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Use of different $tRNA^{Ser}$ isoacceptor species *in vitro* to discriminate between the expression of plasmid genes

(*in vitro* protein synthesis/ribosomal proteins L10 and L12/dipeptide synthesis)

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ABSTRACT **A simplified translation system coupled to DNA transcription that involves assaying the synthesis of the first dipeptide of a gene product has been described recently [Robakis, N., Meza-Basso, L., Brot, N. & Weissbach, H. (1981) *Proc. Natl. Acad. Sci. USA* 78, 4261–4264]. Using this dipeptide system, we have investigated the expression of genes carried on plasmids coding for β-lactamase, ribosomal protein L12, and the chloroplast large subunit (LS) of ribulosebisphosphate carboxylase (RbuBPCase). Although all three nascent gene products begin with the sequence fMet-Ser, the formation of fMet-Ser can be used to distinguish between the synthesis of β-lactamase and either L12 or the LS of RbuBPCase by using different serine isoacceptor tRNA species. In β-lactamase, the serine codon is AGU, which utilizes the serine isoacceptor species $tRNA_3^{Ser}$; in L12 and the LS of RbuBPCase, the serine codewords are UCU and UCA, respectively, both of which are recognized by the serine isoacceptor species $tRNA_1^{Ser}$. By using either pure $tRNA_1^{Ser}$ or pure $tRNA_3^{Ser}$, the expression of each gene can be quantitated. In this system, guanosine-5′-diphosphate-3′-diphosphate inhibits the expression of the β-lactamase and L12 genes but stimulates the synthesis of the LS. In addition, the ratio of fMet-Ser/fMet-Ala (L12/L10) synthesized was about 1 as compared with the ratio of 4 that has been obtained previously *in vivo* or in *in vitro* protein-synthesizing systems, in which the entire gene product was measured.**

A simplified DNA-directed coupled protein-synthesizing system has been developed to study the regulation of the synthesis of ribosomal protein L10 (1). In these initial experiments, plasmid pNF1337 DNA (2) was used as template, and the formation of the first dipeptide (fMet-Ala) of protein L10 was determined. An important advantage of this DNA-dependent system is that it can be constructed with only five highly purified proteins, i.e., RNA polymerase, initiation factor 1 (IF-1), IF-2, IF-3, and elongation factor Tu (EF-Tu). By using fMet-tRNA and the appropriate aminoacyl-tRNA species for the second amino acid, a specific dipeptide gene product can be selectively synthesized. Because dipeptide formation from a DNA template involves accurate transcription and proper initiation of translation, this system is ideally suited for studies on the regulation of gene expression.

The ideal templates are plasmids or purified mRNAs that direct the synthesis of a limited number of products with different second amino acids. Plasmid pBR322 and recombinant plasmids derived from pBR322 contain the β-lactamase gene (3), which is efficiently expressed. Thus, the amino-terminal fragment of β-lactamase (fMet-Ser) will be synthesized in the dipeptide system when fMet-tRNA and Ser-tRNA are present. A difficulty arises when a plasmid has another gene that also contains the serine codeword as the second amino acid. This is, in fact, the case with two recombinant plasmids derived from pBR322 that are described in this study. Plasmid pNF1337 contains, in addition to the β-lactamase gene, the genes for L10, L12, and part of the β subunit of RNA polymerase (2). These latter proteins begin with fMet-Ala (L10), fMet-Ser (L12), and fMet-Val (β subunit). Thus, the detection of the synthesis of L12, as measured by fMet-Ser formation, is complicated by any fMet-Ser synthesis from the β-lactamase gene. Similarly, plasmid pJEA4 contains the spinach chloroplast gene for the large subunit (LS) of ribulosebisphosphate carboxylase (RbuBPCase), which also begins with fMet-Ser (4). One solution to the problem is to modify the system and measure tripeptide formation if the third amino acid is different. However, a simpler approach would be to take advantage, wherever possible, of differences in the amino acid codewords in the respective genes. In the present study, we describe experiments with different serine tRNA isoacceptor species to distinguish gene products with the dipeptide assay.

MATERIALS AND METHODS

Escherichia coli JF943 containing plasmid pNF1337 (2) was supplied by J. Friesen (University of Toronto, ON, Canada). The plasmid DNA was isolated from this transformant and used to transform *E. coli* K-12 strain RRI (5). Strain RRI also was used as host for plasmid pBR322 (6) and pJEA4. Plasmid pJEA4 was prepared by J. Erion in this laboratory and has a 2.2-kilobase-pair (kbp) fragment that contains the gene for the LS of spinach RbuBPCase. This plasmid was constructed by ligation into pBR322 of a DNA fragment obtained by digestion of the plasmid pSoe3101 (7) with the restriction endonucleases *Ava* I/*Bam*HI.

L-[^{3}H]Alanine and L-[^{3}H]serine were obtained from New England Nuclear and L-[^{35}S]methionine was from Amersham/Searle. Purified $tRNA_f^{Met}$ was purchased from Boehringer Mannheim, whereas purified $tRNA_1^{Ser}$, $tRNA_3^{Ser}$, and $tRNA_3^{Ala}$ species were prepared as described (8). Met-tRNA synthetase was obtained from C. J. Bruton (Imperial College of Science and Technology, London) and Ser-tRNA and Ala-tRNA synthetases were purified as described (9). The acylation and transformylation reactions were carried out as described elsewhere (10–13). After the acylation reaction, the acylated tRNA species were purified by phenol extraction and alcohol precipitation as described (1). The fraction of a crude *E. coli* enzyme preparation that eluted from a DEAE-cellulose column at 0.25 M salt (14) was used as the source of N^{10}-formyl-H_4-folate-Met-tRNA transformylase.

N-Formyl-L-methionine, *N*-formyl-L-methionyl-L-alanine, and L-methionyl-L-serine were purchased from Sigma. *N*-For-

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Abbreviations: RbuBPCase, ribulosebisphosphate carboxylase; LS, large subunit of RbuBPCase; $tRNA_1^{Ser}$ and $tRNA_3^{Ser}$, isoacceptor serine tRNA species; IF, initiation factor; EF, elongation factor; ppGpp, guanosine-3′-diphosphate-5′-diphosphate; kbp, kilobase pair(s).

myl-L-methionyl-L-serine was synthesized as described (1). Precoated thin-layer chromatography plates (silica gel G; 250 μm) were obtained from Analtech (Newark, DE).

Preparation of 1.6-kbp DNA Fragment Containing the L10 and L12 Genes. A 1.6-kbp DNA fragment was prepared by digesting pNF1337 DNA with restriction endonuclease *Hin*cII. The reaction mixture (600 μl) contained 10 mM Tris·HCl (pH 7.9), 1 mM dithiothreitol, 30 mM NaCl, 1 mM $MgCl_2$, 600 μg of pNF1337 DNA, and 1000 units of the *Hin*cII enzyme. The mixture was incubated at 37°C for 3 hr. One of the fragments generated encompasses nucleotides 1273–2893 of the ribosomal protein gene cluster from the transducing phage λ*rif*^{d}18 (15). This fragment contains the genetic information for several amino acids at the carboxyl terminus of L1 and the genes for L10 and L12; it terminates within the leader sequence of the β subunit of RNA polymerase. To obtain this fragment, the incubation mixture after *Hin*cII digestion was subjected to electrophoresis on a 1.5% agarose slab gel (Seaplaque, FMC, Rockland, ME), and the area of the agarose gel containing the 1.6-kbp DNA fragment was cut out of the slab. The agarose slices were melted at 68°C and then extracted with phenol at 37°C. The aqueous solution was concentrated in a Speed-Vac concentrator (Savant), and the DNA was precipitated by ethanol at −20°C in the presence of 0.3 M sodium acetate. The precipitation step was repeated twice more. The DNA fragment (50 μg) was finally dissolved in 10 mM Tris·HCl (pH 7.5).

DNA-Directed Dipeptide Synthesis. The *in vitro* incubation mixture (35 μl) was the same as described (1) with the following modifications: polyethylene glycol was omitted, the Mg^{2+} concentration was 12 mM, and 6 μg of RNA polymerase was used. The reaction mixture, containing [^{35}S]fMet-tRNA and [^{3}H]Ser-tRNA or [^{3}H]Ala-tRNA, was incubated at 37°C for 60 min, and the reaction was stopped by the addition of 2.5 μl of 1 M NaOH. After an additional 10-min incubation at 37°C, 30 μg of fMet and fMet-Ala or fMet-Ser, or both, were added in a total volume of 6 μl. Then 4 μl of 2 M HCl was added to the mixture, and the resulting precipitate was discarded after centrifugation. The supernatant was concentrated in a Speed-Vac concentrator to approximately 12 μl. Half of the concentrate was applied to a silica gel G thin-layer plate, and the plate was developed with ethyl acetate/hexane/acetic acid, 8:3:1 (vol/vol). The detection and analysis of the dipeptides have been described earlier (1).

RESULTS

Table 1 summarizes the plasmid templates used in the present experiments and the amino-terminal dipeptide for each gene product as well as the DNA sequence and the expected isoacceptor species for the second amino acid. It is seen that three of the protein products (L12, LS, and β-lactamase) have serine as the second amino acid. Thus, with crude tRNA, the synthesis of fMet-Ser with pNF1337 DNA as template could be due to the combined expression of the genes for L12 and β-lactamase. Similarly, with pJEA4 DNA, the expression of β-lactamase and the LS gene results in fMet-Ser synthesis. Examination of the serine codons indicated a way to distinguish the β-lactamase gene product from either L12 or the LS. As noted in Table 1, the β-lactamase dipeptide should require the $tRNA_3^{Ser}$ isoacceptor species, whereas the LS or L12 dipeptide should use $tRNA_1^{Ser}$. This was experimentally verified by the *in vitro* results shown in Table 2. With pBR322 DNA containing the β-lactamase gene as template, only $tRNA_3^{Ser}$ was active in fMet-Ser formation. In contrast, when the 1.6-kbp *Hin*cII DNA restriction fragment of pNF1337 (containing only genes L10 and L12) was used, $tRNA_1^{Ser}$ and not $tRNA_3^{Ser}$ was active. With pNF1337 and pJEA4, each isoacceptor species individually yielded product as would be expected because these templates code for both β-lactamase and either L12 or the LS. It is also seen in Table 2 that, with these plasmids as templates, a mixture of both isoacceptor species yields a result that is close to additive. These results clearly show that $tRNA_3^{Ser}$ is the isoacceptor species used for fMet-Ser formation directed by the β-lactamase gene, whereas $tRNA_1^{Ser}$ is used for the synthesis of fMet-Ser from the L12 and the LS genes.

Table 2. Utilization of two $tRNA^{Ser}$ isoaccepting species in the dipeptide assay

Template	Protein products containing fMet-Ser	Synthesis of fMet-Ser, pmol: $tRNA_1^{Ser}$	$tRNA_3^{Ser}$	$tRNA_1^{Ser}$ + $tRNA_3^{Ser}$
pBR322	β-Lactamase	0	4.0	—
*Hin*cII fragment	L12	1.2	0	—
pNF1337	L12, β-lactamase	2.4	1.6	3.4
pJEA4	LS (RbuBPCase), β-lactamase	3.7	1.3	6.0

The reactions were carried out as described in the text, with 2 μg of plasmid DNA (pBR322, pNF1337, and pJEA4) or 0.2 μg of the 1.6-kbp *Hin*cII DNA fragment.

An interesting application of the present system is in the study of the effect of guanosine-3′-diphosphate-5′-diphosphate (ppGp) on the expression of these genes. This unique nucleotide, which accumulates when cells are starved for an amino acid, is known to inhibit ribosomal protein synthesis (16) and recently was shown to inhibit the *in vitro* DNA-directed synthesis of β-lactamase (17). Fig. 1 summarizes the effect of ppGp on the synthesis of fMet-Ser using either $tRNA_1^{Ser}$ or $tRNA_3^{Ser}$ with pBR322, pNF1337, and pJEA4 DNA as templates. With pBR322 DNA (β-lactamase only), ppGpp inhibits the synthesis of fMet-Ser with $tRNA_3^{Ser}$. With pNF1337 DNA as template, ppGp reduces fMet-Ser formation with either $tRNA_1^{Ser}$ or $tRNA_3^{Ser}$, indicating that the synthesis of both β-lactamase and L12 is inhibited by ppGp. With pJEA4 DNA, however, there is a differential effect of ppGp. It inhibits the synthesis of fMet-Ser from $tRNA_3^{Ser}$ (β-lactamase) but stimulates

Table 1. Different plasmids used and peptide products

Plasmid	Gene product	NH_2-terminal sequence	Nucleotide coding sequence	Isoacceptor tRNA
pNF1337	Ribosomal protein L10	fMet-Ala	AUG GCU	Ala_3 or Ala_2
pNF1337	Ribosomal protein L12	fMet-Ser	AUG UCU	Ser_1 or Ser_2
pJEA4	Large subunit of RbuBPCase	fMet-Ser	AUG UCA	Ser_1
pNF1337* pJEA4 pBR322	β-Lactamase	fMet-Ser	AUG AGU	Ser_3

* pNF1337 contains a truncated β-lactamase gene because the ribosomal gene cluster from λ*rif*^{d}18 is inserted into pBR322 at the *Pst* I restriction endonuclease site (2).

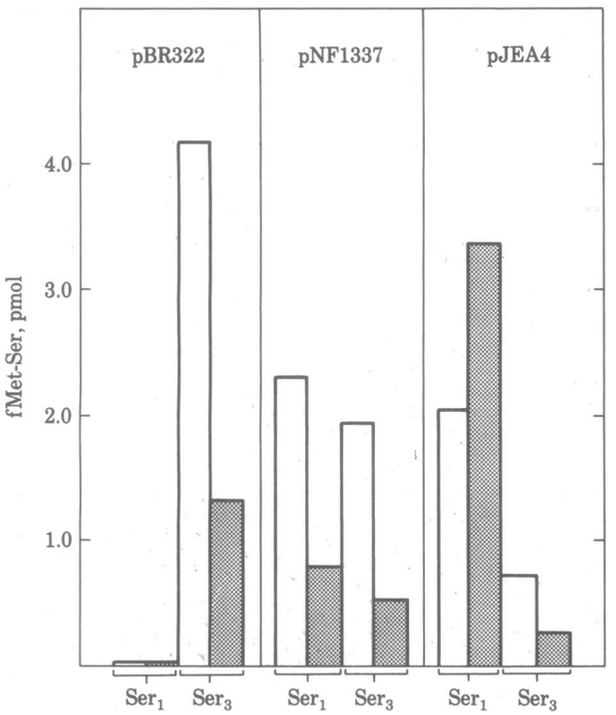

FIG. 1. Effect of ppGpp on fMet-Ser synthesis with different $tRNA^{Ser}$ isoaccepting species and different plasmid DNAs as template. Details of the incubations are described in the text. ▩, Addition of 200 μM ppGpp; Ser_1, $tRNA_1^{Ser}$; Ser_3, $tRNA_3^{Ser}$.

the incorporation from $tRNA_1^{Ser}$ (LS) by about 40%.

It is known from *in vivo* (18) and other *in vitro* experiments (19, 20) that the ratio of L12/L10 synthesized is about 4. In the initial study (1) on dipeptide formation with unfractionated tRNA as the source of all $tRNA^{Ser}$ isoacceptor species, it was reported that the ratio of fMet-Ser/fMet-Ala synthesized with pNF1337 DNA as template was about 1.2. Because it was assumed that both the L12 and β-lactamase genes were being expressed in those experiments, it was not possible to determine what portion of the fMet-Ser formed was due to L12 expression. These experiments were repeated with the $tRNA_1^{Ser}$ isoacceptor species to specifically measure L12 synthesis and with $tRNA_3^{Ala}$ to measure the synthesis of fMet-Ala from the L10 gene. As seen in Table 3, the ratio of fMet-Ser/fMet-Ala is still about 1, with either pNF1337 DNA or the *Hin*cII DNA restriction fragment. In the light of these results with pure $tRNA^{Ser}$ isoacceptor species, the ratio of about 1 reported earlier (1) with an unfractionated tRNA preparation appears low. However, because the amount of each $tRNA^{Ser}$ isoacceptor species in the unfractionated tRNA preparations was not determined, it is not possible to evaluate the previous results.

DISCUSSION

As initially described, the formation of specific dipeptides provides a simple procedure to study gene expression in a highly defined system. The use of different isoacceptor species now makes it possible to distinguish two different gene products that begin with the same dipeptide. Specifically, the formation of fMet-Ser from the β-lactamase gene can be distinguished from fMet-Ser directed by the L12 or LS genes. This has now permitted quantitation of the individual gene products at the dipeptide level.

Table 3. Synthesis of fMet-Ser and fMet-Ala in the presence of $tRNA_1^{Ser}$ and $tRNA_3^{Ala}$

DNA	fMet-Ser, pmol	fMet-Ala, pmol
pNF1337	1.3	1.1
*Hin*cII DNA fragment	1.2	1.0

See legend to Table 2 and text for details.

The expression of the LS gene has been reported using a heterologous system (21), a crude *E. coli* extract (22), and a highly defined system (7). The DNA sequence of the LS gene from corn and spinach is known (4, 23), and, although the mature proteins from spinach and corn have alanine at the NH_2 terminus (4, 22), the DNA sequence indicates that the nascent proteins begin with fMet-Ser. The present results on dipeptide formation verify this and point out another potential use of this system. In cases where there may be some doubt from the DNA sequence where a particular protein initiates, dipeptide formation could provide the answer.

The ratio of L12/L10 formed in the present experiments is also of extreme interest. Both *in vivo* (18) and in DNA-directed *in vitro* studies in coupled systems, the L12/L10 ratio was observed to be about 4 (19, 20). Yet, in the present dipeptide system, the ratio of fMet-Ser/fMet-Ala was about 1. These *in vitro* findings suggest that the 4:1 ratio is not due to an overproduction of L12 mRNA but may be related to the known autogenous regulation of L10 synthesis by L10 (24) or some translational event after the formation of the first dipeptide.

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