THE TWO SITE MODEL OF RIBOSOMAL FUNCTION: A TEST USING DEGENERATE SERINE CODONS IN BACTERIOPHAGE f_2 mRNA

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SUMMARY. Degenerate serine codons occurring early in the f_2 coat and polymerase cistrons have been used to distinguish between multi-site models of ribosomal function. These studies indicate that only two tRNA's are bound to the ribosome prior to translocation.

A number of useful models for protein biosynthesis specify that two tRNA's are bound to the ribosome prior to the translocation reaction (1-11). One of these tRNA's is thought to be the most recently recognized aminoacyl-tRNA and the other, the nascent peptidyl-tRNA. Reasonable modifications of the two site model have been suggested in which additional sites are required either for initiation (12) or sequential alignment of oncoming aminoacyl-tRNA's (13-18). In order to distinguish between these multi-site models we have used mRNA derived from the bacteriophage f_2 and have taken advantage of two degenerate serine codewords which, by analogy to the related virus R17, occur in the third and second positions of the coat and RNA polymerase cistrons, respectively (19). Further, we have employed a purified E. coli protein synthetic system which differs from others (15,20,21) in that translocation is completely inhibited by the addition of small amounts of anti-G factor antibody (18). Under these conditions only the dipeptides initiating f_2 coat, maturation and RNA polymerase proteins, Fmet-ala, Fmet-arg and Fmet-ser, respectively, are synthesized.

Since the initial sequence of f_2 coat protein synthesized in vitro is Fmet-ala-ser-aspn..... (22) and synthesis is limited to the initial dipeptide, the binding properties of the first pre-translocation intermediate permit us

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to distinguish between two or three (or greater) site models for ribosomal function. We have previously reported that unfractionated Fmet-, ala-, and ser-tRNA's are bound to ribosomes in the presence of f_2 mRNA, whereas aspn-tRNA is not (18). A fourth binding site is thus eliminated, and the question arises as to whether the ser-tRNA binding is in response to the third codon of the coat cistron (UCU) or the second codon (UCG) of the polymerase cistron (19).

In this paper we distinguish between recognition of these degenerate serine codons using two countercurrent fractions of ser-tRNA. One fraction responds only to the codon UCU (ser-$tRNA_{UCU}$); the other to UCG and UCA as well as to UCU (ser-$tRNA_{UCG,A,U}$). Incorporation of serine from these tRNA's into initial f_2 coat and polymerase peptides is consistent with the nucleotide sequence data obtained from the related bacteriophage, R17, by Steitz (19). Further, of the two ser-tRNA's only ser-$tRNA_{UCG,A,U}$ is bound to the ribosome prior to translocation. Its binding can only be in response to the second polymerase codon, UCG, since the third coat codon, UCU, is not available for the binding of ser-$tRNA_{UCU}$. The result is consistent with the binding of no more than two tRNA's to the ribosome prior to translocation.

MATERIALS AND METHODS

Crude E. coli B tRNA was purchased from General Biochemical Corporation; ^{3}H-serine (spec. act. 3800mC/mM) and Liquifluor from New England Nuclear Corporation; benzoylated DEAE-cellulose and phenoxyacetyl-N-hydroxysuccinate from Schwarz BioResearch; standard peptides, which were subsequently formylated by the procedure of Sheehan and Yang (23), from Mann Research Laboratories.

Isoaccepting met- and ala-tRNA's were prepared as described by Gillam et al. (24) using activating enzymes purified according to Muench and Berg (25). The resulting tRNA's were >95% pure for acceptance of the desired amino acids. Ser-tRNA acceptance peaks were isolated from a 375 transfer countercurrent distribution of E. coli B tRNA (26).

Purified reaction components isolated from E. coli MRE600 have been described elsewhere (27,28). Ribosomes containing initiation factors were washed five times in buffer containing 0.05 M Tris-acetate, pH 7.2, 0.05 M NH_4Cl, 0.01 M $Mg(OAc)_2$, and 0.1 mM dithiothreitol until they were virtually free of G factor. Residual G factor was inactivated by pre-treating the ribosomes with anti-G factor immunoglobulin (29), purified from rabbit anti-serum by DEAE-cellulose column chromatography. The trinucleoside diphosphates corresponding to serine codons, UpCpU, UpCpA, and UpCpG, were the generous gift of Drs. C. T. Caskey, M. N. Nirenberg, and Mrs. T. M. Caryk. Assays were carried out in duplicate as detailed in legends.

RESULTS AND DISCUSSION

As shown in Table 1, two countercurrent fractions of $tRNA_{ser}$ differ in their recognition of the degenerate serine codons UpCpU, UpCpA, and UpCpG.

TABLE 1

CODON RECOGNITION BY COUNTERCURRENT FRACTIONS OF SER-tRNA

ADDITION	RIBOSOMAL BINDING ^{3}H-ser-tRNA FRACTION	
	A	B
	ΔμμMOLES	
UpCpU	1.65	2.54
UpCpA	0.07	4.09
UpCpG	-0.13	2.99

Binding assays were carried out as previously described by Nirenberg and Leder (30). Each 0.05 ml reaction contained: 0.05 M Tris acetate, pH 7.2; 0.02 M $Mg(OAc)_2$; 0.05 M NH_4Cl; 1.2 A^{260} ribosomes; UpCpU, 0.2 A^{260}; UpCpA, 0.11 A^{260}; or UpCpG, 0.12 A^{260}; and 10 μμmoles of ^{3}H-ser-tRNA. Reactions were incubated at 23°C for 20 min. Control binding values without mRNA, subtracted from the complete reaction binding values, were 1.78 μμmoles for fraction A and 3.66 μμmoles for fraction B. All assays were performed in duplicate.

Fraction A binds to ribosomes only in response to UpCpU, while fraction B responds to all three codons tested. Although neither fraction necessarily contains a pure species of $tRNA_{ser}$, it is clear that fraction A (ser-$tRNA_{UCU}$) does not contain at least one species of tRNA present in fraction B (ser-$tRNA_{UCG,A,U}$). According to the Wobble Hypothesis (31), one might expect a single tRNA species to respond to the codons UpCpA and UpCpG but not to these codons and UpCpU.

The cistron specificities of the ser-tRNA fractions are shown by electrophoretic analyses of the radioactive peptides synthesized in reaction mixtures containing f_2 mRNA and purified Fmet-, ala-, and ^{3}H-ser-tRNA's (Figure 1). Only in the presence of G factor is serine from ser-$tRNA_{UCU}$ (Fig. 1A) incorporated into the third position of the f_2 coat peptide, Fmet-ala-ser. There is no incorporation from this tRNA into the second position of the polymerase peptide, Fmet-ser, either in the presence or absence of G factor. Serine from ser-$tRNA_{UCG,A,U}$ (Fig. 1B), however, is incorporated into the polymerase dipeptide, Fmet-ser, in the absence of G factor. In the presence of G factor, ser from this tRNA is incorporated into both the polymerase dipeptide and the coat tripeptide.

Knowing that ser-$tRNA_{UCU}$ responds only to the third codon of the coat cistron, the availability of this codon at a third ribosomal binding site can be tested directly. The f_2 RNA-directed binding of ala- and both ser-tRNA's to ribosomes in the presence and absence of G factor is shown in Table 2. Ala-tRNA responding to the second coat codon is bound in the presence and absence of G factor. Ser-$tRNA_{UCU}$ does not bind to ribosomes in the absence of G factor. This tRNA species is bound only if translocation occurs (plus G factor) making the third coat codon, UCU, available for translation. Ser-$tRNA_{UCG,A,U}$, which responds to codons in both cistrons, is bound both before and after translocation has occurred (minus and plus G factor). Because of the codon specificity of these $tRNA_{ser}$ fractions, binding of ser-$tRNA_{UCG,A,U}$ to ribosomes prior to translocation must be in response to the second codon

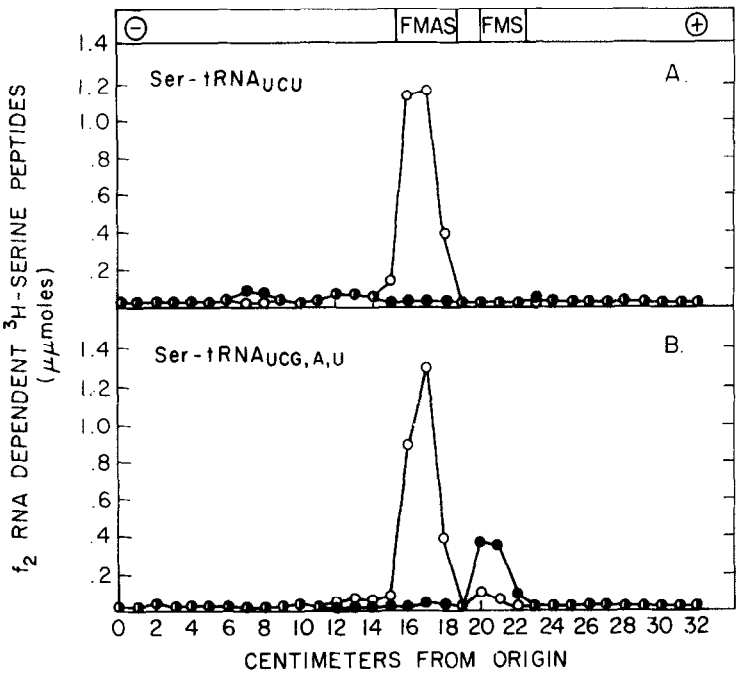

Figure 1. Electrophoretic analysis of the initial serine containing peptides synthesized in response to f_2 mRNA. Reaction mixtures contained the following components in a 0.1 ml volume; 0.05 M Tris acetate, pH 7.2; 0.005M $Mg(OAc)_2$; 0.05 M NH_4Cl; 5 A^{260} ribosomes pretreated with 44 µgms of anti-G factor; 15 µgms T factor; 1.0 A^{260} f_2 mRNA; ^{12}C-Fmet and ala-tRNA's, 0.07 and 0.06 A^{260}, respectively; and 40 µµmoles of ^{3}H-ser-tRNA. Reactions were incubated at 37°C for 20 min, and terminated by the addition of 0.01 ml 1 M NH_4OH. Continued incubation at 37°C for 30 min hydrolysed aminoacyl- and peptidyl-tRNA's. Reaction mixtures were lyophilized, redissolved in water, and spotted on adjacent lanes of Whatman 3MM paper. Electrophoresis in 1 M pyridine acetate, pH 3.5, was carried out for 6 Kv-hours. After developing the standard formyl-peptide spots (32), lanes were cut into 1 x 3 cm strips and analyzed by scintillation counting. Background values obtained from analyses of reaction mixtures incubated without f_2 mRNA have been subtracted. No appreciable radioactivity was associated with the F-met peptides in these control studies.

A, analysis of ^{3}H-ser-tRNA$_{UCU}$ product. B, analysis of ^{3}H-ser-tRNA$_{UCG,A,U}$ product. Closed circles (•---•) represent reactions carried out in the absence of G-factor; open circles (o---o), reactions in the presence of 2.6 µgms of pure G-factor, an amount sufficient to reverse the anti-G factor inhibition. Electrophoretic standard peptides: FMAS, Fmet-ala-ser, and FMS, Fmet-ser.

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of the polymerase cistron.

Inasmuch as Kuriki et al., (33) and Lucas-Lenard and Haenni (34) have reported that deacylated tRNA is released from the ribosome only after translocation, our data are consistent with the ribosomal binding of only

TABLE 2

F_2 mRNA-DIRECTED BINDING OF PURIFIED AMINOACYL-tRNA to PRE- AND POST-TRANSLOCATION RIBOSOMES

ADDITION	RIBOSOMAL BINDING ^{3}H-Ala-tRNA	^{3}H-Ser-tRNA$_{UCU}$	^{3}H-Ser-tRNA$_{UCG,A,U}$
		ΔμμMOLES	
NONE (PRE-TRANSLOCATION)	1.17	-0.05	0.14
G-FACTOR (POST-TRANSLOCATION)	0.81	1.10	0.40

Reaction mixtures contained the components listed in the legend to Fig. 1, but final volumes were 0.05 ml. 20 μμmoles of ^{3}H-ala-tRNA or ^{3}H-ser-tRNA were added to each mixture. Incubation was at 37°C for 20 minutes, and duplicate reactions were assayed according to Nirenberg and Leder (30). Control binding values without f_2 mRNA of 0.43 μμmoles for tRNA$_{UCU}$, 0.55 μμmoles for tRNA$_{UCG,A,U}$ and 0.08 μμmoles for ^{3}H ala-tRNA have been subtracted.

two tRNA's in response to the coat cistron prior to translocation. This conclusion is supported by our studies with mRNA from the bacteriophage Qβ (35) and the studies of Skogerson (personal communication), which demonstrate the presence of deacylated tRNA$^F_{met}$ on pre-translocated ribosomal intermediates.

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