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Biochemistry

In vitro synthesis of the first dipeptide of the β subunit of *Escherichia coli* RNA polymerase

(gene expression/β-subunit synthesis/dipeptide synthesis/secondary promoter)

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ABSTRACT **Plasmids pNF1337 and pNF1341, which contain part of the L10 operon including the RNA polymerase β-subunit gene, have been used as templates *in vitro* to investigate expression of the β-subunit gene. For these studies, the synthesis of the first dipeptide of the β subunit, fMet-Val, was measured instead of that of the entire protein. By using this dipeptide system, we studied the effects of RNA polymerase holoenzyme and L factor (*nus A* gene product) on fMet-Val synthesis and compared the relative effects of the primary and secondary promoters in the L10 operon on expression of the β-subunit gene. The results show that the inhibitory effect of RNA polymerase on β-subunit synthesis and the stimulatory effect of L factor occur before formation of the first dipeptide bond. In this *in vitro* system, the secondary promoters account for about 50% of the total fMet-Val synthesized. Although the primary promoter is sensitive to guanosine 5′-diphosphate 3′-diphosphate *in vitro*, the secondary promoters are not affected by this nucleotide.**

The DNA coding for the β subunit of RNA polymerase is part of a polycistronic operon that also contains the genes for ribosomal proteins L10 and L12 and the β′ subunit of RNA polymerase (1–3). A major promoter for all of these genes is located about 380 base pairs upstream from the L10 gene (1, 4, 5), although evidence for secondary promoters (5–10) that could influence the expression of the L12 and β- and β′-subunit genes has been obtained. This operon has received special attention because of the unique regulation that controls the expression of these gene products. In previous studies *in vitro*, the ratio of synthesis of the four products, L10/L12/β subunit/β′ subunit, was about 1:4:<0.1:<0.1 (11). It is still not entirely clear how the synthesis of these proteins is regulated, but both transcriptional and translational controls are involved. In addition to the secondary promoters, it has been demonstrated *in vitro* that (*i*) L10 inhibits its own synthesis (autoregulation) at the level of translation (12, 13); (*ii*) RNA polymerase inhibits β- and β′-subunit synthesis (11, 14–16); (*iii*) a transcriptional attenuator site is present in the intracistronic region between L12 and the β subunit (6, 17); and (*iv*) L factor (18), which is the *nus A* gene product (19), stimulates β-subunit synthesis (11).

We previously studied the DNA-dependent *in vitro* synthesis of the β subunit, L10, and L12 by using a crude protein-synthesizing system (7, 8, 20) as well as a highly defined system containing more than 30 purified factors (11). In those earlier studies, DNA from the transducing phage λ*rif*^{d}18 or from the plasmids pNF1337 and pNF1341 was used as template. Using pNF1337, we recently developed a simplified *in vitro* system to study the regulation of L10 synthesis (21). In this procedure, formation of the first dipeptide (fMet-Ala in the case of L10) was measured in lieu of that of the entire protein. In the present study, this *in vitro* system was used to investigate the synthesis of the β subunit by assaying for the formation of fMet-Val, the first dipeptide of the β subunit of RNA polymerase (4, 22).

MATERIALS AND METHODS

Plasmids pNF1337 and pNF1341 (23) were obtained from J. Friesen, University of Toronto, and the transducing phage λ*rif*^{d}18 was obtained from J. B. Kirschbaum, Harvard University.

Materials. Unfractionated *Escherichia coli* tRNA and purified $tRNA_f^{Met}$ were purchased from Boehringer Mannheim; the purified tRNA isoacceptor species $tRNA_1^{Ser}$, $tRNA_3^{Ser}$, and $tRNA_3^{Ala}$ were kindly supplied by B. Reid, University of Washington, Seattle, WA. L-[^{3}H]Alanine (87 Ci/mmol; 1 Ci = 3.7 × 10^{10} becquerels), L-[^{3}H]serine (17 Ci/mmol), and L-[^{3}H]valine (12.2 Ci/mmol) were purchased from New England Nuclear. Guanosine 5′-diphosphate 3′-diphosphate (ppGpp) was purchased from P-L Biochemicals or synthesized by A. Cook, Hoffmann–La Roche. A 0.25 M DEAE-salt eluate (18) was used as a source of enzymes to acylate the tRNA species. The formation of fMet-tRNA using 10-formyltetrahydrofolate:L-methionyl-tRNA *N*-formyltransferase has been described (24). For the experiments described here, the following acylated tRNA species were used: $fMet\text{-}tRNA_f^{Met}$; $Ala\text{-}tRNA_3^{Ala}$, 7,000 cpm/pmol; $Ser\text{-}tRNA_1^{Ser}$, 4,500 cpm/pmol; $Ser\text{-}tRNA_3^{Ser}$, 3,000 cpm/pmol; and $Val\text{-}tRNA^{Val}$ (unfractionated), 9,000 cpm/pmol. Except for some experiments (see Table 2) in which $Ser\text{-}tRNA_3^{Ser}$ was used (β-lactamase expression), all experiments in which fMet-Ser was synthesized used $Ser\text{-}tRNA_1^{Ser}$. L factor was purified as described (18).

DNA-Directed Dipeptide Synthesis. The incubation conditions used for the *in vitro* synthesis of dipeptides have been described (21); the components are given in Table 1. They are similar to those reported previously (21), with the exception that each reaction mixture contained 9 pmol of unlabeled $fMet\text{-}tRNA_f^{Met}$ and 9 pmol of one of the [^{3}H]aminoacyl-tRNA species. Synthesis of the dipeptide was linear for more than 60 min but, in the present study, the reactions were allowed to proceed for 40 min at 37°C. The reactions were stopped by addition of 2.5 μl of 1 M NaOH and the mixtures were incubated for an additional 10 min at 37°C to hydrolyze any dipeptidyl-tRNA. The mixtures were placed at room temperature, and 0.5 ml of 0.5 M HCl was added followed by 3 ml of ethyl acetate. This mixture was shaken and centrifuged at low speed, a 2.5-ml aliquot of the ethyl acetate phase was transferred to a scintillation vial, and the radioactivity was determined. Under these conditions, formylmethionine and the formylmethionyl dipeptides are extracted into the organic phase whereas the free amino acids are not. Since the formylmethionine was unlabeled, the extractable radioactivity represented dipeptide formation. Under the ex-

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Abbreviation: ppGpp, guanosine 5′-diphosphate 3′-diphosphate.

Table 1. Dependencies for fMet-Val synthesis using pNF1337 DNA

System	fMet-Val, pmol
Complete	0.9
Lacking	
DNA	0
Initiation factor 1	0.6
Other factors (initiation factors 2 and 3, elongation factor Tu, ribosomes, polymerase)	0
Val-tRNA	0
fMet-tRNA	0
Lacking DNA but including pBR322 DNA	0

The complete incubation mixture contained 2 μg of DNA; 2 μg of RNA polymerase; 0.3, 0.5, and 0.6 μg, respectively, of initiation factors 1, 2, and 3; 0.5 μg of elongation factor Tu; 12 pmol of 70S ribosomes; 9 pmol of [^{3}H]Val-tRNA; 9 pmol of fMet-tRNA; and NTPs, salts, and buffers as described in ref. 21. fMet-Val synthesized was measured by an extraction procedure.

traction conditions used, 60%, 75%, and 33% of fMet-Val, fMet-Ala, and fMet-Ser, respectively, can be extracted into the ethyl acetate phase. These values were determined by using radioactive dipeptide standards that had been synthesized *in vitro* and isolated by TLC (21). The values presented here are corrected for losses due to extraction and the aliquots that were used. To verify the results obtained by this extraction procedure, the formylmethionyl dipeptides were also identified and quantitated by TLC as described (21). In the case of fMet-Val, the solvent system ethyl acetate/1-butanol/*n*-hexane/acetic acid (12:10:1:2) was used.

For some experiments, the reactions were uncoupled; i.e., transcription was separated from translation. In these experiments, the components required for transcription (DNA, RNA polymerase, NTPs) were incubated for 20 min, 1 μg of rifampicin (to stop further transcription) and the necessary factors for translation (see Table 1) were added, and the mixtures were incubated for another 40 min. fMet-Val formation was assayed as described above.

RESULTS

***In Vitro* Synthesis of fMet-Val.** The region of the L10 operon present in plasmids pNF1337 and pNF1341 is shown in Fig. 1.

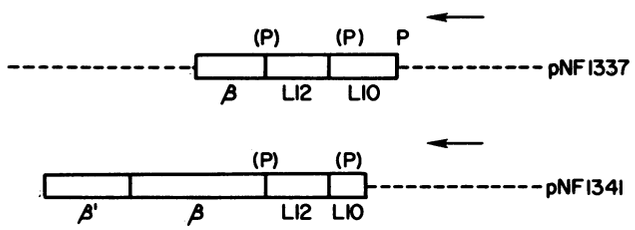

FIG. 1. Map of the bacterial inserts in plasmids pNF1337 and pNF1341. These plasmids contain DNA obtained by cloning partial *Pst* I digests of λ*rif*^{d}18 DNA (23). The bacterial insert of pNF1337 starts at codon 106 of ribosomal protein L11, continues through ribosomal proteins L1, L10, and L12, and ends within the gene coding for the β subunit of RNA polymerase. The bacterial DNA from pNF1341 begins at codon 24 of ribosomal protein L10, includes the complete genes for L12 and the β subunit of RNA polymerase, and terminates within the RNA polymerase β'-subunit gene. The primary promoter, P, for the L10/L12/β-subunit/β'-subunit transcription unit is located in front of the L10 gene on pNF1337 but is absent on pNF1341. Transcription can also be initiated from secondary promoters (P) located within the L10 structural gene and in the intracistronic region between the L12 and β-subunit genes present on both plasmids. ←, Direction of transcription of the insert (which is opposite to that of the β-lactamase gene into which it is cloned).

pNF1337 contains the primary promoter for the L10 operon (1), a secondary promoter located in the L10 structural gene (7, 8), and a possible promoter in the intracistronic region between the L12 and β-subunit genes (6, 9, 10). The entire L10 and L12 genes and part of the β-subunit gene are present. pNF1341 contains an insert that is lacking the genetic information for the primary L10 promoter and the beginning of the L10 gene but still contains the two secondary promoters described above. This plasmid contains the entire L12 and β-subunit genes plus part of the β'-subunit gene. DNA from the transducing phage λ*rif*^{d}18 contains a much larger segment of the bacterial chromosome that includes the entire L10 operon, the genes for L11, L1, elongation factor Tu, and ribosomal RNA (1).

Both pNF1337 and pNF1341 directed the synthesis of fMet-Val in the *in vitro* system. As an example, the effect of DNA concentration on the synthesis of fMet-Val using pNF1337 as template is shown in Fig. 2. With pNF1337, as well as pNF1341, about 2 μg of DNA are saturating. The dependencies for the *in vitro* synthesis of fMet-Val using pNF1337 as template are shown in Table 1. Except for initiation factor 1, complete dependencies were obtained for all of the components examined, and the parent plasmid, pBR322, did not direct the synthesis of fMet-Val. Partial dependencies on initiation factor 1 have also been observed in studies of β-galactosidase and L10 synthesis (21, 25).

Since fMet-Val is also synthesized using pNF1341 as template, an attempt was made to determine how important the secondary promoters are in β-subunit synthesis. To compare the relative template activity of the two plasmids, it was necessary to measure independently the expression of an unrelated gene present on both plasmids. Since both plasmids were derived from pBR322 and contain an identical segment of the β-lactamase gene (23), the synthesis of fMet-Ser in the presence of $tRNA_3^{Ser}$ isoacceptor species was used as a measure of β-lactamase synthesis (26). Under conditions in which equal amounts of fMet-Ser (β-lactamase) are formed from both plasmids, the amount of fMet-Val synthesized from pNF1337 was twice that obtained using pNF1341 as template (Table 2). Since pNF1341 does not contain the primary L10 promoter, these results indicate that, under the conditions used, the secondary promoters located in the L10 gene and in the L12–β intracistronic region can account for about 50% of the total amount of β-subunit synthesized. Also, when Ser-$tRNA_1^{Ser}$ was used, about twice as much fMet-Ser (measure of L12 synthesis) is formed with pNF1337 as template compared with pNF1341. The absence of the primary L10 promoter in pNF1341 is confirmed by the lack of synthesis of fMet-Ala, the first dipeptide of L10, from this template.

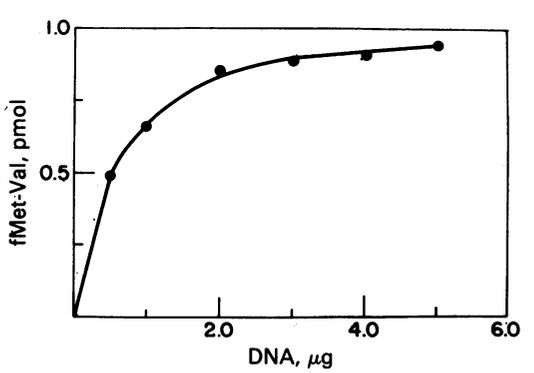

FIG. 2. Effect of DNA concentration on synthesis of fMet-Val using pNF1337 as template. Incubation conditions were as described in ref. 21 and in *Materials and Methods*.

Table 2. Comparison of dipeptide synthesis using pNF1337 and pNF1341 as templates

Gene product	Dipeptide	Synthesis, pmol	
		pNF1337	pNF1341
β Subunit	fMet-Val	0.8	0.4
L12	fMet-Ser	4.2	2.4
L10	fMet-Ala	2.4	—

Under the incubation conditions used, about 6 pmol of fMet-Ser was formed from Ser-tRNA$_3^{Ser}$ when 2 μg of pNF1337 or 2.2 μg of pNF1341 was added. This value represents expression of the β-lactamase gene in both plasmids. For other experiments, purified fMet-tRNA$_f^{Met}$, Ser-tRNA$_1^{Ser}$, and Ala-tRNA$_3^{Ala}$ isoacceptor species were used but Val-tRNA was prepared by using an unfractionated *E. coli* tRNA preparation. Values presented are those from a typical experiment.

Regulation of fMet-Val Synthesis. The synthesis of fMet-Val provides a rapid procedure to study regulation of the expression of the β-subunit gene. Previous *in vitro* results using λ*rif*^{d}18 transducing phage DNA as template showed that high levels of RNA polymerase holoenzyme inhibit synthesis of the β subunit (11, 14–16) and that L factor stimulates synthesis of the β subunit (11). The effect of RNA polymerase on fMet-Val synthesis is shown in Fig. 3. In these experiments, both λ*rif*^{d}18 DNA and pNF1337 were used as templates. Although the inhibition of fMet-Val synthesis by RNA polymerase with λ*rif*^{d}18 DNA as template is somewhat greater than that with pNF1337 as template, a similar overall effect of RNA polymerase is observed. Maximum synthesis of fMet-Val is seen with 2–5 μg of RNA polymerase and significant inhibition is seen with 10–15 μg of RNA polymerase. As a control for these experiments, expression of the L12 gene was determined by measuring the formation of fMet-Ser (using Ser-tRNA$_1^{Ser}$) under similar conditions. As seen in Fig. 3, there is no inhibitory effect of high levels of RNA polymerase on fMet-Ser synthesis using pNF1337 as template.

The effect of L factor on fMet-Val synthesis using pNF1337 is shown in Fig. 4. The addition of 0.1–0.3 μg of L factor results in two- to threefold stimulation of fMet-Val synthesis. Similar

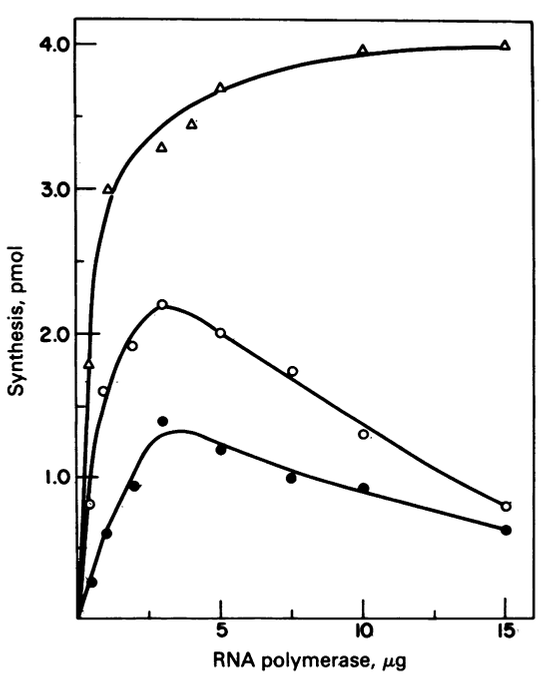

FIG. 3. Effect of RNA polymerase on fMet-Val (●) and fMet-Ser (△) synthesis using 2 μg of pNF1337 as template and on fMet-Val (○) synthesis using 2 μg of λ*rif*^{d}18 DNA as template.

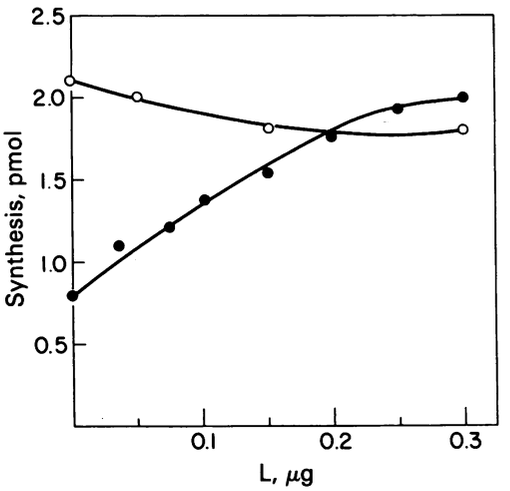

FIG. 4. Effect of L factor on fMet-Val (●) and fMet-Ser (○) synthesis using pNF1337 as template.

results were obtained with λ*rif*^{d}18 DNA and pNF1341 as template (data not shown). Fig. 4 also shows that L factor does not stimulate fMet-Ser formation using pNF1337 as template. The lack of an effect on fMet-Ser formation is in agreement with previous results showing that both L factor and RNA polymerase had no effect on the *in vitro* synthesis of several proteins coded for by λ*rif*^{d}18 or λ*fus*3 transducing phages (11).

By using an uncoupled system, we studied whether RNA polymerase and L factor were functioning at transcription or translation. In a typical incubation mixture, addition of excess RNA polymerase either before or after transcription resulted in 60–70% inhibition of fMet-Val synthesis. These results indicate that excess RNA polymerase inhibits translation of the β-subunit mRNA. In contrast, L factor stimulated fMet-Val synthesis (≈twofold) only when it was added before transcription, indicating that L factor was affecting transcription.

Although the synthesis of L10 and L12 is inhibited by ppGpp (20), previous results on the effect of ppGpp on β-subunit synthesis have varied from no effect to significant inhibition by ppGpp (27–30). By using pNF1337 and pNF1341, we examined the effect of ppGpp on the *in vitro* synthesis of fMet-Val from primary and secondary promoters. As shown in Fig. 5, when pNF1337 is used as template, 50% inhibition of fMet-Val synthesis is observed at a ppGpp concentration of 150 μM. How-

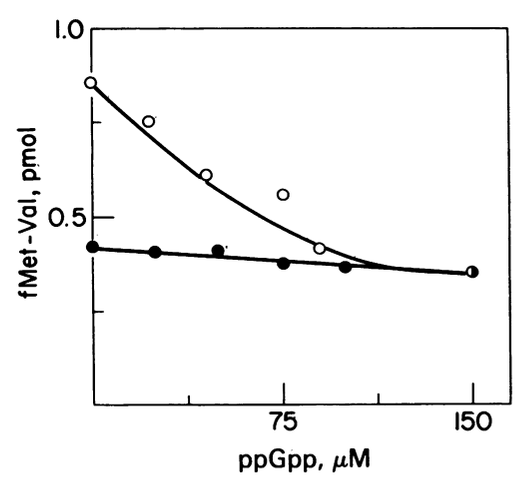

FIG. 5. Effect of ppGpp on synthesis of fMet-Val using pNF1337 (○) or pNF1341 (●) as template.

ever, the nucleotide has no significant effect on fMet-Val synthesis using pNF1341 as template.

DISCUSSION

Dipeptide formation provides a relatively rapid and simple system to study gene expression using plasmids that contain a limited number of genes. This system has been used to study L10 synthesis (fMet-Ala) using pNF1337 as template (21) and, in the present study, we have used fMet-Val synthesis as a measure of expression of the β-subunit gene. Because valine is infrequently seen as the second amino acid in *E. coli* proteins, fMet-Val synthesis can be used as a measure of β-subunit synthesis even with templates containing many more genes, such as $\lambda rif^d 18$ DNA. The evidence that fMet-Val synthesis arises from expression of the β-subunit gene in $\lambda rif^d 18$ DNA is provided by the results on the effects of RNA polymerase and L factor on dipeptide formation (Figs. 3 and 4). The effects of these proteins on β-subunit synthesis *in vitro* are rather specific, as shown previously in studies using $\lambda rif^d 18$ as template (11).

The data in Figs. 3 and 4 show that both RNA polymerase and L factor exert their effects at or before formation of the first peptide bond. It has been suggested that RNA polymerase inhibits β-subunit synthesis at the translation level (15) and this was confirmed here. Based on the present results, it appears that the initiation step of translation is affected and that L factor functions at the level of transcription in stimulating fMet-Val synthesis. Since L factor is thought to affect transcription by modulating RNA polymerase at pause or termination sites (31), a logical site of action of L factor would be the known attenuator in the intracistronic region between the L12 and β-subunit genes (17). However, direct evidence for this is still lacking.

We also tried to compare the activity of the various promoters that could influence β-subunit synthesis. In the *in vitro* system used here, the secondary promoters account for as much as 50% of the synthesis obtained when all three promoters are present (i.e., synthesis of fMet-Val with pNF1341 compared with pNF1337 as template). This high level of fMet-Val synthesized from the secondary promoters was unexpected but this conclusion was supported indirectly by the results with ppGpp (Fig. 5). This nucleotide inhibited fMet-Val synthesis using pNF1337 as template by about 50% but had no effect when pNF1341 was used as template, which is consistent with specific inhibition by ppGpp of the primary L10 promoter. In earlier *in vitro* experiments in which L12 synthesis was measured, it also appeared that the internal promoter in the L10 gene was not sensitive to ppGpp (7). These studies indicate that the differential effect of ppGpp provides a way to distinguish the primary L10 promoter from the secondary promoter in the L10 operon.

Of the two potential β-subunit secondary promoters, it appears that the promoter in the L10 gene (Fig. 1) is responsible for the bulk of the synthesis of fMet-Val from pNF1341. This conclusion comes from the finding that the synthesis of both fMet-Val and fMet-Ser decrease by one-half when pNF1341 is used as template compared with pNF1337 (Table 2). If the promoter in the intracistronic region between the L12 and β-subunit genes exerted a significant role, preferential synthesis of fMet-Val would have been observed with pNF1341.

In addition, this dipeptide system should permit studies on synthesis of the individual β and β′ subunits. The former protein begins with fMet-Val whereas the latter protein has fMet-Lys at the amino terminus (22). Other procedures currently available to individually measure the synthesis of these two closely related proteins are long and quite difficult.

Recently, Bruckner and Matzura (32) have presented *in vivo* evidence for the presence of a polycistronic mRNA for ribosomal proteins L11, L1, L10, and L12. If so, the L11 promoter would be the primary promoter for the synthesis of L11, L1, L10, L12, and the β and β′ subunits. In that case, the L10 promoter that we refer to in this study as the "primary" promoter would be a secondary promoter. However, the interpretation of the present results should not be affected by the location of the primary promoter.

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