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Translational control of ribosomal protein L10 synthesis occurs prior to formation of first peptide bond

(autogenous regulation/*in vitro* protein synthesis)

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ABSTRACT **A simplified DNA-directed *in vitro* system has been developed to study the regulation of the synthesis of ribosomal protein L10 by measuring the formation of the first dipeptide, fMet-Ala. The results show that the inhibition of L10 synthesis by L10 (autoregulation) occurs at or prior to the formation of the first peptide bond.**

It is now known that the synthesis of *Escherichia coli* ribosomal proteins is under both transcriptional and translational control. At the level of transcription, ribosomal protein synthesis is under stringent control mediated by the unique nucleotide guanosine-3′-diphosphate-5′-diphosphate (ppGpp) (1). More recently, it has been shown that the synthesis of certain ribosomal proteins can be autoregulated at the level of translation. In this process, specific ribosomal proteins inhibit their own synthesis and, in some cases, the synthesis of other ribosomal proteins whose genes are on the same operon. Thus, ribosomal proteins S4 (2, 3), S7 (4), S8 (2), L1 (2, 3, 5), L4 (2, 6), and L10 (7–10) have been shown to be autoregulators, functioning at the level of translation.

Our laboratory has recently studied the effect of L10 on its own synthesis in DNA- and mRNA-directed *in vitro* systems (7). Although considerable progress has been made in using defined components in these *in vitro* systems (11, 12), their complexity to some extent limits their usefulness in studying the mechanism of autoregulation.

The present study describes a simplified *in vitro* system that can be used to study the regulation of gene expression at transcription or translation initiation by measuring the formation of NH_2-terminal small peptides characteristic of the gene product. Evidence is presented that the inhibition by L10 of its own synthesis occurs at, or prior to, the formation of the first peptide bond of L10.

MATERIALS AND METHODS

E. coli containing $\lambda rif^d 18$ phage was obtained from J. B. Kirschbaum (Harvard University, Cambridge, MA). *E. coli* JF943 containing either plasmid pNF1337 or 1341 were kindly supplied by J. Friesen (York University, Ontario, Canada). Ribosomal protein L12 was purified as described (13), and ribosomal protein L10 was a generous gift of J. Dijk (Max-Planck-Institut für Molekulare Genetik, Berlin).

Unfractionated *E. coli* tRNA and purified $tRNA_f^{Met}$ were purchased from Boehringer Mannheim, L-[^{3}H]alanine and L-[^{3}H]serine were obtained from New England Nuclear, and L-[^{35}S]methionine was obtained from Amersham/Searle. Purified Met-tRNA synthetase was obtained from C. J. Bruton (Imperial College of Science and Technology, London). A 0.25 M salt eluate from a DEAE-cellulose column was used as the source of Ala-tRNA and Ser-tRNA synthetase (14). N^{10}-Formyl-H_4-folate-Met-tRNA transformylase was purified by a described procedure (15). The acylation and transformylation reactions were carried out as described (15–18). At the end of the incubations, the reaction mixtures were extracted with phenol and the acylated tRNA species were precipitated with alcohol and dialyzed overnight against 2 mM K acetate (pH 5.5).

For Ala- and Ser-tRNA synthesis, unfractionated *E. coli* tRNA was used; purified $tRNA_f^{Met}$ was used to prepare fMet-tRNA. *N*-Formyl-L-methionine, *N*-formyl-L-methionyl-L-alanine, and L-methionine-L-serine were purchased from Sigma. *N*-Formylmethionylserine was synthesized as follows (19). To 71 mg of L-methionyl-L-serine dissolved in 1.26 ml of 98% formic acid was added dropwise 0.42 ml of acetic anhydride at 10°C. The mixture was stirred for 1 hr at room temperature, and 0.5 ml of ice-water was added. The mixture was brought to dryness under vacuum and the white product was recrystallized from isopropanol. The purity of the product *N*-formyl-L-methionyl-L-serine formate ester was checked by thin-layer chromatography and confirmed by NMR spectroscopy. Acid hydrolysis of the ester in 1 M HCl at room temperature yielded *N*-formyl-L-methionyl-L-serine. Precoated thin-layer chromatography plates (silica gel G, 250 μm) were obtained from Analtech (Newark, DE).

DNA-Directed Dipeptide Synthesis. The *in vitro* incubation mixture was a modification of a described *in vitro* system (11) that included only those components that would be required for dipeptide formation (Fig. 1). The complete system (35 μl) contained 30 mM Tris acetate (pH 7.5), 10 mM Na dimethylglutarate (pH 6.0), 36 mM NH_4 acetate, 2 mM dithiothreitol 9.2 mM Mg acetate, 2.9 mM ATP, 0.7 mM CTP, GTP, and UTP, 29 mM phospho*enol*pyruvate, 0.5 μg of pyruvate kinase, 39 mM K acetate, 0.8 mM spermidine, 2.5 mg of polyethylene glycol 6000, 0.3 μg of IF-1, 0.5 μg of IF-2, 0.6 μg of IF-3, 8.0 μg of EF-Tu, 2 μg of RNA polymerase, 2 μg of plasmid DNA, 12 pmol of NH_4Cl-washed 70S ribosomes, and 9 pmol of [^{35}S]fMet-$tRNA_f^{Met}$ (6500 cpm/pmol). The system was supplemented with 9 pmol of [^{3}H]Ala-tRNA (5500 cpm/pmol) or [^{3}H]ser-tRNA (1700 cpm/pmol) as indicated. The reaction mixture was incubated at 37°C for 60 min, and the reaction was stopped by adjusting the pH of the mixture to 9.5 by the addition of 2.5 μl of 1 M NaOH. The mixture was incubated for an additional 10 min at 37°C, and then 30 μg of fMet and either fMet-Ala or fMet-Ser were added in a total volume of 3 μl. Then, 4 μl of 2 M HCl was added to the mixture to bring the pH to 2.5, and the precipitate was discarded after centrifugation. An aliquot of the supernatant (usually 24 μl) was applied to a silica gel G thin-layer plate. The plate was developed with ethyl acetate/hexane/acetic acid, 8:4:1 (vol/vol), and the me-

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Abbreviations: IF, initiation factor; EF, elongation factor.

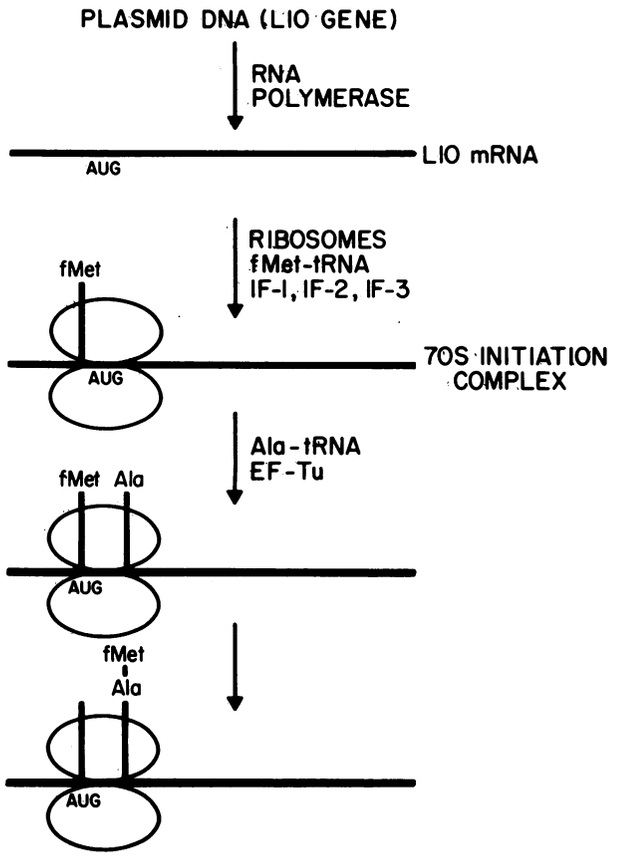

FIG. 1. Outline of steps leading to dipeptide formation in the *in vitro* system.

thionine-containing areas were visualized by exposing the plates to iodine vapor. The yellow spots were scraped off the plates into scintillation vials and the silica scrapings were extracted with 1 ml of water for about 5 min. Scintillation fluid was added and the radioactivity was assayed in a Beckman liquid scintillation spectrometer. In the solvent system used, fMet, fMet-Ala, and fMet-Ser yield R_F values of 0.46, 0.34, and 0.15, respectively. The free methionine, alanine, and serine remain at the origin.

RESULTS

In an attempt to pinpoint the step at which ribosomal protein L10 inhibits its own synthesis, we used a modified DNA-directed protein synthesis system that will permit only the synthesis of the first dipeptide of L10, fMet-Ala. As template in these reactions, we used DNA from the plasmid pNF1337 which contains a bacterial insert (cloned into pBR322) that starts at codon 106 of ribosomal protein L11, contains all of the genetic information for ribosomal proteins L1, L10, and L12, and terminates within the gene coding for the β subunit of RNA polymerase (Fig. 2) (20). It has been shown (21) that ribosomal protein L1 cannot be synthesized from pNF1337 DNA because it is transcribed from the L11 promoter which has been deleted from this plasmid. Thus, the only bacterial genes expressed are L10, L12, and an NH_2-terminal fragment of the β gene. DNA sequence studies of this genetic region (22) have revealed that the first two amino acids of these three proteins are Met-Ala, Met-Ser, and Met-Val, respectively. Because fMet-tRNA initiates protein synthesis in prokaryotes, the corresponding formylated species should be synthesized *in vitro*—e.g., fMet-Ala in the case of L10. When pNF1337 DNA was incubated in this defined system in the presence of [^{35}S]fMet-tRNA and [^{3}H]Ala-

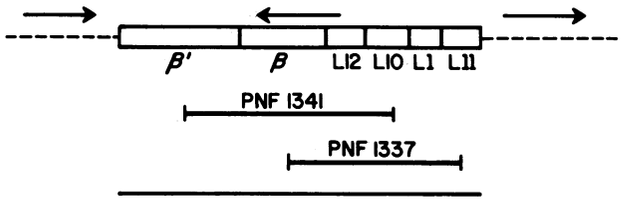

FIG. 2. Map of the bacterial inserts on plasmids pNF1337 and pNF1341. These plasmids contain fragments derived from the transducing phages λrif^d18 by limited digestion with *Pst* I (20). Dashed lines, plasmid DNA; arrows, direction of transcription. Figure adapted from Goldberg *et al.* (21).

tRNA, the dipeptide [^{35}S]fMet-[^{3}H]Ala was synthesized in a reaction that was linear for 60 min (Fig. 3). The comigration of the radioactive product with fMet-Ala and the presence of stoichiometric amounts of [^{35}S]fMet and [^{3}H]Ala in the product provide strong support that the dipeptide fMet-Ala was synthesized.

In addition to its simplicity, one of the advantages of the present system is that it contains a limited number of highly purified factors. For the synthesis of fMet-Ala, there was an absolute requirement for each of the components shown in Table 1 with the exception of IF-1, for which there is only a partial dependency. This may be due to the presence of IF-1 as a contaminant in IF-3 or a reflection of the actual requirement for IF-1 under the experimental conditions used.

Like pNF1337, pNF1341 also contains a fragment of λrif^d18 DNA but the insert begins at codon 26 of the L10 gene and extends through L12 and the β subunit of RNA polymerase (Fig. 2). This DNA therefore lacks both the promoter and NH_2-terminal fragment of L10. When DNA from the plasmid pNF1341 or pBR322 was used as template, no synthesis of fMet-Ala was observed (Table 1). These results further support the conclusion that the synthesis of fMet-Ala from pNF1337 DNA results from the transcription of the L10 gene and beginning of translation of the L10 mRNA.

Previous results, using both DNA and RNA as templates for the synthesis of L10 *in vitro*, demonstrated that the synthesis of L10 was under autogenous control (7, 9). Fig. 4 shows the effect of L10 on fMet-Ala synthesis in the present system. A 50–70% inhibition of fMet-Ala formation was observed when 150

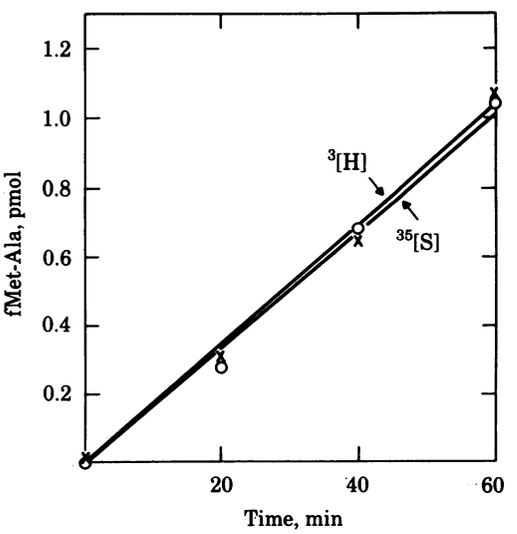

FIG. 3. Time course for the synthesis of fMet-Ala. The incubation conditions and assay are described in the text. The incubation mixtures contained [^{35}S]fMet-tRNA and [^{3}H]Ala-tRNA.

Table 1. Components required for the synthesis of fMet-Ala

Omission	fMet-Ala, pmol
None	1.7
RNA polymerase	0
Ribosomes	0
IF-1	0.7
IF-2	0
IF-3	0
EF-Tu	0
−1337 DNA + 1341 or pBR322 DNA	0

pmol of L10 was present in the reaction mixture. Several controls were run to show the specificity of this effect. When 240 pmol of ribosomal protein L12 was added to a reaction mixture, <15% decrease in the synthesis of fMet-Ala was observed (data not shown). In addition, the inhibitory effect of L10 on the synthesis of another dipeptide, fMet-Ser was investigated. This dipeptide contains the first two nascent amino acids in the proteins L12 and β-lactamase (22, 23), whose genes are present on pNF1337. Although L10 inhibited the synthesis of fMet-Ala by about 70%, it had no effect on the synthesis of fMet-Ser (Table 2).

It was also found that the synthesis of fMet-Ala was inhibited about 60% when 100 μM ppGpp, but not GDP, was added to the reaction mixtures (Fig. 5). Thus, this simplified *in vitro* system, in which a dipeptide is formed, can also be used to study transcriptional control of gene expression.

DISCUSSION

In studies designed to reveal the mechanism of the autoregulation of the synthesis of ribosomal protein L10, we used a simplified *in vitro* system that uses a limited number of purified components. The fact that both transcription and translation initiation occur in this system makes it ideally suited for studies on the regulation of gene expression. Although protein synthesis as described here cannot proceed past the formation of the first peptide bond, knowledge of the amino acid sequence permits specificity with regard to product formation. In the case of L10, dipeptide formation is sufficient for specificity because no other plasmid-encoded proteins begin with fMet-Ala. However, the system can be easily adapted to measure tripeptides

Table 2. Effect of L10 on synthesis of fMet-Ala and fMet-Ser

	Synthesis, pmol	
L10	fMet-Ala	fMet-Ser
Absent	1.6	1.8
Present	0.6	1.8

In these experiments, 150 pmol of L10 was used.

or larger peptides, as required. It is most applicable to *in vitro* studies (such as those described in this report) in which a limited number of genes are expressed and the sequence of the protein products are known. The control studies showing that fMet-Ala was not formed when pBR322 or pNF1341 DNA was used, as well as the specificity of the L10 effect, demonstrated the validity of the present assay system.

The present results using the dipeptide assay are consistent with previous *in vitro* findings (8) in which it was shown that the synthesis of ribosomal protein L10 was inhibited by exogenous L10 at the level of translation. The site of L10 regulation appears to be located at either the formation of the 70S initiation complex or the formation of the first peptide bond. Because the inhibition by L10 is very specific, it is unlikely that L10 is interfering with either the EF-Tu-dependent binding of Ala-tRNA to the ribosome or the subsequent peptidyl transferase reaction yielding the dipeptide fMet-Ala. Recent reports by Yates *et al.* (2) and Fukuda (9) have suggested that the leader region of the mRNA is involved in the autogenous control of the synthesis of ribosomal proteins L1, L11, and L12. In support of this suggestion is the observation that the binding sites for S4 and S7 on 16S RNA and L1 on 23S RNA have a striking homology to the nucleotide sequence at or close to the initiation codon of their respective mRNAs (4, 24). If L10 inhibits the formation of the 70S initiation complex containing the L10 mRNA, the present system should be useful in determining what step in the initiation reaction (e.g., mRNA binding to 30S subunit, formation of the 30S initiation complex, or joining of the 50S subunits to the 30S initiation complex) is sensitive to L10.

Under the conditions used in the present studies, the dipeptide (or dipeptidyl-tRNA) appears to be released from the mRNA·ribosome complex, permitting the mRNA to recycle. If it were possible to stabilize the dipeptide on the mRNA, then the amount of dipeptide formed would be an accurate measure of the amount of specific mRNA present.

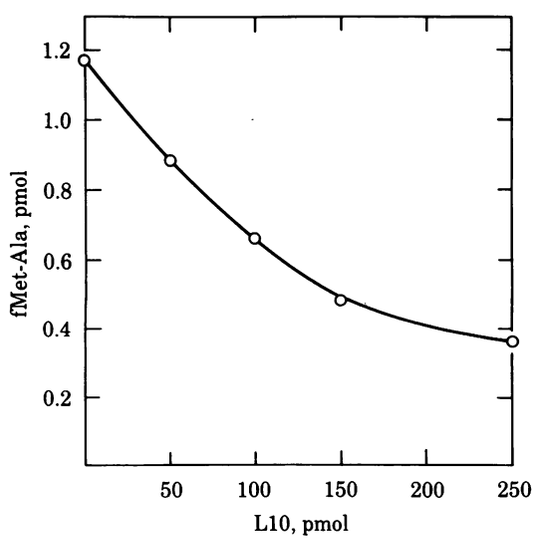

FIG. 4. Effect of protein L10 on fMet-Ala synthesis. See text for details.

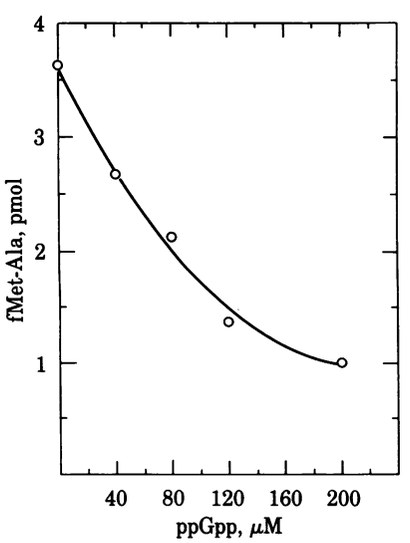

FIG. 5. Effect of ppGpp on fMet-Ala synthesis. See text for details.

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