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Peptide Chain Termination with Mammalian Release Factor

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Abstract. We report a method for the *in vitro* study of peptide chain termination in mammals. A proteinaceous release factor has been isolated from rabbit reticulocyte extracts. This factor and a polyribonucleotide template containing the bases U and A stimulate the release of *N*-formylmethionine from mammalian [*N*-formylmethionyl-tRNA^f·ribosome] intermediates. Our studies suggest that UAA is a terminator codon for mammalian cells.

Introduction. Release of *N*-formylmethionine (fMet) dependent on terminator codon and a protein release factor, R, from [fMet-tRNA^f·AUG·ribosome], the complex which initiates protein synthesis in *E. coli*, has been used for the *in vitro* study of peptide chain termination in bacteria.[1] This communication describes how a similar approach has been used for examining peptide chain termination in mammalian extracts.

Several reports indicate that mammalian cells contain two species of tRNA^{Met} (references 2, 3). The major species, tRNA^{fMet}, resembles the bacterial initiator tRNA in its codon recognition (AUG and GUG),[4] ability of its methionine to be formylated by the bacterial transformylase,[2,5] and its high affinity for the *E. coli* ribosomal site of initiation (P-site).[4] In the present studies, we find that fMet-tRNA^f can interact with the initiator site of rabbit reticulocyte ribosomes, resulting in the formation of a [fMet-tRNA^f·ribosome] intermediate. Release of fMet from this intermediate requires a specific RNA template and a protein release factor from reticulocytes, and is stimulated by GTP.

Materials and Methods. Preparation of reticulocyte relase factor and ribosomes: The high speed supernatant fraction (105,000 × *g* for 3 hr) is prepared from a rabbit reticulocyte lysate by the procedure of Allen and Schweet,[6] except that the lysis buffer contained 2 mM $MgCl_2$; 0.1 mM EDTA; and 3 mM DTT. Factor R is partially purified from the high speed supernatant fraction by ammonium sulfate (0–40%) precipitation; column chromatography on Sephadex G-150 (R activity occurs in the excluded volume); and DEAE-Sephadex column chromatography (R elutes with 0.4 M KCl). These purification steps remove an enzyme which converts fMet-tRNA^f to fMet in the absence of either ribosomes or poly (U,A). A detailed description for R purification will be the subject of a separate communication.

The ribosomal pellet from the high speed centrifugation is suspended in 0.1 volume of the original lysate and stirred for 16 hr at 4°C in a solution containing 0.5 M KCl; 0.05 M Tris-chloride, pH 7.4; 0.002 M $MgCl_2$; 0.03 M β-mercaptoethanol; and 15% glycerol, then centrifuged at 105,000 × *g* for 3 hr. The ribosomal pellet is then suspended in a

buffer containing 0.25 M sucrose; 0.002 M $MgCl_2$; 0.03 M KCl; 0.05 M Tris-chloride, pH 7.4; 0.003 M β-mercaptoethanol and is stored at −170°C after a low speed centrifugation (30,000 × *g* for 15 min). This procedure is a modification of one previously described by McKeehan and Hardesty.[7]

Preparation of f[^{3}H]-Met-tRNA^f (*E. coli* or mammalian) and [^{3}H]-Met-tRNA^f (*E. coli* or mammalian): The tRNA^{fMet} (*E. coli*) is supplied by an interagency agreement through the National Institute of General Medical Sciences (NIGMS), and tRNA^{fMet} (mammalian) is prepared from unfractionated tRNA of guinea pig liver[4] by benzoylated DEAE-cellulose column chromatography.[3] The acylation and formylation of the tRNA^{fMet} (*E. coli* or mammalian) has been described previously.[2] Leucovorin is omitted in the preparation of Met-tRNA^f. The methionine attached to f[^{3}H]-Met-tRNA^f (*E. coli* or mammalian) is completely formylated; the [^{3}H]-Met-tRNA^f (*E. coli* or mammalian) is less than 3% formylated. Methionine acceptance by tRNA^{fMet} (*E. coli*) and (mammalian) is 1443 and 46 pmol/A_{260} unit, respectively. The tRNA^{Val} (*E. coli*) and tRNA^{Phe} (*E. coli*) mentioned in the results section were supplied through NIGMS and accepted 1310 and 880 pmol per A_{260} unit, respectively.

Preparation of reticulocyte ribosomal intermediates: The f[^{3}H]-Met-tRNA^f (*E. coli*)·ribosome intermediates are prepared with reticulocyte ribosomes. The complex is formed during a 12 min incubation at 24°C in a 0.1 ml reaction mixture containing the following components: 0.06 M Tris-chloride, pH 7.2; 0.055 M $MgCl_2$; 0.085 M NH_4Cl; 12 A_{260} units of reticulocyte ribosomes; and 46 pmol f[^{3}H]-Met-tRNA^f (*E. coli*). Under these conditions, 42 pmol of f[^{3}H]-Met-tRNA^f (*E. coli*)·ribosome intermediate is formed, as determined by the method of Nirenberg and Leder.[8] An aliquot of this intermediate (10 μl) remains stable and 100% reactive with puromycin at 24°C, for at least 30 min, when diluted to a final volume of 0.05 ml and a final concentration of 0.02 M Mg^{2+}.

Assay of fMet-puromycin and Met-puromycin: The quantitation of f[^{3}H]-Met-puromycin and [^{3}H]-Met puromycin formed is by the method of Leder and Bursztyn.[9]

fMet release assay: Each reaction is incubated for 20 min at 24°C and contains, in a final volume of 0.05 ml; 0.05 M Tris-chloride, pH 7.2; 0.02 M $MgCl_2$; 0.05 M NH_4Cl; 0.1 mM GTP; 4.2 pmol of f[^{3}H]-Met-tRNA^f (*E. coli*)·reticulocyte ribosome intermediate; 6–60 μg protein of a partially purified R factor from reticulocytes; and 0.225 A_{260} units poly (U,A_3) as indicated. The f[^{3}H]-Met released is determined as previously described,[1] except that samples are counted in Bio-solv scintillation fluid (Beckman Instruments, Inc.) at an efficiency of 60%.

Materials. The [^{3}H-methyl]-methionine isotopes (5.4 Ci/mmol and 3.3 Ci/mmol) were purchased from Amersham/Searle Corp. and from Schwarz BioResearch respectively. Poly U, poly A, and poly C were purchased from Miles. Other polyribonucleotides were synthesized by the method of Singer and Guss.[10] Each polynucleotide used in these studies was shown to be an active template for directing the binding of the appropriate radioactive aminoacyl tRNA to *E. coli* ribosomes (experiments not shown). Preparation and sequence determination of trinucleotides is by a previously described method.[11]

Results. Both mammalian and *E. coli* Met-tRNA^f and fMet-tRNA^f bind strongly to reticulocyte ribosomes at 0.055 M Mg^{2+} without added oligo- or polyribonucleotide mRNA templates. Highly purified Val-tRNA (*E. coli*) and Phe-tRNA (*E. coli*) bind 8- and 4-fold less well under identical conditions (unpublished data), indicating a specificity of tRNA species for this ribosomal interaction. We have compared the puromycin reactivity of fMet- or Met-tRNA^f·ribosome intermediates (Fig. 1). Less than 10% of the ribosomal-bound Met-tRNA^f reacts with puromycin to form Met-puromycin while all of the ribosomal-bound fMet-tRNA^f is converted to fMet-puromycin in less than 5 min. No detectable difference between the puromycin reactivity of mammalian and *E. coli* fMet- or Met-tRNA^f is observed.

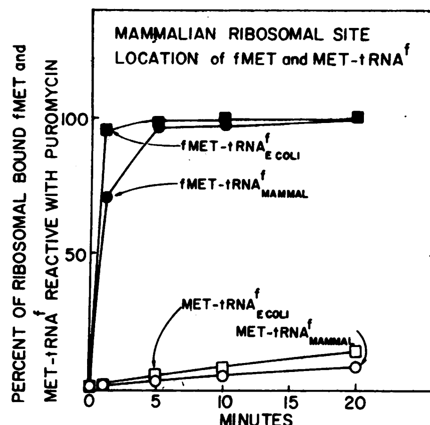

FIG. 1.—Mammalian ribosomal site localization of fMet- and Met-tRNA^f. The [fMet- or Met-tRNA^f·ribosome] intermediates are formed as described in *Methods* except for the following: 0.72 A_{260} unit reticulocyte ribosomes; and, as indicated, 1.43 pmol f[^{3}H]-Met-tRNA^f (mammalian), 1.39 pmol [^{3}H]-Met-tRNA^f (mammalian), 1.95 pmol f[^{3}H]-Met-tRNA^f (*E.* coli), or 1.10 pmol [^{3}H]-Met-tRNA^f (*E. coli*). The amount of [fMet- or Met-tRNA^f·ribosome] intermediate formed for the four reactions, determined as described in *Methods*, is, respectively, 1.10 pmol f[^{3}H]-Met-tRNA^f (mammalian); 0.92 pmol [^{3}H]-Met-tRNA^f (mammalian); 1.95 pmol f[^{3}H]-Met-tRNA^f (*E. coli*); and 1.10 pmol [^{3}H]-Met-tRNA^f (*E. coli*)·ribosome intermediates.

Each puromycin reaction is incubated at 24°C for the indicated time, and contains in a final volume of 0.05 ml the following components: 0.05 M Tris-chloride, pH 7.2; 0.02 M $MgCl_2$; 0.05 M NH_4Cl; 10^{-3} mM puromycin; and the above indicated [f[^{3}H]-Met- or [^{3}H]-Met-tTNA^f·ribosome] intermediate added in 0.01 ml. The 100% puromycin value corresponds to the amount (pmol) of [f[^{3}H]-Met- or [^{3}H]-Met-tRNA^f·ribosome] intermediate added above.

Requirements for the release of f[^{3}H]-Met from f[^{3}H]-Met-tRNA^f·ribosomes are shown in Table 1. Release of fMet is dependent upon ribosomes, R, and a specific polyribonucleotide template. GTP stimulates release 2-fold, while the GTP analog, GDPCD, completely inhibits release. The addition of GDP, GMP, and ATP has no effect on fMet release. In experiments not shown, the addition of GTP (0.1 mM, final) did not affect the rate of formation of fMet-puromycin, suggesting that the GTP stimulation of fMet release is related to events in peptide chain termination and not to the [fMet-tRNA^f·ribosome] intermediate. Under the conditions used for release reactions, we find that R and poly (U,A) stimulate release from fMet-tRNA^f·ribosomes and not from Met-

TABLE 1. *Requirements for fMet release.*

Reaction	f[^{3}H]-Met, Δ pmol
Expt. 1	
Complete	1.35
– Poly UA	0.14
– Ribosomes	0.02
– R	0.04
– Poly UA, – R	0.00
– Poly UA, – ribosomes	0.00
Expt. 2	
Complete	1.13
– Poly UA	0.12
– GTP	0.53
– GTP, + GDP	0.55
– GTP, + GMP	0.48
– GTP, + GDPCP	0.08
– GTP, + ATP	0.53

In Expt. 1, the complete release reaction is carried out as described in *Methods* with 30 μg R; omissions are indicated. The fMet extracted at zero time (0.58 pmol) is subtracted from each value.

In Expt. 2, the indicated nucleotides are added at 10 μM. The fMet extracted in the absence of R (0.50 pmol) is subtracted from each value.

tRNA^f·ribosomes. These observations are similar to those in *E. coli*, and indicate that in order to participate in polypeptide chain termination, peptidyl-tRNA or fMet-tRNA^f must be in the ribosomal site that permits reactivity with puromycin[12-14].

The fMet-tRNA^f of either mammalian or *E. coli* origin can be used for the study of fMet release *in vitro* by mammalian R factor and mammalian ribosomes, as well as for study of fMet-puromycin formation by mammalian ribosomes (Table 2). We have routinely used [fMet-tRNA^f (*E. coli*)·ribosome] intermedi-

TABLE 2. *Comparison of reticulocyte ribosomal intermediates.*

	f[^{3}H]-Met-tRNA^f Ribosomal Intermediates	
Reaction	Mammalian fMet-tRNA^f, Δ pmol	*E. coli* fMet-tRNA^f, Δ pmol
fMet-tRNA^f, ribosomal binding	1.38	2.78
fMet-puromycin formation	1.39	2.70
fMet release, poly UA-dependent	0.42	1.00

Ribosomal binding: Each reaction is carried out as described in *Methods* except for the following: 0.72 A_{260} unit ribosomes; and, as indicated, 1.50 pmol f[^{3}H]-Met-tRNA^f (mammalian) or 2.80 pmol f[^{3}H]-Met-tRNA^f (*E. coli*). Ribosomal-bound fMet-tRNA^f determined by the method of Nirenberg and Leder.[8]

fMet-puromycin formation: Each reaction is carried out as described in Fig. 1 except for the following: as indicated, 1.38 pmol [f[^{3}H]-Met-tRNA^f (mammalian)·ribosome] intermediate or 2.78 pmol [f[^{3}H]-Met-tRNA^f (*E. coli*)·ribosome] intermediate added in 0.01 ml. Radioactivity extracted in the absence of puromycin (0.03 pmol) is subtracted from each value.

fMet release: Each reaction is carried out as described in *Methods* except for the following: 10 μM GTP; 45 μg reticulocyte R factor; and as indicated, 1.38 pmol [f[^{3}H]-Met-tRNA^f (mammalian)·ribosome] intermediate or 2.70 pmol [f[^{3}H]-Met-tRNA^f (*E. coli*)·ribosome] intermediate added in 0.01 ml. The fMet extracted in the absence of poly (U,A_3) (0.28 pmol, mammalian; 0.42 pmol, *E. coli*) is subtracted from each value.

ates since more fMet-tRNA^f (*E. coli*) is bound to a fixed level of ribosomes than with the less purified fMet-rRNA^f (mammalian).

The quantity of R factor affects both the rate (unpublished data) and extent of fMet release. The extent of release is proportional to the amount of R between 0 and 40 μg protein (Fig. 2). We routinely measure release at 20 min. At saturating levels of R, 65% of the ribosomal-bound fMet-tRNA^f is converted to fMet.

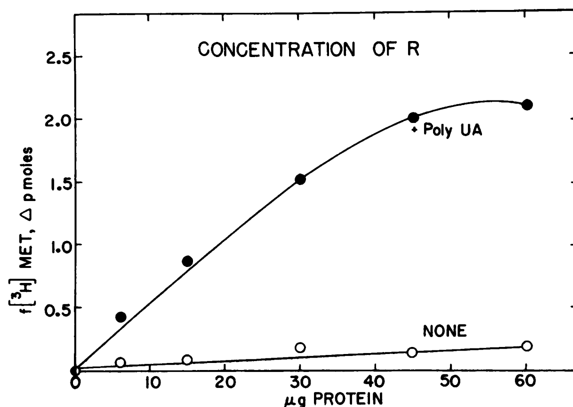

FIG. 2.—Relation between concentration of R and extent of fMet release. Each release reaction is carried out as described in *Methods* with 6–60 μg R as indicated. The fMet extracted (0.54 pmol) in the absence of R is substracted from all values.

The sensitivity of R to inactivation is shown in Table 3. R is inactivated by incubation at 55°C, phenol extraction, and *N*-ethylmaleimide alkylation. This data suggests that the R preparation contains at least one protein with an essential sulfhydryl group.

The codon specificity for fMet release is shown in Table 4. Three polymers,

TABLE 3. *Inactivation of reticulocyte R factor.*

Treatment of R	Poly (U,A)-dependent f[^{3}H]-Met released, Δ pmol
1. Control	1.55
55°C, 10 min	0.21
2. Control	0.95
Phenol treatment	0.00
3. Control	1.04
N-Ethylmaleimide treatment	0.13

Each release reaction is carried out as described in *Methods* with 60 μg R. The fMet extracted in the absence of poly (U,A_3) (0.50 pmol) is subtracted from each value. In Expt. 2, 300 μg R, in a volume of 0.10 ml, is extracted with an equal volume of water-saturated phenol and 0.1 volume of 20% potassium acetate. The aqueous phase of the above mixture is dialyzed for 4 hr against 0.02 M Tris-chloride, pH 7.5–3mM DTT buffer, and 0.020 ml is then assayed. The control is treated identically, except for the omission of phenol. In Expt. 3, 300 μg R, in a volume of 0.10 ml, is incubated with 10mM *N*-ethylmaleimide for 15 min at 24°C in a final volume of 0.105 ml, then β-mercaptoethanol is added (0.3 M, final concentration); incubation is continued for 5 min before assay for R activity. The control is treated identically, except the *N*-ethylmaleimide added was inactivated with β-mercaptoethanol before addition.

TABLE 4. *Polyribonucleotide specificity for mammalian release.*

Poly- or oligoribonucleotide	f[^{3}H]-Met, Δ pmol		
	Expt. 1	Expt. 2	Expt. 3
(None)	(0.22)	(0.08)	(0.50)
Poly (U,A_3)	0.73	1.07	0.96
Poly (U,A_3, $G_{0.3}$)	0.77		
Poly (U,G_2)	0.25		
Poly U	0.00		
Poly A	0.01		
Poly (A_6,G)	0.05		
Poly (A,C)	0.03		
Poly C		0.00	
Poly (U,C_2)		0.02	
Poly (U_4,C_2, G)		0.00	
Poly (A_3,C_5, G)		0.00	
UAA			−0.16
UAG			−0 07
UGA			−0.03

Reactions are carried out as described in *Methods* except for the following: 0.225 A_{260} unit poly- or trinucleotide and 30 μg R (Expts. 1 and 2) or 45 μg R (Expt. 3). fMet release that occurs in the absence of oligo- or polyribonucleotide (enclosed in parentheses), as well as fMet extracted at zero time (0.45 pmol), is subtracted from each value. Poly (U,G_2) had no detectable A content (detection level 0.5%) upon alkaline degradation and base analysis.

poly (U,A_3), poly (U,A_3,$G_{0.3}$), and poly (U,G_2), stimulate release, whereas a number of other randomly ordered polyribonucleotides have no detectable effect. In experiments not shown, poly (U,G_2) is 30% as active as poly (U,A_3) in directing fMet release from *E. coli* [fMet-tRNA^f·AUG·ribosome] intermediates with either *E. coli* R1 or R2. Since *E. coli* R1 and R2 recognize terminator codons that contain U and A (R1, UAA or UAG; R2, UAA or UGA) but not trinucleo-

tides that contain U and G (UGU, UGG, GUG, UUG, GGU and GUU) (refs. 1 and 15 and unpublished data), and poly (U,G_2) response both in *E. coli* and mammalian extracts probably represents ambiguous codon recognition.

Ribosomal specificity for mammalian and bacterial R factors has been examined in Table 5. Reticulocyte R factor has no detectable release activity with *E. coli* ribosomes. We are, however, able to detect a small but reproducible fMet release with *E. coli* R factors and reticulocyte ribosomes.

The effect of antibiotics on release and peptide bond formation with mammalian ribosomes is compared in Table 6. Sparsomycin and gougerotin, antibiotics

TABLE 5. *Ribosomal specificity for R factors.*

Polynucleotide	f[³H]-Met, Δ pmol Source of fMet-tRNA·Ribosome Intermediate: *E. coli*	Reticulocyte
	Reticulocyte R Factor	
None	0.35	0.50
Poly UA	0.35	1.47
	E. coli R1	
None	0.06	0.01
Poly UA	2.50	0.04
	E. coli R2	
None	0.01	0.03
Poly UA	1.30	0.11

Release from *E. coli* ribosomal intermediate: Each release reaction is incubated at 24°C for 20 min and contains in 0.05 ml: 0.05 M Tris-chloride, pH 7.2; 0.02 M $MgCl_2$; 0.05 M NH_4Cl; 0.1 mM GTP; 0.225 A_{260} unit poly (U,A_3); 4.2 pmol *E. coli* [f[³H]-Met-tRNA^f·AUG·ribosome] intermediate,[1] and as indicated, 30 μg reticulocyte R, 15.3 μg *E. coli* R1 (Fraction VI),[22] or 17.8 μg *E. coli* R2 (Fraction VII).[22] The fMet extracted in the absence of R (0.80 pmol) is subtracted from each value

Release from reticulocyte ribosomal intermediate: Each release reaction is carried out as described in *Methods* with 30 μg reticulocyte R, 15.3 μg *E. coli* R1 (Fraction VI),[22] or 17.8 μg *E. coli* R2 (Fraction VII).[22] The fMet extracted in the absence of R (0.50 pmol) is subtracted from each value.

TABLE 6. *Antibiotic effects on release and peptide bond formation with reticulocyte ribosomes.*

Antibiotics added	% Control: Poly (U,A)-dependent f[³H]-Met	f[³H]-Met-puromycin
None	100	100
Sparsomycin	0	0
Gourgerotin	62	47
Lincomycin	105	86
Amicetin	102	87
Chloramphenicol	96	94
Erythromycin	102	87
Streptomycin	99	112
Tetracycline	104	95
Fusidic acid	104	95

Assays are carried out as described in *Methods* with 30 μg R. The poly (U,A_3)-dependent f[³H]-Met released in the absence of antibiotics is 1.00 pmol. The fMet extracted in the absence of R (0.70 pmol) is subtracted from each value.

Puromycin reactions are incubated at 24°C for 4 min and contain, in 0.05 ml: 0.05 M Tris-chloride, pH 7.2; 0.02 M $MgCl_2$; 0.05 MNH_4Cl; 4.2 pmol [f[³H]-Met-tRNA^f (*E. coli*)·ribosome] intermediate; antibiotics as indicated; and 60 μM puromycin. 2.3 pmol of f[³H]-Met-puromycin is formed in the absence of antibiotics. Radioactivity extracted in the absence of puromycin (0.13 pmol) is subtracted from each value.

Final antibiotic concentration for both reactions is 10^{-4} M, except for gougerotin and fusidic acid which are assayed at 10^{-3} M. There was no effect of these antibiotics on the [fMet-tRNA^f·ribosome] intermediate, as determined by the method of Nirenberg and Leder.[8]

shown earlier by Monro to inhibit peptidyl transferase activity of 60S ribosomal subunits,[16] also inhibit mammalian peptide chain termination.

Discussion. Mammalian peptide chain termination appears to possess characteristics fundamentally similar to those described for bacteria. In both rabbit reticulocyte and *E. coli* extracts, the terminal event in protein synthesis requires terminator codon recognition, involves protein release factor(s), and occurs on ribosomes.

On the basis of genetic and biochemical data[17,18] three terminator codons have been identified for *E. coli:* UAA, UAG, and UGA. In the present studies, three polyribonucleotide templates—poly (U,A), (U,A,G), and (U,G)—stimulate mammalian peptide chain termination. The data suggest that the poly (U,G) response represents an example of a translational error that involves R factor. If such translational errors occur *in vivo,* the result would be peptide chain termination independent of terminator codons. One terminator codon for mammalian cells can be assigned from the present studies. The minimal base composition of this codon is U and A, but the possible occurrence of mammalian terminator codons consisting of U, A, and G cannot be excluded. Three of the six RNA codons containing only A and U—UAU (*tyr*), AUA (*ile*), and AUU (*ile*)—have been demonstrated to correspond to amino acids in mammalian cells.[19] Two additional codons, AAU and UUA, can be indirectly assigned to aspartic acid and leucine, since their synonym codon assignments, AAC (*asp*) and UUG (*leu*), have been determined for mammalian cells.[20,21] UAA is the only one of the six codons that is unassigned, and therefore probably corresponds to a terminator codon in mammal. In other studies with rabbit reticulocyte extracts, Gupta found that the codons UAG and UGA did not correspond to any of 20 amino acids.[21] Thus, in addition to UAA, UAG and UGA may also be mammalian terminator codons. The existence of terminator codons for mammalian cells makes predictable the occurrence of premature chain termination mutations (nonsense). Furthermore, since suppressors are known for correction of nonsense mutations (UAA, UAG, UGA)[17] in bacteria, it is likely that mammalian cells possess this same potential.

Three protein factors are known to participate in *E. coli* chain termination: the two codon-specific release factors (R1, UAA or UAG; and R2, UAA or UGA)[21] and S factor,[22,23] which stimulates the release of fMet in the presence of GTP.[24] Since fMet release by our reticulocyte R preparation is stimulated by GTP, this preparation, by analogy, probably contains a stimulatory as well as a release factor(s).

Peptide release in *E. coli* is inhibited by antibiotics which inhibit the peptidyltransferase, an enzyme associated with the 50S ribosomal subunit.[12-14] Similarly, our studies on mammalian ribosomes indicate that antibiotics which inhibit peptidyl transferase activity also inhibit release activity. These data add further support to the idea,[25-27] but do not prove, that peptidyl transferase participates in the hydrolysis of peptidyl tRNA that occurs upon peptide chain termination.

While it is clear that the substrate used in these studies, fMet-tRNA^f·reticulocyte ribosomes, is suitable for examining *in vitro* mammalian peptide chain termination, the significance of this intermediate in mammalian initiation is less

certain. To date, no mammalian enzyme capable of converting Met-tRNA^f to fMet-tRNA^f has been identified. Furthermore, recent reports indicate that Met-tRNA^f, and not fMet-tRNA^f, initiates protein synthesis in mammalian extracts.[3,5]

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Abbreviations used: DTT, dithiothreitol; GDPCP, 5′-guanylylmethlenediphosphate; fMet, *N*-formylmethionine.

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