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Biochemistry

DNA dependent synthesis of protein L12 from *Escherichia coli* ribosomes, *in vitro*

(guanosine-5′-diphosphate-3′-diphosphate/λrif^{d}18 DNA)

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ABSTRACT **The *in vitro* synthesis of ribosomal protein L12 has been obtained in a coupled system with DNA extracted from the transducing phage λrif^{d}18 as template. In addition, a second protein (molecular weight of 16,000) with immunological, chemical, and ribosome-binding characteristics similar to L12 is formed in this *in vitro* system. The synthesis of both proteins is depressed by one-half when guanosine-5′-diphosphate-3′-diphosphate is added to the reaction mixture.**

Recent studies have demonstrated that *Escherichia coli* ribosomes contain multiple copies of ribosomal protein L12*, whereas all other proteins are present in stoichiometric amounts (1–5). In addition, it has been suggested from studies using stringent and relaxed organisms that ribosomal protein and ribosomal RNA synthesis appear to be regulated coordinately (6, 7). A previous report (8) has described the DNA-dependent synthesis of L12 *in vitro* with DNA isolated from the transducing phage, λrif^{d}18 (9). This phage has been found to contain the structural genes for ribosomal RNA, $tRNA_2^{Glu}$, RNA polymerase subunits β and β′, elongation factor Tu, and several ribosomal proteins, which include L12 (10–12).

The present investigation also describes the synthesis of L12, with λrif^{d}18 DNA as a template, in an *in vitro* system similar to that for β-galactosidase synthesis (13). L12 synthesis is inhibited by guanosine-5′-diphosphate-3′-diphosphate (ppGpp) which indicates that this *in vitro* system can be used to study the stringent response to this metabolite. In addition, another protein with L12-like properties has been identified.

MATERIALS AND METHODS

Uniformly ^{14}C-labeled L-amino acid mixture (54 Ci/atom of carbon) was purchased from Amersham/Searle; L-[^{3}H]tryptophan (5.2 Ci/mM) and L-[^{3}H]histidine (9.6 Ci/mM) were obtained from New England Nuclear Corp. Cesium chloride (99.9% pure) was purchased from Fisher Scientific Co. ppGpp was kindly provided by Alan Cook of Hoffmann-La Roche. Tritiated ribosomal protein L12 was prepared as previously described (14).

Preparation of Phage DNA. *E. coli* H105, (λCI857S7, λCI857S7drif^{d}18), obtained from J. B. Kirschbaum, was used as the source of λrif^{d}18 DNA. The phage were induced by heat treatment of a growing culture and then purified by CsCl equilibrium centrifugation. The phage DNA was then extracted by the method of Thomas and Abelson (15). The λdlac DNA (λh80dlacp^{s}CI857t68) was prepared from *E. coli* RV cells by the same method (16).

Preparation and Fractionation of the S-30 Extract. An S-30 extract was prepared from *E. coli* Z19i^q (gal-Sm^R), kindly provided by G. Zubay, Columbia University, by the method of Zubay *et al.* (17). The ribosomal wash, washed ribosomes, and a supernatant fraction were prepared from the S-30 extract as previously described (13, 16). The supernatant fraction was further fractionated by chromatography into 0.25 M and 1.0 M DEAE salt eluates from DEAE-cellulose columns as described elsewhere (13).

Protein Synthesis. The complete system (70 μl) for L12 synthesis contained 3 μmol of Tris·acetate at pH 8.2, 1.0 μmol of magnesium acetate, 1.9 μmol of ammonium acetate, 3.8 μmol of potassium acetate, 0.1 μmol of dithiothreitol, 0.065 μmol each of CTP, GTP, and UTP, 0.26 μmol of ATP, 1.4 μmol of phospho*enol*pyruvate, 0.5 μg of pyruvate kinase, 0.35 μmol of adenosine 3′:5′-cyclic monophosphate, 13 μg of *E. coli* B tRNA, 3.0 μg of Ca leucovorin, 1.3 mg of polyethylene glycol-6000, 13 μg of λrif^{d}18 DNA, 1.2 A_{260} units of NH_4Cl-washed ribosomes, 100 μg of ribosomal wash, 220 μg of 0.25 M eluate and 15 μg of 1 M eluate from DEAE-cellulose columns, 1.25 μCi of uniformly ^{14}C-labeled L-amino acid mixture, and 0.016 μmol each of asparagine, glutamine, cysteine, methionine, tryptophan, and histidine. The reaction mixture was incubated at 37° for 90 min. At the end of the incubation, the reaction mixture was rapidly chilled and the ribosomes were removed by sedimentation at 100,000 × *g* for 1 hr. An aliquot of the supernatant was removed, total protein synthesis determined by hot CCl_3COOH precipitation, and the remainder of the supernatant subjected to immunoprecipitation with antiserum against L12 (see below).

The β-galactosidase synthesizing system and assay have been described elsewhere (13).

Assay for L12 by Immunoprecipitation. The complete reaction (1 ml) contained 0.05 M Tris·HCl at pH 7.4, 0.5 M NaCl, 1% (vol/vol) Triton X-100, 100 μl of antiserum to L12 (18), (which also crossreacts with L7), 10 μg of [^{3}H]L12 (300–500 cpm/μg) and various amounts of the ribosome-free reaction mixture. The ^{3}H-labeled L12 served as an internal standard for calculating the recovery (50–70%) of the synthesized product after immunoprecipitation and the subsequent washing of the precipitate. All values have been normalized to 100% of L12 recovery. After incubation at 37° for 90 min, the immunoprecipitation mixture was further incubated at 4° for 15–30 min. The immunoprecipitate was washed three times in 1 ml of 0.05 M Tris·Cl at pH 7.4, 0.5 M NaCl, and 1% Triton X-100 (vol/vol) and dissolved in a solution of 4 M urea containing 50% acetic acid. An aliquot was assayed for radioactivity in a Beckman liquid scintillation spectrometer.

Polyacrylamide Gel Electrophoresis. Aliquots of the solu-

Abbreviations: ppGpp, guanosine-5′-diphosphate-3′-diphosphate; $NaDodSO_4$, sodium dodecyl sulfate.

* Because L7 is an acetylated form of L12, no attempt will be made to distinguish between the two forms in this study. The term L12 will refer to either or both of these species which migrates with authentic L7L12. [^{3}H]L12 also refers to a mixture of [^{3}H]L7 and [^{3}H]L12. L12A will be used to designate the heavier or altered form of the protein.

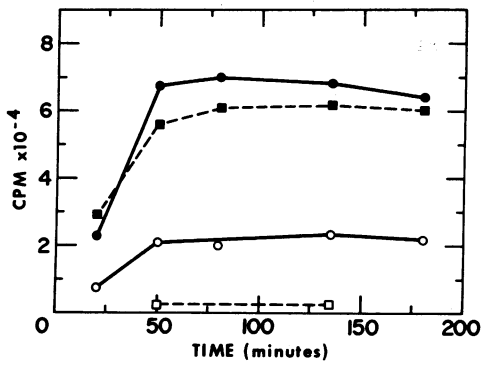

FIG. 1. Kinetics of synthesis of L12. Aliquots from the *in vitro* incubations containing either λrif^{d}18 DNA or λdlac DNA, as template, were removed at various times and the amount of radioactivity incorporated into total protein (●—● λrif^{d}18 DNA, ■ - - - ■ λdlac DNA), and into L12 immunoprecipitable material (○—○ λrif^{d}18 DNA, □ - - - □ λdlac DNA) was determined. Details are described in the *text*.

bilized immunoprecipitates were lyophilized and then dissolved in either 1% sodium dodecyl sulfate ($NaDodSO_4$) or 4 M urea. $NaDodSO_4$ electrophoresis was carried out by the procedure of Laemmli (19) with 15% polyacrylamide gels. The gels were then sliced into 1 or 2 mm sections (Gilson Aliquogel Fractionator) which were extracted with 0.7 ml of 0.1% $NaDodSO_4$ for 1 hr at 80°, and then assayed for radioactivity in 5 ml Instabray (Yorktown Research, N.J.). Albumin (67,000), ovalbumin (45,000), chymotrypsinogen (25,000), and cytochrome *c* (12,400) were used as migration standards for molecular weight determinations.

For electrophoresis at pH 4.5 (20), the urea-solubilized immunoprecipitate was subjected to electrophoresis in 7.8% polyacrylamide gels. The gels were cut as described above, solubilized in 0.5 ml of 30% H_2O_2 by heating at 55° for 15 hr, and assayed for radioactivity in 10 ml of Aquasol (New England Nuclear).

Binding of L12 to Ethanol-NH_4Cl Extracted 70S Ribosomes. ^{14}C-Labeled L12, synthesized *in vitro*, was assayed for binding to ethanol-NH_4Cl extracted (L12-depleted) 70S ribosomes as described previously (18), except that the binding was carried out at 37° for 5 min. The ribosomes were recovered by centrifugation at 200,000 × *g* for 1 hr. The supernatant was aspirated, and the amount of bound [^{14}C]L12 in the ribosome pellet per A_{260} unit was determined by immunoprecipitation.

Protein Determination. Protein concentration was determined by the method of Lowry *et al.* (21). Crystalline bovine serum albumin was used as standard.

RESULTS

Kinetics of synthesis of L12

Fig. 1 shows the time course for the incorporation of radioactivity into total protein and the L12 immunoprecipitate. Incorporation is linear for the first hour of incubation. It was calculated that about 30% of the incorporated radioactivity was immunoprecipitated by antiserum against ribosomal protein L12 which indicates that this is a major product of the *in vitro* incubations. The phage DNA specificity for L12 synthesis is shown by the results of experiments in which λdlac DNA is used. It is seen that the amount of immunoprecipitated radioactivity is sharply reduced (Fig. 1) and analysis of the immunoprecipitate obtained from the λdlac DNA incubation failed to show any protein peaks on $NaDodSO_4$ electrophoresis. No

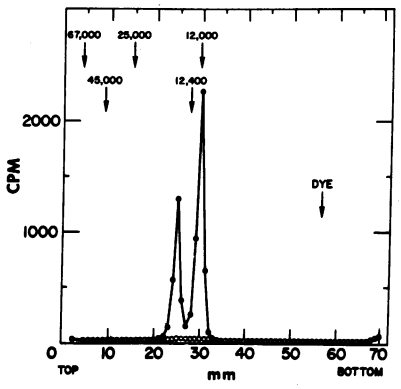

FIG. 2. $NaDodSO_4$ gel electrophoresis of *in vitro* synthesized L12. An aliquot of the *in vitro* synthesis mixture was immunoprecipitated, solubilized and analyzed by electrophoresis in $NaDodSO_4$. The gels were sliced and assayed for radioactivity. Details are described in the *text*. Marker proteins with molecular weight in parentheses are albumin (67,000), ovalbumin (45,000), chymotrypsinogen (25,000), cytochrome c (12,400), L12 (12,000). Proteins were either analyzed with ^{14}C-labeled amino acids (●) or with [^{3}H]tryptophan and [^{3}H] histidine (○).

radioactivity was immunoprecipitated in the absence of DNA (data not shown).

Characterization of the immunoprecipitate

Analysis of the solubilized immunoprecipitates by $NaDodSO_4$ gel electrophoresis revealed the presence of two radioactive peaks (Fig. 2). The smaller polypeptide comigrated with authentic L12 and has a molecular weight of about 12,000, whereas the larger polypeptide (L12A)* migrated with a molecular weight of about 16,000. Histidine and tryptophan, two amino acids which are absent in L12, were not incorporated into either of the two immunoprecipitable products which suggests a similarity in structure between the two proteins. Similarly, two peaks of activity were observed when the solubilized immunoprecipitates were electrophoresed at pH 4.5 (Fig. 3). The more acidic peak comigrated with authentic L12.

Kinetics of L12 and L12A formed during *in vitro* synthesis

The kinetics of synthesis of both L12 and L12A were studied to determine the relationship between them. Fig. 4 shows that about 38% of the immunoprecipitable material is in the form of L12 after 20 min of incubation but that this value increases to about 60% after 50 min and then remains constant up to 3 hr. The increase in L12 synthesized with time is observed consistently and is unaffected by the presence of ppGpp (data not shown). It is to be noted that this change in the ratio of L12

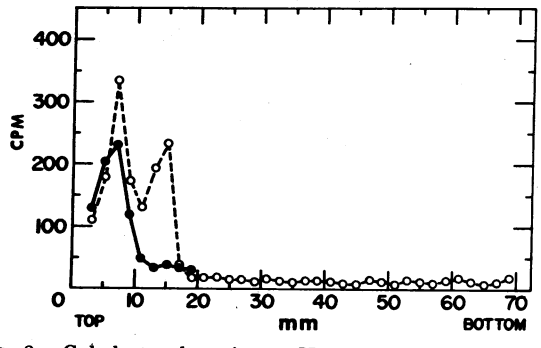

FIG. 3. Gel electrophoresis, at pH 4.5, of the *in vitro* product. Details are described in the *text*. ○---○, [^{14}C]L12 synthesized *in vitro*; ●—●, ribosomal [^{3}H]L12.

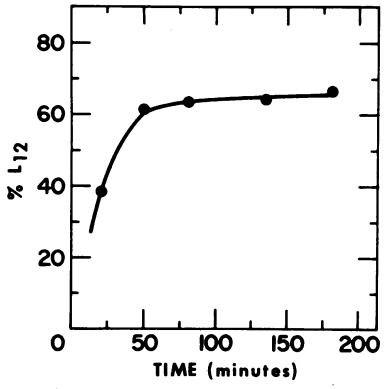

FIG. 4. Time course of the *in vitro* synthesis of L12 and L12A. Aliquots of the incubation mixture were removed at various times of incubation, immunoprecipitated, solubilized, and analyzed by electrophoresis in $NaDodSO_4$. The radioactivity in each of the peaks was determined (see Fig. 2) and the data are expressed as (cpm L12)/(cpm L12 + cpm L12A) × 100.

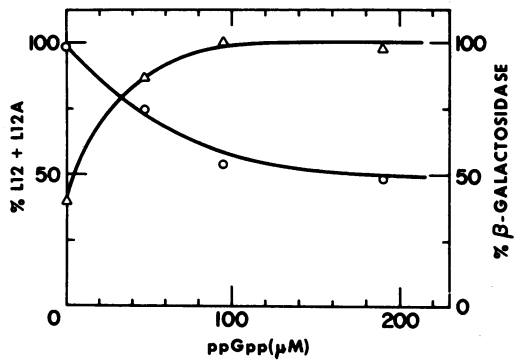

FIG. 5. Effect of ppGpp on the *in vitro* synthesis of L12 and β-galactosidase. The incubation conditions and assays are described in the *text*. Values (100%) for L12 represent about 25% of the total radioactivity incorporated into protein. Values (100%) for β-galactosidase represent an enzyme activity of 0.25 A_{420}/hr (13). O—O, L12 + L12A synthesized; Δ—Δ, β-galactosidase synthesized.

synthesized occurs while L12 is still being synthesized (Fig. 1); thus, it is not clear whether L12A is being converted to L12 during this period of time or whether more than one gene product is involved and there is a preferential synthesis of L12 during the latter part of the incubation.

Binding of *in vitro* synthesized L12 to depleted ribosomes

The ribosome-binding activity of the L12 synthesized *in vitro* was assayed by determining its ability to bind to L12-depleted ribosomes. Table 1 shows that when a reaction mixture containing *in vitro* synthesized L12 (and L12A) was incubated with L12 depleted ribosomes, radioactivity was associated with the ribosome. The specificity of the binding can be seen by the fact that the binding was greatly reduced in the presence of a large excess of L12 or when nondepleted ribosomes were employed. Other experiments have shown that the efficiency of binding of the *in vitro* synthesized L12 was comparable to that observed with authentic L12 (data not shown).

Because two species of an L12-like protein were synthesized, we investigated whether both forms were capable of binding to depleted ribosomes. We found that when incubation mixtures containing different L12/L12A protein ratios were incubated with L12-depleted ribosomes, both L12 and L12A proteins were found associated with the ribosomes. However, the L12 form appeared to bind somewhat better since there was an increase in the L12/L12A ratio found on the ribosome compared to the ratio in the incubation mixture (data not shown). It is also possible that both proteins bind equally well but that the ribosome is capable of converting one form to the other.

Effect of ppGpp on synthesis of L12

It was recently shown with *E. coli* total DNA as template, that ribosomal RNA synthesis *in vitro* was inhibited by ppGpp (22, 23). Because it is known that, *in vivo*, ribosomal RNA and ribosomal proteins are synthesized coordinately (24, 25), one would expect ppGpp to inhibit the *in vitro* synthesis of L12. Fig. 5 shows the effect of increasing concentrations of ppGpp on the synthesis of L12. Although this nucleotide (100 μM) did not significantly alter total protein synthesis (data not shown), it did depress the synthesis of L12 by about one-half. An analysis of the product of the incubation revealed that ppGpp had no effect on the relative amounts of the L12 and L12A forms synthesized (L12/L12A = approximately 1.7 with and without ppGpp). In contrast to the effect of ppGpp on L12 synthesis, Fig. 5 shows that ppGpp stimulates the *in vitro* synthesis of β-galactosidase when λdlac DNA was substituted for λrif^{d}18 DNA. Previous studies (26–28) have indicated that the stimulation of β-galactosidase synthesis is due to an effect on the transcription of the lactose operon.

DISCUSSION

The present report corroborates an earlier study (10) which showed that the synthesis of ribosomal protein L12 can be obtained in an *in vitro* system directed by λrif^{d}18 phage DNA. The results presented here indicate that two different species of L12-like proteins are formed during the *in vitro* synthesis. One of these proteins has a molecular weight and electrophoretic mobility which is very similar to L12, whereas the other is slightly larger and appears to be somewhat more basic. The L12 character of these proteins is further suggested by the fact that both proteins can be immunoprecipitated by L12 antibodies, and they appear to lack tryptophan and histidine, two amino acids which are absent from L12. In addition, the synthesis of both proteins is inhibited by the addition of ppGpp to the reaction mixture. It has also been shown that both species

Table 1. Binding of *in vitro* synthesized L12 to L12-depleted ribosomes

Ribosome	[^{14}C]L12 bound (cpm/A_{260})
Depleted	38
Depleted + L12	2
Control	5

[^{14}C]L12 was synthesized *in vitro* as described in the *text*, and at the end of the incubation the ribosomes were removed by centrifugation. An aliquot of the supernatant was removed and then further incubated for 5 min at 37° in the presence of 460 pmol of [^{3}H]L12 and an excess of either depleted or control ribosomes (540 pmol containing about 1600 L12 binding sites). Where indicated, 9 nmol of unlabeled L12 protein were added before the addition of the ribosomes. The binding assay was carried out as described in the *text* except that the L12 plus L12A were reisolated from the ribosomes by immunoprecipitation. It was found that about the same percentage of [^{3}H]L12 and [^{14}C]L12 was bound to the depleted ribosomes. Control ribosomes are untreated and were isolated as described in *Materials and Methods*. A blank of 10 cpm/A_{260} has been subtracted from each value. This represents nonspecific binding which was observed when an *in vitro* incubation containing ^{14}C-labeled proteins, synthesized with λdlac as template, was used for the binding reaction.

may be biologically active since they bind to L12-depleted ribosomes. The exact relationship between these two proteins is not clear at present but it is possible that L12 is first synthesized as a larger molecule which then undergoes processing. The increase in the amount of L12 synthesized with time of incubation (Fig. 4) reinforces this possibility.

A number of studies (6, 7) have shown that the *in vivo* synthesis of both ribosomal RNA and ribosomal proteins are depressed in a stringent, but not in a relaxed, organism which had been starved for an amino acid. Because it is known that ppGpp accumulates under stringent conditions, it was suggested that the synthesis of ribosomal RNA and ribosomal proteins are regulated in a similar fashion. The present results, which show that ppGpp inhibits the *in vitro* synthesis of ribosomal protein L12, provide direct evidence that this unique nucleotide also affects the synthesis of ribosomal proteins. It is of interest to note that ppGpp stimulates the synthesis of β-galactosidase directed by λdlac DNA (Fig. 5; refs. 26–28), under similar incubation conditions. It has recently been shown (29) that the synthesis of elongation factors Tu and G are also depressed in a stringent organism when starved for an amino acid. The genes for these proteins have been found to map very close to the genes for a large number of other ribosomal proteins. Thus, it now appears that ppGpp may affect the synthesis of a large number of other macromolecules.† The DNA-dependent *in vitro* synthesis of L7L12 provides a convenient system for studying the molecular mechanism by which the synthesis of these proteins is regulated by ppGpp.

One of us (F.C.) is a Fellow of the National Research Council of Canada.

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† The ability of ppGpp to inhibit the *in vitro* synthesis of a number of proteins directed by DNA extracted from λrif^{d}18 and λfus transducing phages has been observed by Nomura and coworkers (personal communication).