L22, is analyzed in the next experiment. ∑A proteins and three out of the four ∑B proteins are added to the 1.1 incubation, and the omitted protein is added to the 1.2 incubation. As shown in Table II, experiment 2, the omission of L20 leads to a complete loss of activity and that of L21 induces a weak effect (23% loss of activity), whereas the omission of L13 and L22 has no effect at all. It is clear that L20 is the initiator protein present in group ∑B.

A corresponding analysis of group ∑A (Table II, experiment 3) reveals that the omission of L3 causes the most severe effect (70% reduction of activity), whereas the omission of L4 and L23 shows an intermediate effect (36 and 38% reduction, respectively) and that of L29 no effect. It is clear that, next to L20, protein L3 has the strongest effect on the initiation of assembly.

In the last experiment we test whether L20 and L3 are indeed capable of forming competent initiation complexes. Accordingly, we put together a new protein group, ∑C, which comprises all the proteins of both ∑A and ∑B, with the exception of L20, L3, and L29. As expected, ∑C plus L3 and L20 show good activity when present in the 1.1 incubation (Table II, experiment 4), and the ∑C proteins are inactive, but surprisingly L3 and L20 alone yield only low activity (21%). One possible explanation is that the initiation complex

with L3 and L20 is not stable enough to resist an 8 molar excess of rRNA. To test this, L3 and L20 were incubated according to the 1.1 step, and then the ∑C proteins were added in the cold (5 min at 0 °C), before the rRNA excess (8×) was established for the 1.2 step. Now significant activities were found (52%), whereas the reversed sequence of additions (∑C proteins in the 1.1 incubation, with L3 and L20 being added in the cold before establishing the rRNA excess) was ineffective. Since L4 and L23 show a low but significant effect in the single-omission experiment (Table II, experiment 3), these proteins probably play a major role in stabilization of the nonstandard initiation complex consisting of 23S rRNA and L3 and L20. We conclude that L3 and L20 are the initiator proteins at 36 °C in the absence of L24.

Discussion

The initiation of the assembly of the 50S subunit strictly depends on two initiator proteins. When only L3 is effective (in the absence of L24 at 44 °C), the output of active particles is reduced to only 10%. Decreasing the temperature of the first step in the absence of L24 improves the output, and this effect is paralleled by the appearance of a second initiator protein (Herold et al., 1986) Here we demonstrate that the new assembly-initiator protein is L20, which replaces L24, since L3 also functions as initiator protein at lower temperatures. The need for two initiator proteins represents an optimum, since the number must on the one hand be as small as possible to guarantee a significant output of active particles in the presence of an rRNA excess (unfavorable growth conditions) whereas on the other hand the number must be larger than one to enable translational control to operate under the same conditions [for further discussion, see Nowotny and Nierhaus (1982)].

It is likely that the requirement for two initiator proteins reflects a bilobal structure of the assembly process, as is evident for the 30S assembly (see preceding paper). This view is supported by a number of observations: (1) L3 binds near the 3′-region of 23S rRNA, whereas both L24 and L20 bind to the 5′-end (Zimmermann, 1980). (2) L3 is not essential (but stimulatory) for the formation of the $RI^*_{50}(1)$ particle (Spillmann et al., 1977). (3) L3 is not found on the first precursor particle of the in vivo 50S assembly (Nierhaus et al., 1973). A possibly decisive comparison with the quaternary structure of the 50S subunit cannot be made at present, since our knowledge of the spatial distribution of the ribosomal proteins is too sparse.

The effect of L20 exceeds that of L3 (Table II, experiments 2 and 3), the previously established initiator protein. The assembly of the 5′-assembly domain (L24, L20) obviously dominates that of the 3′-assembly domain (L3) in the course of the 50S assembly.

L20 is well suited to replace L24 since, as mentioned above, it also binds to the 5′-end of 23S rRNA. Furthermore, like L24 it is a mere assembly protein, which is essential for the early assembly but plays no role either in the late assembly or in the ribosomal functions (Nowotny & Nierhaus, 1980). However, L20 cannot fully compensate the lack of L24 as is strikingly indicated by the severe growth defects observed with the temperature-sensitive mutant lacking L24. In the absence of L24 the maximal output of active 50S subunits is only 50% of that observed in the presence of L24, and even at permissive temperatures L24 decreases the activation energy for $RI^*_{50}(1)$ formation from about 80 to 40 kcal/mol (Herold et al., 1986). Furthermore, L3 and L20 cannot form stable initiation complexes (Table II, experiment 4), in contrast to L3 and L24 (Nowotny & Nierhaus, 1982). Possibly, L20 is part of an "emergency" system which maintains 50S assembly, and therefore guarantees survival of the cell, if the optimal assembly initiation with L24 fails.

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