DNA-DEPENDENT IN VITRO SYNTHESIS OF ESCHERICHIA COLI RIBOSOMAL PROTEIN L10 AND THE FORMATION OF AN L10L12 COMPLEX

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SUMMARY

The in vitro synthesis of ribosomal protein L10 has been demonstrated using λrif^{d}18 DNA as template. The L10 synthesized in vitro forms a complex with ribosomal protein L12 and the L10 in this complex can be immunoprecipitated with L12 antiserum.

It is now well established that the DNA from the transducing phage λrif^{d}18 (1) contains the genetic information for Escherichia coli ribosomal proteins L1, L10, L11 and L12[1] (2-4). Although it has recently been shown that this DNA directs the in vitro synthesis of ribosomal proteins L1, L11 and L12, the evidence for the synthesis of ribosomal protein L10 is not as convincing (4). A previous report from this laboratory (5) showed that when λrif^{d}18 DNA was incubated in an in vitro protein synthesizing system containing radioactive amino acids, two radiolabeled proteins of molecular weights about 12,000 and 16,000 could be immunoprecipitated by antibody raised against purified ribosomal protein L12. The smaller protein was identified as L12, and it was suggested that the other immunoprecipitable protein might be a precursor form of L12. Another possibility was that the 16,000 dalton protein was ribosomal protein L10, especially since it has recently been shown (6) that L10 can form a very strong complex with L12. The present studies show that this, in fact, is the case. Not only does λrif^{d}18 DNA direct the in vitro synthesis of ribosomal

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[1]Since no attempt will be made to distinguish between ribosomal protein L12 and its acetylated form L7, the term L12 will refer to either or both of these species.

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protein L10, but the newly synthesized L10 forms a tight complex with L12, which can be quantitatively immunoprecipitated by L12 antibody. Additional data are presented which show that when purified ribosomal proteins L10 and L12 are incubated together, a complex is formed which can be immunoprecipitated with either L10 or L12 antiserum.

MATERIALS AND METHODS

Uniformly ^{14}C-labeled L-amino acid mixture (54 Ci/atom of carbon) was purchased from Amersham/Searle and concentrated 25-fold by lyophilization. Guanosine-5'diphosphate-3'diphosphate (ppGpp) was synthesized and provided by Dr. Alan Cook of Hofmmann-La Roche. Ribosomal protein L12 was isolated and purified from NH_4Cl-washed 70S ribosomes of *E. coli* B cells as described previously (7-9). Purified L10 was a kind gift of Dr. H.G. Wittmann. [^{14}C]L12, [^{3}H]L12 and [^{3}H]L10 were prepared by reductive methylation of the purified proteins (10) using either ^{14}C or ^{3}H-labeled formaldehyde (New England Nuclear Corp.). *E. coli* H105 was obtained from Dr. J.B. Kirschbaum and used as the source of $\lambda rif^{d}18$ DNA (1). Ribosomal wash, washed ribosomes and the 0.25 M and 1.0 M salt eluates from a DEAE-cellulose fractionation of an S-100 extract were prepared as described elsewhere (11,12). Antibodies to purified L10 and L12 were raised in rabbits with the aid of Freund's complete adjuvant.

Protein synthesis. The complete system for protein synthesis using $\lambda rif^{d}18$ DNA as template and ^{14}C-labeled amino acids was as described previously (5) with several minor modifications (13). After incubation, the synthesis was stopped by the addition of 0.7 µg pancreatic ribonuclease and the reaction mixture was chilled and centrifuged for 10 min at 7000 x g. Five µl of the supernatant were precipitated with Cl_3CCOOH (20 min at 90°) and the radioactivity in the precipitate was determined. Protein concentrations were determined by the method of Lowry *et al.* (14).

Radioimmunoprecipitation reaction. Five µg of [^{3}H]L10 (700 cpm/µg) and 150 µl of L10 antiserum were added to 30 µl of the ribonuclease-treated clarified reaction mixture. The immunoprecipitation was performed in a buffer containing 0.05 M Tris-HCl, pH 7.4, 0.5 M NaCl and 1% triton X-100 (Buffer A) (15). After 90 minutes at 37° the incubation mixture was chilled at 4° for 15 min and centrifuged. Five µg of [^{3}H]L10 was again added to the supernatant and the remaining L10 was precipitated with 150 µl of L10 antiserum. This procedure was found necessary to completely precipitate the *in vitro* synthesized [^{14}C]L10. The immunoprecipitates were washed four times in 0.5 ml of Buffer A dissolved (2 min at 90°) in 30 µl of 4 M urea containing 50% acetic acid and combined. A 10 µl aliquot was assayed for radioactivity in a Beckman liquid scintillation counter. The remainder of the solubilized immunoprecipitate was lyophilized and analyzed by gel electrophoresis as described below. The same procedure was used for the immunoprecipitation of L12 except that 5 µg [^{3}H]L12 (1000 cpm/µg) and 50 µl of L12 antiserum were used and only a single immunoprecipitation was necessary.

Polyacrylamide gel electrophoresis. For electrophoresis in the presence of sodium dodecylsulfate (SDS) the lyophilized immunoprecipitate was dissolved in 30 µl of 1% SDS (2 min at 90°) and subjected to disc gel electrophoresis using 15% polyacrylamide gels and 0.1% SDS (5,16).

For disc gel electrophoresis at pH 4.5 (17), the urea-containing lyophilized immunoprecipitate was solubilized in 30 µl of H_2O and electrophoresed in 7.8% polyacrylamide gels containing 6 M urea.

The gels were sliced into 1 mm (SDS gels) or 2 mm (urea gels) sections (Gilson Aliquogel Fractionator), extracted with 0.7 ml of 0.1% SDS for 1 hr at 80°, and then assayed for radioactivity in 5 ml of Instabray (Yorktown Research, N.J.).

A two-dimensional polyacrylamide slab gel electrophoresis (18) capable of detecting microgram quantities of ribosomal proteins (19) was used for the further identification of the synthesized products. The system was modified so that only the acidic proteins were electrophoresed in the first dimension (5 mA/gel for 4 hr) and the slab gel for the second dimension (30 V for 14 hr) contained 12% polyacrylamide. After electrophoresis, the slab gels were stained with Coomassie Brilliant Blue (R-250, 0.1% in 7.5% acetic acid containing 50% methanol) for 20 min at 50° and then destained at 50° with a solution of 7.5% acetic acid containing 5% methanol. The stained spots were punched out, digested in 0.5 ml of 30% H_2O_2 at 55° for 6 hr and assayed for radioactivity in 10 ml aquasol (New England Nuclear). The recovery of the tritiated standards was between 15-20%.

Purification of synthesized products. The synthesized products from a ten-fold scaled up reaction mixture (5,13) were precipitated with either L10 or L12 antiserum and the solubilized immunoprecipitates were electrophoresed in SDS as described above. The gels were sliced into 1 mm sections, extracted with 0.5 ml of 0.1% SDS and 50 µl aliquots were assayed for radioactivity to locate the radioactive peaks. The fractions under each of the peaks were pooled. The gel particles were allowed to settle and the supernatant was carefully aspirated. Bovine serum albumin (0.5 mg/ml) was added to the extracted ^{14}C-labeled proteins and the SDS was removed by precipitating the protein with two volumes of ice cold acetone. The precipitated proteins were washed once with acetone and lyophilized. The lyophilized powder was dissolved in 8 M urea containing 6 mM β-mercaptoethanol and subjected to both disc gel (pH 4.5) and two-dimensional polyacrylamide gel electrophoresis.

Assay for complex formation between purified ribosomal proteins L10 and L12. The incubation mixture for complex formation (250 µl) contained 0.01 M Tris·HCl pH 7.4, 0.01 M $MgCl_2$, 0.01 M NH_4Cl and various concentrations of [^3H]L10 and [^{14}C]L12. After 5 min at 37°, 125 µl of a solution containing 0.2 M Tris-HCl pH 7.4, 2 M NaCl and 4% Triton X-100 were added, followed by the addition of either L10 (120 µl) or L12 (60 µl) antiserum. The total reaction mixture was diluted to 0.5 ml and then incubated at 37° for 60 min. The reaction was stopped by diluting to 2 ml with a cold solution containing 0.05 M Tris-HCl pH 7.4, 0.5 M NaCl and 1% Triton X-100. The mixture was filtered through a nitrocellulose filter (Millipore 0.45 µM) which had been previously washed with the above detergent solution. The incubation tube was then rinsed with four 2 ml aliquots of the detergent solution and the rinses filtered as above. The filter was then placed into a vial containing 0.5 ml H_2O, 10 ml of Instabrays and assayed for radioactivity in a Beckman liquid scintillation spectrometer. It is important to emphasize that the filter must be washed with the detergent solution prior to use, since the unwashed filter will bind the unprecipitated ribosomal proteins. In the absence of antibody essentially no radioactivity is bound to the filter. This method avoids the lengthy and tedious washings of the immunoprecipitate and has the advantage that many samples can be assayed very rapidly. If desired, the immunoprecipitate can be recovered from the filter by rinsing the filter with an SDS solution. The recoveries range from 30 to 50%.

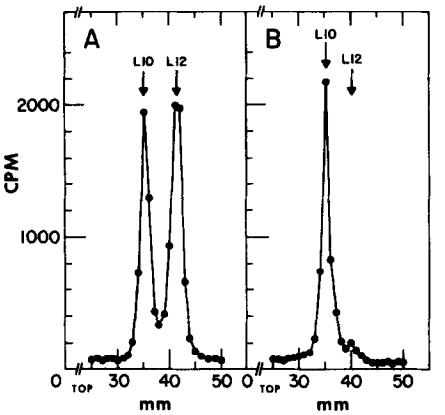

Fig. 1: SDS gel electrophoresis of *in vitro* synthesized products. Duplicate aliquots of an *in vitro* synthesis were immunoprecipitated with

(A) L12 antiserum or (B) L10 antiserum. Details of the immunoprecipitation and electrophoresis are described in the text. The gels were sliced and assayed for radioactivity. The arrows show the positions of marker proteins L10 and L12.

RESULTS

Identification of the 16,000 dalton protein. We have previously described the *in vitro* synthesis of ribosomal protein L12 using λrif^{d}18 DNA as template (5). It was observed at that time that antiserum against ribosomal protein L12 also immunoprecipitated another protein of about 16,000 daltons from the *in vitro* incubation. Since, as noted above, this protein might be ribosomal protein L10, an attempt was made to characterize it. The *in vitro* synthesized products were immunoprecipitated with antiserum against either ribosomal protein L12 (Fig. 1A) or L10 (Fig. 1B) and electrophoresed. Two peaks of radioactivity are observed in each gel. One peak comigrates with ribosomal protein L12 and the other with ribosomal protein L10. It can be seen that while the total amount of the 16,000 dalton protein (comigrating with L10) precipitated with either antiserum was comparable, the quantity of L12 precipitated with the L10 antiserum was much lower than with the L12 antiserum (Figs. 1A and 1B). The amount of L12 precipitated by the L10 antiserum varied between 5-30% of

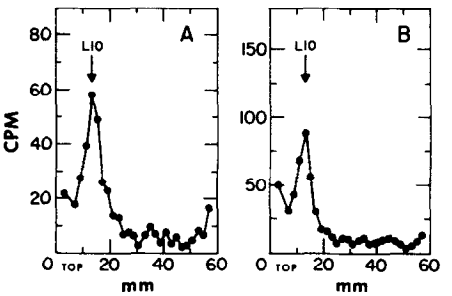

Fig. 2: Gel electrophoresis at pH 4.5 of *in vitro* synthesized product: (A) L10 immunoprecipitate, and (B) purified [^{14}C]L10 after L12 immunoprecipitation and SDS gel electrophoresis. Details are described in the text. The marker protein is ribosomal protein L10.

the total immunoprecipitable radioactivity. It is to be noted that while the L10 antiserum precipitated about 80% of the carrier ribosomal L10, it only reacted with about 50% of the *in vitro* synthesized product. It was found that a second immunoprecipitation was required to completely immunoprecipitate all of the *in vitro* synthesized L10 (see Methods). There was no detectable cross reaction of either antiserum with the heterologous purified protein.

The 16,000 dalton protein was purified by SDS gel electrophoresis (see Methods) and then further identified as ribosomal protein L10 by its electrophoretic mobility at pH 4.5 (Figs. 2A and 2B) and by its ability to comigrate with authentic L10 in a two-dimensional gel electrophoresis system (data not shown). It was found that all of the 16,000 dalton protein immunoprecipitated by L12 antiserum and isolated from the SDS gel could be accounted for by the radioactivity recovered in the L10 area after two-dimensional gel electrophoresis.

Quantitative immunoprecipitation of L10 by L12 antiserum. The data in Figure 1 suggest that the *in vitro* synthesized L10 forms a complex with L12 since it can be immunoprecipitated by L12 antiserum. Additional evidence that the immunoprecipitation of L10 by L12 antiserum was quantitative was observed in experiments in which the supernatant from the L12 immunoprecipitate

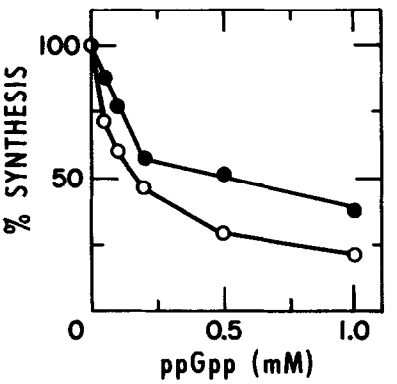

Fig. 3: Effect of ppGpp on the in vitro synthesis of ribosomal proteins L10 and L12. The conditions for the synthesis and immunoprecipitation of the products by L12 antiserum are described in the text. The [^{14}C]labeled L10 and L12 in the immunoprecipitates were resolved by SDS gel electrophoresis and their respective peak areas were quantitated. 100% values for L10 (-●-) and L12 (-o-) represent about 7% and 10%, respectively, of the total radioactivity incorporated into protein.

was then further incubated with L10 antiserum and carrier L10. Very little [^{14}C]L10 was found in the immunoprecipitate (data not shown). These results suggest that all of the in vitro synthesized L10 was bound to L12 and was precipitated by the L12 antiserum.

Effect of ppGpp on L10 and L12 synthesis. Previous work (5) showed that L12 synthesis and the synthesis of the 16,000 dalton protein were decreased when ppGpp was added to the in vitro incubation. The results in Figure 3 corroborate and extend these findings and show that the synthesis of L12 appears to be somewhat more sensitive to inhibition by ppGpp than that of L10. These in vitro results agree with the in vivo findings of Dennis and Nomura (20,21) who showed differences in the relative rates of synthesis of the ribosomal proteins during amino acid starvation of a stringent strain of E. coli.

Formation of an L10L12 complex. In an attempt to gain further information about the formation of an L10L12 complex, ribosomal proteins L10 and L12 were

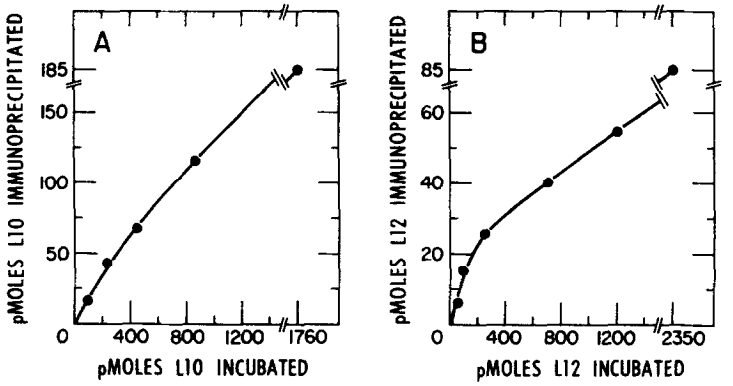

Fig. 4: Formation of an L10L12 complex.

(A) Various concentrations of [^{3}H]L10 were incubated with 470 pmoles of [^{14}C]L12 and immunoprecipitated with L12 antiserum.

(B) Various concentrations of [^{14}C]L12 were incubated with 220 pmoles of [^{3}H]L10 and immunoprecipitated with L10 antiserum. Details of the incubation and immunoprecipitations are given in the text. The efficiencies of the immunoprecipitations were between 65-80% and all values have been normalized to 100% recovery.

purified (7-9) and labeled by reductive methylation (10). The [^{3}H]L10 and [^{14}C]L12 were incubated together and then treated with either L10 or L12 antiserum. The assay used for complex formation was based on the selective retention of the immunoprecipitate by a nitrocellulose filter.

Figure 4A shows that when increasing concentrations of [^{3}H]L10 are incubated with [^{14}C]L12 and the reaction mixture immunoprecipitated with L12 antiserum, increasing amounts of L10 are precipitated with the L12 antiserum. Similarly, when increasing concentrations of [^{14}C]L12 are incubated with [^{3}H]L10, increasing amounts of L12 are immunoprecipitated with the L10 antiserum (Fig. 4B). It is to be noted that with either antiserum only about 15-20% of the incubated heterologous protein could be found in the immunoprecipitated complex. This is in contrast to the quantitative precipitation of the *in vitro* synthesized L10 by L12 antiserum (Fig. 1). In addition, the same amount of [^{14}C]L12 or [^{3}H]L10 was immunoprecipitated by the homologous antiserum in the

presence or absence of the other protein, suggesting that the antibodies recognized the free and complexed proteins equally well.

DISCUSSION

The present study presents data which show that ribosomal protein L10 is synthesized in an in vitro protein synthesizing system using λrif^{d}18 DNA as template. The protein has been identified on the basis of its mobility in a number of electrophoretic systems and by its ability to react with L10 antiserum. The in vitro synthesized L10 formed a complex with ribosomal protein L12 and could be quantitatively precipitated, presumably as an L10L12 complex, by L12 antiserum. On the other hand, although the newly synthesized L10 was immunoprecipitated by L10 antiserum, two successive immunoprecipitations were required to quantitatively remove the in vitro synthesized material. In contrast about 80-90% of the ribosomal carrier L10 was recovered in a single immunoprecipitation. These results suggest that the in vitro synthesized L10 might be slightly different from the carrier protein (which was used as antigen) and that the in vitro product does not recognize the antibody with the same affinity as the carrier L10. In addition, it appears that the in vitro synthesized L12 binds poorly to the carrier L10 since relatively little of the L10L12 complex was immunoprecipitated by L10 antiserum. It is possible that the L10 isolated from the ribosome has either been post-translationally modified or has been slightly altered during its purification so that it reacts differently than the newly synthesized L10 with L10 antibody and L12. It has recently been reported (22) that an L10L12 complex was isolated which consists of four molecules of L12 and one molecule of L10. This ratio would appear to be of physiological significance since it is believed that the same stoichiometry exists on the ribosome. Since details of the formation of the complex were not reported, it is difficult to compare those results with the data in the present study. However, although the stoichiometry of L10L12 complex was not analyzed in the experiments shown in Fig. 4A,

it can be seen that 165 pmoles of L10 were immunoprecipitated in the presence of 470 pmoles of L12, yielding a maximum L12/L10 ratio of 2.8. The possible significance of the different L12/L10 ratios is not understood.

The *in vitro* formation of this complex may be characteristic of its function on the ribosome. Thus, it has been shown that the presence of L10 on the 50S ribosomal subunit is required for the subsequent binding of L12 (23,24). In addition, experiments using a bifunctional crosslinking reagent have demonstrated that L10 and L12 are in close proximity (within 6.3 A°) to each other on the 50S ribosomal subunit (25). These results suggest that these two proteins may interact directly on the ribosome.

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