Sequential Translation of Trinucleotide Codons for Peptide Bond Formation, Translocation, and Termination

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Abstract. The addition of GUA, valyl-tRNA, T factor, GTP, and ethanol to reactions containing fMet-tRNA·AUG·ribosome intermediate results in the synthesis of fMet-valyl-tRNA which can be translocated if G factor is also present. The translocated peptidyl-tRNA can interact with puromycin to form fMet-Val-puromycin. Release of fMet-valine can also take place after translocation if a terminator trinucleotide and its specific release factor are present.

Introduction. There are at least four sequential events that occur during the translation of mRNA and the synthesis of protein: initiation, peptide bond formation and elongation, translocation, and termination. Analysis of the interrelationships of these reactions *in vitro* has required as template either synthetic polynucleotides of defined sequence or phage mRNA. The earlier observation that trinucleotides can be used for the sequential initiation and termination of protein synthesis¹ has been extended in this communication. It is shown that all the individual events of mRNA translation can be sequentially examined *in vitro* using trinucleotide codons as template.

Materials and Methods. Preparation of ribosomes and protein factors: *Escherichia coli* B or *MRE 600* ribosomes washed in 1.0 M ammonium chloride were prepared as previously described.² Purified release factors R1 (Fraction VI) and R2 (Fraction VII), transfer factors T (Tu + Ts) and G were partially purified from *E. coli* B supernatant enzymes (S-100) by described methods.^{3,4} The fMet-tRNA·AUG·ribosome intermediates were formed as before^{1,4} and were stored in liquid nitrogen until use.

Peptide synthesis: Synthesis of peptide was conducted at 4° or 24°C in 0.050-ml reactions which contained: 0.013 M magnesium acetate, 0.1 M ammonium acetate, 0.05 M Tris-acetate (pH 7.2), 0.001 M GTP, 4.5–5.1 pmole f[¹⁴C]Met-tRNA·AUG·ribosome intermediate, 6.0 µg T (Tu + Ts) factor, 3.0–6.0 pmole [³H]Val-tRNA^{val}, 0.12–0.15 A₂₆₀ GUA, and were initiated by the addition of ethanol to a final concentration of 10% (v/v). Reactions were stopped by the addition of 0.1 ml 1.0 N KOH, after which they were incubated at 65°C for 15 min to deacylate all tRNA. After cooling for 5 min at 4°C, 0.2 ml 1.0 N HCl was added, followed by 1.5 ml ethyl acetate; the tubes were agitated, spun for 1–2 min in a desk top centrifuge, and the radioactivity in a 1.0-ml aliquot of the ethyl acetate phase was determined by scintillation counting.⁵ The amount of [³H]valine extracted under these conditions represents the amount of f[¹⁴C]Met-[³H]valine formed (see Fig. 2). Values were multiplied by 2.14 to correct for extraction efficiency and sample volume.¹

Product identification: The peptide f[¹⁴C]Met-[³H]valine was identified after ethyl acetate extraction of the reaction mixture. Paper electrophoresis was performed on Whatman no. 1 (53 V/cm; 75 min) in 0.05 M pyridine acetate, pH 3.5.⁶ Paper

strips 1×2.4 cm were counted in 10 ml Triton-POPOP-toluene scintillation fluid to which was added 1.0 ml water in order to partially elute the product from the paper (counting efficiency $^{14}\text{C} = 38.4\%$; $^3\text{H} = 41.2\%$). Markers (methionine, fmethionine, valine, fMet-ethyl ester) were electrophoresed simultaneously with the product.

Materials: The [^{14}C]methyl-methionine (218 mCi/mmole) and [^3H]valine (6.0 Ci/mmole) were purchased from Schwarz BioResearch. Purified tRNA^{fMet} and tRNA^{val} were obtained from Oak Ridge National Laboratory under the AEC-NIGMS inter-regional agreement and distributed by the NIGMS. Puromycin was purchased from Nutritional Biochemicals Co.; fusidic acid was the gift of Dr. Philip Leder.

Results. Synthesis of formyl-methionyl-valine: Release of fmethionine from fMet-tRNA·AUG·ribosome intermediates occurs upon translation of the appropriate terminator codon by R1 or R2.⁸ The data of Figure 1 demonstrates that

TABLE 1. Requirements for fMet-valine synthesis.

Components	f[^{14}C]Met-[^3H]valine (pmoles)
Complete	2.11
-GUA	0.65
-T factor	0.37
-GTP	0.57
-Ribosomes	0.11
-Ethanol	0.11

Each reaction was incubated at 4°C for 30 min and contained the components described in *Materials and Methods* except for the indicated deletions. In the reactions where ribosomes were deleted, 6.2 pmole f[^{14}C]Met-tRNA were added.

FIG. 1.—Rate of fMet-valine synthesis: Each reaction was incubated at 4°C and contained in 0.050 ml: 4.5 pmoles f[^{14}C]Met-tRNA·AUG·ribosome intermediate, 6.2 pmoles [^3H]Val-tRNA; 0.26 ammonium acetate, and other components as described in *Materials and Methods*.

this same ribosome intermediate can also interact with a nonterminator trinucleotide, GUA, and valyl-tRNA to form the peptide fMet-valine. It is possible, therefore, to use trinucleotide codons for the sequential initiation of protein synthesis and either peptide bond formation or termination. Synthesis of fMet-valine requires ribosomes, GUA, transfer factor T (Tu + Ts), GTP, and ethanol (Table 1). The peptide can be formed when T factor and GTP are omitted from the reaction (9) but this requires much higher concentrations of Val-tRNA or ethanol (20% v/v) and results in a decrease in codon specificity (unpublished data). This confirms the observation that translation of internal codons by aa-tRNA requires T factor and GTP (6, 10).

Identification of the products of the peptide bond-forming reaction is shown in Figure 2. There are two major peaks of radioactivity, one of which migrates with the marker fmethionine. The second peak migrates at the appropriate rate for an N-blocked monocarboxylic peptide⁸ and has a $^3\text{H}/^{14}\text{C}$ ratio of 0.74, presumptively identifying it as f[^{14}C]methionyl-[^3H]valine. A very small peak of doubly labeled material migrates with the marker fMet-ethyl ester and

FIG. 2.—Product identification: The reaction was incubated at 4°C for 30 min and contained in 0.25 ml: 0.013 M magnesium acetate, 0.11 M ammonium acetate, 0.05 M Tris acetate (pH 7.2), 22.5 pmoles $f[^{14}\text{C}]\text{Met-tRNA}\cdot\text{AUG}\cdot\text{ribosome intermediate}$, 30 μg T factor, 46 pmoles $[^3\text{H}]\text{Val-tRNA}$, 0.75 A_{260} GUA, 0.001 M GTP, and 10% (v/v) ethanol. The reaction was stopped by the addition of 0.5 ml 1.0 N KOH, after which it was incubated at 65°C for 15 min. After cooling, 1.0 ml 0.1 N HCl and 1.5 ml ethyl acetate were added. The mixture was agitated, centrifuged, and the ethyl acetate phase removed; this was evaporated to dryness, redissolved in 0.1 ml water and spotted on Whatman no. 1 paper. Conditions of electrophoresis and counting method are described in *Materials and Methods*. All values have been corrected to yield total product present.

probably represents a combination of this product and small amounts of $f[^{14}\text{C}]\text{Met}\cdot[{}^3\text{H}]\text{valyl-ethyl ester}$. The formation of fMet-ethyl ester in reactions containing fMet-tRNA, ribosomes, tRNA, and ethanol has been demonstrated previously (9). It would appear likely that fMet-Val-ethyl ester could also be synthesized in reactions containing these components and fMet-Val-tRNA.

FIG. 3.—Cation requirements: Each reaction was incubated at 4°C for 5 min and contained the components described in *Materials and Methods*.

concentrations. Increasing Val-tRNA results in a linear increase in fMet-valine synthesis (Fig. 4). Increasing ethanol concentrations up to 20% (v/v) also increases the yield of peptide. However, codon-dependent fMet-valine synthesis has a sharp optimum at 10–12% ethanol and decreases abruptly at higher concentrations (unpublished data). The amount of peptide formed is also dependent on the concentration of T (Tu + Ts) factor, as shown in Figure 5.

Translocation and release of fmethionyl-valine: Translocation of $f[^{14}\text{C}]\text{Met}\cdot[{}^3\text{H}]\text{valyl-tRNA}$, as detected by the formation of fMet-Val-puromycin, occurs only when G factor is added to the reactions (Table 2). If the terminator codon UAA and R2 are substituted for puromycin, terminator codon directed release of fMet-valine also occurs in the presence of G factor (Table 2), confirming previous

The optimal conditions for fMet-valine synthesis using sequential trinucleotides resemble those of oligopeptide synthesizing systems with polynucleotides used as templates,^{10,11,12} except for the absolute requirement for ethanol. Figure 3 demonstrates the ammonium and magnesium requirements. Although maximal fMet-valine synthesis occurs at 0.2 M ammonium, the greatest codon dependency is observed at 0.1 M ammonium, which is the level employed for most of the reported experiments. Other conditions which affect the amount of peptide formed are the Val-tRNA and ethanol

FIG. 4.—tRNA concentration: Each reaction was incubated at 4°C for 20 min and contained in 0.05 ml the components described in *Materials and Methods*, including 3.9 pmoles $f[^{14}\text{C}]\text{Met-tRNA}\cdot\text{AUG}\cdot\text{ribosome intermediate}$.

FIG. 5.—T factor concentration: Each reaction was incubated at 4°C for 4 min and contained in 0.05 ml: 4.5 pmoles $f[^{14}\text{C}]\text{Met-tRNA}\cdot\text{AUG}\cdot\text{ribosome intermediate}$, 4.45 pmoles $[^3\text{H}]\text{Val-tRNA}$, and other components described in *Materials and Methods*.

TABLE 2. Requirements for release of fMet-valine.

Components	$f[^{14}\text{C}]\text{Met-}[^3\text{H}]\text{valine}$ (pmoles)	$f[^{14}\text{C}]\text{Met-}[^3\text{H}]\text{-Val-}$ puromycin (pmoles)
Complete	0.78	1.07
-G factor	0.39	0.24
-R2	0.34	...
-UAA	0.39	...

Reactions for formation of $f[^{14}\text{C}]\text{Met-}[^3\text{H}]\text{valine}$ were incubated at 4°C for 30 min and contained in 0.05 ml the components described in *Materials and Methods* and 9.3 pmoles $[^3\text{H}]\text{Val-tRNA}$. At the end of the initial incubation the following components were added where indicated: 5.1 μg G factor, 0.1 A_{260} UAA, 3.25 μg R2, or 10^{-3} M puromycin. Incubation was continued at 24°C for 30 min; reactions were stopped by the addition of 0.25 ml 0.1 N HCl (termination reactions) or 1.0 ml 0.1 M K-phosphate, pH 8.0 (puromycin reactions); extraction with ethyl acetate was performed as described.⁷

FIG. 6.—Rate of release of fMet-valine: Reactions for formation of $f[^{14}\text{C}]\text{Met-}[^3\text{H}]\text{valine}$ were incubated at 4°C for 30 min, and contained the components designated in *Materials and Methods* and G factor (5.1 μg). Fusidic acid (2×10^{-3} M final concentration) was added to half the reactions. At the end of the initial incubation period, each 0.05 ml reaction received either puromycin (final conc. 1×10^{-3} M) or R1 (10.0 μg) and UAA (0.1 A_{260}). Incubation was continued at 24°C and reactions were stopped by the addition of either 1.0 ml 0.1 M K-phosphate, pH 8.0 (puromycin reaction), or 0.25 ml 0.1 N HCl (termination reactions). Extraction into ethyl acetate was performed as previously described.^{1,7} The values for R1-directed release represent the amount of $f[^{14}\text{C}]\text{Met-}[^3\text{H}]\text{valine}$ released because of added terminator codon.

observations that both termination and puromycin release require peptidyl-tRNA to be in the ribosomal P site.^{13,18,19} Figure 6 demonstrates that fusidic acid inhibits both R1-dependent release of fMet-valine and formation of fMet-Val-puromycin, suggesting that translocation of fMet-Val-tRNA is a specific function of G factor.^{12,14}

Discussion. These studies demonstrate that all the events required for translation of mRNA (initiation, elongation, translocation, and termination) can be analyzed sequentially *in vitro* with trinucleotides used as templates. Under appropriate conditions, AUG-directed binding of fMet-tRNA to ribosomes results in a stable ribosomal intermediate.¹ When GUA, valyl-tRNA, T (Tu + Ts) factor, GTP, and ethanol are added to the fMet-tRNA·AUG·ribosome intermediate, a single peptide bond is formed producing fMet-Val-tRNA. Addition of factor G (translocase) causes translocation of fMet-Val-tRNA to the ribosomal P site after which a second peptide bond (fMet-Val-puro) can be formed or fMet-valine can be released upon addition of a terminator trinucleotide and the appropriate release factor.

There are several advantages of this experimental system. First, each event in protein synthesis can be studied independently and also in sequence. Second, the use of trinucleotides permits varied combinations of codons and aa-tRNA to be employed for the peptide bond forming step. Third, experiments can be performed without preparing long polynucleotides of defined sequence.

It has been previously demonstrated by Monro^{15,16} that N-blocked oligopeptides can be synthesized in the absence of mRNA codons in reactions containing ethanol (33% v/v), fMet-tRNA, ribosomes, and other charged tRNA species. Ethanol is also required for release factor recognition of terminator codons¹⁷ and for R-factor mediated release of fmethionine in the absence of terminator codons.¹³ The ethanol concentration determines the degree of codon dependency in both the termination reaction and in peptide bond formation (unpublished results). High codon dependency is observed at 10% ethanol whereas 20% ethanol abolishes the requirement for codon. It is likely that ethanol stabilizes the interaction of the ribosome, mRNA codon, and either aa-tRNA or release factor.

Earlier studies^{13,19} showed that release of fmethionine from fMet-tRNA·AUG·ribosome intermediates by terminator trinucleotide and release factor required positioning of fMet-tRNA in the ribosomal P site. Release of fMet-valine from ribosomes carrying fMet-Val-tRNA also requires peptidyl-tRNA to be translocated from the A site to the P site of the ribosome. This translocation depends on addition of factor G (translocase) and is inhibited by fusidic acid, a specific antibiotic inhibitor of the translocase enzyme.^{12,14} Translocation of fMet-valyl-tRNA in the trinucleotide directed system demonstrates that covalently linked mRNA codons are not required for translocation of peptidyl-tRNA.

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