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Mammalian Peptide Chain Termination, II. Codon Specificity and GTPase Activity of Release Factor

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ABSTRACT ***In vitro* peptide chain termination with release factor preparations from rabbit reticulocytes, guinea pig liver, or Chinese hamster liver is directed with UAAA, UAGA, or UGAA, suggesting that UAA, UAG, and UGA are terminator codons for mammalian cells. Purified release factor from rabbit reticulocytes has ribosomal-dependent GTPase activity, which is stimulated by UAAA. GTP hydrolysis appears requisite for *in vitro* peptide chain termination in mammals.**

The process of peptide chain termination has been studied in mammalian extracts by slight modifications of the formylmethionine (fMet) release assay (1) described for bacterial extracts (2). The release of fMet from fMet-tRNA·ribosome (rabbit reticulocyte) intermediates is directed by mRNA [poly(U,A) or poly(U,A,G)], requires reticulocyte release factor (R), and is stimulated by GTP (1).

In this paper, we use tetranucleotides of defined sequence to study chain termination in mammalian extracts. The use of small, defined oligonucleotides has enabled the assignment of the mammalian terminator codons. Purified rabbit reticulocyte R was found to have an associated ribosomal-dependent GTPase activity.

MATERIALS AND METHODS

Release assay

The f[^{3}H]Met-tRNA$_f$·ribosome intermediates are prepared as described (1), with *Escherichia coli* f[^{3}H]Met-tRNA$_f$ and rabbit reticulocyte ribosomes. The intermediates are stable when stored at −170°C. A typical release reaction incubated 10 min at 24°C contains, in 0.05 ml: 0.02 M Tris-chloride, pH 7.4; 0.06 M potassium chloride; 11 mM magnesium chloride; 3.0 pmol of f[^{3}H]Met-tRNA$_f$·ribosome intermediates; R from a mammalian extract; 0.1 mM GTP; 0.1 A_{260} unit of oligonucleotide; and other additions as indicated. Bacterial release reactions and quantitation of f[^{3}H]Met have been described (2).

Polyphenylalanine polymerization

Ribosomes for polymerization studies are washed twice (1). Each reaction incubated 20 min at 37°C contains, in 0.05 ml: 0.02 M Tris-chloride, pH 7.4; 0.10 M potassium chloride; 8 mM $MgCl_2$; 1 mM dithiothreitol (DTT); 0.20 A_{260} unit of poly(U); 15 mM phosphoenolpyruvate; 0.1 mM GTP; 0.2 unit of pyruvate kinase (Calbiochem); 1.0 A_{260} unit of ribosomes; 6.9 pmol of unfractionated [^{3}H]Phe-tRNA (*E. coli*); and rabbit reticulocyte transfer factors as indicated. Phenylalanine polymerized is determined as described (3).

GTP hydrolysis

Hydrolysis of [γ-^{32}P]GTP is determined according to Kolakofsky *et al.* (4) except that 1.5 ml of the organic phase is counted in 9.4 ml of PPO–POPOP–toluene and 0.6 ml of NCS (Amersham/Searle). Reactions incubated 15 min at 24°C contain, in 0.05 ml: 0.010 M Tris-chloride, pH 7.4; 30 mM NH_4Cl; 15 mM $MgCl_2$; 1.5 A_{260} units of twice-washed reticulocyte ribosomes; 9 μM [γ-^{32}P]GTP; reticulocyte R; and other additions as indicated. The ribosomal-dependent GTPase activity of R is optimal at 37°C in 30 mM NH_4Cl–15 mM $MgCl_2$.

Reticulocyte release factor purification

A high-speed supernatant (Fraction I) from rabbit reticulocyte lysate is prepared (1) and R factor is collected by ammonium sulfate precipitation (60% saturation, 42.4 g/100 ml) at 5°C, pH 7.5. The ammonium sulfate precipitate (Fraction II) is dissolved in buffer A [0.1 M potassium chloride–0.02 M Tris-chloride (pH 7.8)–1 mM DTT–0.1 mM EDTA], dialyzed against 50 volumes of buffer A for 12 hr, and applied to a DEAE-Sephadex column equilibrated with buffer A. After sample application, the column is washed with 2.5 column volumes of buffer A before the gradient for elution of R is begun. Typical columns and gradients were 2.5 × 40 cm with 1600 ml and 5 × 50 cm with 2400 ml for 3.36 and 24.9 g of protein, respectively. All gradients are linear from 0.1 to 0.7 M KCl and contain the additional components of buffer A. Active fractions are identified (Fraction III) as described in the legend to Fig. 2*A* and concentrated by pressure filtration (Amicon Co.).

Phosphocellulose (P11, Whatman) is washed twice with 0.5 N KOH, followed by 0.5 N hydrochloric acid. R factor (Fraction III) is dialyzed against buffer B [0.10 M potassium chloride–0.050 M Tris-chloride (pH 7.9)–1 mM DTT–0.1 mM EDTA] and applied to phosphocellulose columns equilibrated in buffer B. After sample application, 1.5 column volumes of buffer B are washed through the column, then gradient elution of R is begun. Typical columns and gradients were 2.5 × 33 cm with 1200 ml and 5 × 33 cm with 1600 ml for 300 and 990 mg of protein, respectively. All gradients are linear from 0.1 to 0.6 M KCl and contain the additional components of buffer B. Active fractions are determined

Abbreviations: DTT, dithiothreitol; GDPCP, 5'guanylylmethylenediphosphonate.

Paper I of this series is ref. 1.

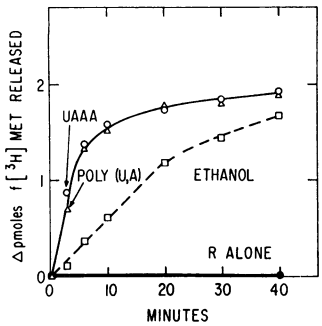

FIG. 1. Reticulocyte R Activity. At the indicated time intervals, f[³H]Met released from a 0.03-ml portion of a 0.25-ml reaction was determined. Each tube was incubated at 24°C and contained: 15.25 pmol of f[³H]Met tRNA$_f$·ribosome intermediates; 65 μg of reticulocyte R (Fraction V); 0.1 mM GTP; 1.0 A_{260} unit poly(A$_3$,U) (△), or 0.5 A_{260} unit UAAA (○), or 20% ethanol (v/v) (□), or no template (●); and other components as described in *Methods*. The zero time value of 0.18 pmol was subtracted from all values.

(Fraction IV) as shown in Fig. 2*B*, pooled, and concentrated as above.

Fraction IV (4.0 ml) is applied to a 2.5 × 92 cm Sephadex G-200 column equilibrated in buffer C [0.05 M KCl–0.02 M Tris-chloride (pH 7.4)–1 mM DTT–0.1 mM EDTA]. The column void volume (elution volume for dextran blue) is 142.5 ml, and R factor is eluted at 213 ml. The R activity is determined as described in the legend to Fig. 2*C*, pooled, concentrated, and stored at −170°C in buffer C (Fraction V).

Guinea pig and Chinese hamster R factor purification

Guinea pig extract was prepared from the livers of male Hartley guinea pigs by homogenization (60 sec, Omnimixer) with 1.5 volumes (ml/gm) of buffer C with 5 mM $MgCl_2$ followed by successive 30,000 × *g* (15 min) and 100,000 × *g* (3 hr) centrifugation. The supernatant from these centrifugations corresponds to Fraction I, and was purified as described through the phosphocellulose column chromatography step. Chinese hamster extract was prepared in identical fashion to that of guinea pig, except that the liver was homogenized with a loose-fitting tube and pestle.

Purification of transfer factors

Transfer factor activities (T_1 and T_2) are determined by phenylalanine polymerization. Transfer factor T_2 (translocase) was isolated from the DEAE-Sephadex chromatography of rabbit reticulocyte R (Fraction II). Transfer factor T_2 elutes prior to R factor. Transfer factor T_1 was partially separated from R by phosphocellulose chromatography of Fraction III. Transfer factor T_1 elutes immediately after R factor. The T_1 was further purified by Sephadex G-200 chromatography to remove any remaining R. T_1 elutes at 143 ml from G-200.

Synthesis and purification of oligonucleotides

The oligonucleotide sets UAA and UAAA, and UGA and UGAA, were isolated from two reactions optimized for enzymatic synthesis of tri- and tetranucleotides with polynucleotide phosphorylase, the doublets UA or UG, and ADP (5). The tetranucleotide UAGA was synthesized in two steps. Initially, UAG was synthesized and isolated as described (6). The UAGA was synthesized with polynucleotide phosphorylase, UAG, and ADP. The preparation of the tetranucleotides UUUU and AAAA was described (7). The nucleotide components of all reactions were purified by DEAE-cellulose chromatography as described by Petersen (8), except that urea was omitted. The tri- and tetranucleotides isolated by this method have less than 2% contamination with ultraviolet-absorbing material (5). T_2 ribonuclease digestion and base-ratio analysis were previously described (5).

Protein was determined by the method of Lowry (9). Amino acid isotopes used were [³H-methyl]L-methionine (7.0 Ci/mmol, Amersham/Searle) and [³H]L-phenylalanine (5.32 Ci/mmol, Schwarz BioResearch Corp). [γ-³²P]GTP, prepared by the method of Glynn and Chappell (10), was supplied by Dr. Michael Cashel. Randomly ordered poly(A,G,U) (1:1:1) and GDPCP were purchased from Miles Laboratories. Sparsomycin was supplied by the National Cancer Institute; fusidic acid by Squibb; anisomycin by Pfizer; cycloheximide by Sigma; gougerotin, rabbit muscle pyruvate kinase, and *Candida krusei* cytochrome *c* by Calbiochem; egg-white ovalbumin by Worthington; and rabbit muscle aldolase by Boehringer–Mannheim.

RESULTS

The activity of release factor from mammalian cells can be determined with randomly ordered mRNA templates, tetranucleotides of defined sequence, or ethanol (Fig. 1). In each case, *in vitro* peptide termination is determined by hydrolysis of f[³H]Met-tRNA (*E. coli*) bound to ribosomes (rabbit reticulocyte). The tetranucleotide UAAA and poly(A$_3$,U) direct release of all available f[³H]Met in 40 min (Fig. 1). Although the template activities of the two appear equal in

TABLE 1. *R-factor codon specificity*

Oligonucleotide added	Source of R: Rabbit reticulocyte	Guinea pig liver	Chinese hamster liver	*E. coli* R1	*E. coli* R2
UAAA	**1.11***	**0.82**	**0.86**	**2.11**	**0.97**
UGAA	**0.85**	**0.84**	**0.92**	0.12	**1.53**
UAGA	**0.48**	**0.50**	**0.34**	**1.75**	0.06
UAA	0.00	0.04	0.05	**2.35**	**1.49**
UGA	0.01	0.05	0.06	0.13	**1.86**
UAG	0.00	0.04	0.06	**2.43**	0.05
UUUU	0.01	0.05	0.06	0.11	0.06
AAAA	0.01	0.05	0.06	0.11	0.05
None	0.00	0.03	0.07	0.09	0.04

* All results given as Δpmol of f[³H]Met released; boldface values are considered significant.

R activity with mammalian factors was determined at 10 min, 24°C, in 0.05-ml reactions containing: 3.05 pmol of f[³H]Met-tRNA$_f$·ribosome (reticulocyte) intermediates; the indicated 0.1 A_{260} unit oligonucleotide, and, where indicated, 2.8 μg of rabbit reticulocyte R (Fraction V); 6.6 μg of guinea pig liver R (Fraction IV), or 20 μg of Chinese hamster liver R (Fraction IV); and other components described in *Methods*.

E. coli R1 and R2 activity were determined at 15 min, 24°C, in 0.05-ml reactions containing: 3.74 pmol of f[³H]Met-tRNA$_f$·AUG·ribosome (*E. coli*) intermediates; and, where indicated, 0.1 A_{260} unit oligonucleotide; 10.2 μg of R1 (Fraction VI) or 11.8 μg of R2 (Fraction VII); other components as described (2). Zero time values (0.25 and 0.17 pmol) were subtracted from all reticulocyte and *E. coli* values, respectively.

TABLE 2. *Reticulocyte R-factor purification*

	Fraction	Protein (mg)	Specific activity*				% Yield
			UAAA	UAGA	UGAA	poly(A,G,U)	poly(A,G,U)
I	S100	14,796	...	...	...	...	...
II	Ammonium sulfate	1,822	...	...	...	...	...
III	DEAE-Sephadex	294	23.0	2.78	29.2	13.9	100
IV	Phosphocellulose	7.2	311	87.2	342	198	35
V	Sephadex G-200	1.2	879	214	817	572	17

* pmol f[^{3}H]Met per mg per 10 min.

For calculation of specific activity, the rate of f[^{3}H]Met release was determined at 7 min, 24°C, in reactions containing limiting levels of R, 2.92 pmol of (f[^{3}H]Met-tRNA$_f$·ribosome) intermediates, 0.1 A_{260} unit tetranucleotide or 0.2 A_{260} unit poly(A,G,U), 0.1 mM GTP, and other components as described in *Methods*. The percent yield is the cumulative recovery based on the activity of Fraction III. Activity was calculated as the increase in f[^{3}H]Met release due to the addition of oligonucleotide.

Fig. 1, UAAA is more effective than poly(A$_3$,U) in stimulating f[^{3}H]Met release in studies where R is limiting. Purified R (Fraction IV) is required to observe f[^{3}H]Met release in the presence of ethanol, methanol (not shown), or acetone (not shown).

The oligonucleotide specificity for peptide chain termination with mammalian and bacterial R preparations is summarized in Table 1. The activity of rabbit reticulocyte, guinea pig liver, and Chinese hamster liver R preparations is determined with f[^{3}H]Met-tRNA·ribosome (reticulocyte) intermediates. *E. coli* R1 and R2 activities are determined with f[^{3}H]Met-tRNA·AUG·ribosome (*E. coli*) intermediates. All of the mammalian R preparations participate in fMet release with the tetranucleotides UAAA, UAGA, and UGAA, but not with AAAA and UUUU. The corresponding terminator trinucleotides UAA, UAG, UGA, and UAG$_p$ (last not shown) have no detectable template activity with these mammalian preparations. The addition of an A residue to the 3′-end of a terminator trinucleotide enables us to determine mammalian terminator codons without apparently altering the specificity of recognition of the terminator trinucleotide portion, as determined with *E. coli* R factors. R1 recognizes UAA, UAAA, UAG, or UAGA. R2 recognizes UAA, UAAA, UGA, or UGAA. The influence of other base residues, such as U, C, or G, on the 3′-end of the terminator tetranucleotides has not been determined. These studies suggest that the codons UAA, UAG, and UGA, which direct peptide chain termination in bacterial cells, also direct the termination of mammalian protein synthesis.

The purification of rabbit reticulocyte R factor was studied in detail (Table 2). The specific activity of R was determined at each purification step with poly(A,G,U), UAAA, UAGA, and UGAA. Activity cannot be detected prior to Fraction III. A 50-fold reduction in protein is achieved from Fraction I to III. The overall purification of R from Fraction III to V has ranged from 30- to 50-fold. The UAGA preparation used in the experiments of Table 2 and Fig. 2 has lower template activity than the preparation used for the experiments in Table 1. This difference can be attributed to a partially inhibitory concentration of ammonium salts in the less-active preparation. The relative template activity for fMet release is therefore considered more reliable in Table 1, and the activity for UAGA in Table 2 and Fig. 2 is partially inhibited.

The purification procedures for rabbit reticulocyte R are illustrated in Fig. 2. In Fig. 2*A*, *B*, and *C*, a single peak of R activity is identified by DEAE-Sephadex, phosphocellulose, and G-200 Sephadex column chromatography. The relative template activity of each of the tetranucleotides appears constant for the fractions with R activity for each column procedure. These studies indicate the presence of a single active fraction for release as determined with mRNA template and ethanol (not shown). Furthermore, attempts to detect a factor complementary to reticulocyte R by the use of rate-limiting amounts of Fraction V have been negative to date. Reticulocyte R has an estimated molecular weight of 255,000, as determined by its position of elution from G-200 Sephadex (11). Studies with bacteria (12, 13) indicate the presence of three smaller proteins, two codon-specific R factors (MW ≈ 40–50,000) and a stimulatory factor, S (MW ≈ 40–50,000).

The transfer factor content of reticulocyte R factor is estimated in Fig. 3. Partially purified transfer factor preparations (T_1 and T_2) do not contain detectable release activity and, furthermore, do not stimulate or inhibit R release activity. Reticulocyte R (Fraction V) has no detectable T_1 or T_2 activity, as measured by its ability to complement T_1- or T_2-dependent polyphenylalanine polymerization. Thus, *in vitro* peptide chain termination does not appear affected by transfer factors. We have reached similar conclusions in our studies of bacterial peptide chain termination. Our most purified preparations of bacterial R and S proteins are devoid of the transfer factors: T_u,T_s, and G. These conclusions differ from those of Capecchi (13), who suggests that the stimulatory protein α(S) is equivalent to the elongation factor T_u.

The addition of GTP stimulates f[^{3}H]Met release directed by UAAA and poly(A,G,U) as shown in Table 3. GDP has slight effect. The analogue of GTP, GDPCP, inhibits f[^{3}H]Met release under all three assay conditions. These studies suggest that gamma-phosphate hydrolysis of GTP is required for peptide chain termination. The GTP hydrolysis was assessed directly (Table 4) by means of gamma-labeled [^{32}P]GTP. GTP hydrolysis occurs in the absence of added f[^{3}H]Met-tRNA in reactions containing reticulocyte ribosomes and reticulocyte R (Fraction V) (Table 4). The ribosomes and R hydrolyze little GTP alone. Hydrolysis of GTP is stimulated 5- to 10-fold in reactions containing R and ribosomes. The hydrolysis is further enhanced by the addition of the tetranucleotide UAAA, but not AAAA. The addition of 20% ethanol also stimulates this GTP hydrolysis. Since both UAAA and ethanol stimulate R factor-dependent ribosomal GTPase, and both have been shown in bacteria to

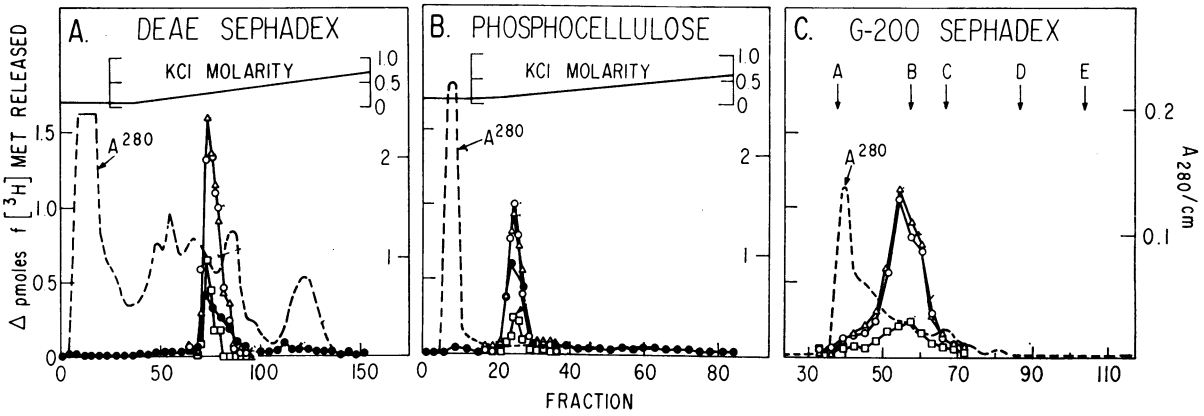

FIG. 2. Reticulocyte R Factor Purification. *A*. Fraction II (3.36 g of protein in 82 ml) was applied to a 43 × 2.5-cm DEAE-Sephadex column; washed with 230 ml of buffer A; and eluted with a KCl gradient (2000 ml). Fractions were 15.5 ml. All release reactions were 10 min, 24°C, and contained: 0.2 A_{260} unit poly(A,G,U) (●); 3.05 pmol of f[^{3}H]Met-tRNA$_f$·ribosome intermediates; 0.1 mM GTP; 0.015 ml of each column fraction, and other components described in *Methods*. Activity was determined with 0.1 A_{260} unit UAAA (○), UGAA (△), or UAGA (□) under the identical conditions except that all fractions were dialyzed against buffer C prior to assay with 0.025 ml of each fraction. Values for f[^{3}H]Met release are stimulation by oligonucleotide. The peak A_{280} value (fraction 12) is 26.5 A_{280}/cm. Note that the left-hand scale for f[^{3}H]Met release is constant in frames *A*, *B*, and *C*, while the right-hand scale for A_{280}/cm is 10-fold less in frame *C* than in *A* and *B*.

B. Fraction III (300 mg of protein in 59 ml) was applied to a 33 × 2.5-cm phosphocellulose column, washed with 100 ml of buffer B, and eluted with a linear (1200 ml) KCl gradient. Fractions, 15.5 ml, were assayed as described in *A* except that 0.015 ml, rather than 0.025 ml, of each dialyzed fraction was assayed with tetranucleotide.

C. Fraction IV (5.84 mg of protein in 4.0 ml) was applied to a 92 × 2.5-cm Sephadex G-200 column and eluted. Fractions were 3.8 ml. Release assays were incubated 10 min, 24°C and contained: 2.92 pmol of f[^{3}H]Met-tRNA$_f$·ribosome intermediates; 0.1 mM GTP; where indicated 0.1 A_{260} unit UAAA (○), UGAA (△), or UAGA (□); 0.025 ml of each fraction; and other components as indicated in *Methods*. The zero time value (0.24 pmol) is subtracted. Each purified protein marker was applied separately in 4.0 ml and its position of elution was determined by absorbance at 280 nm or 416 nm (cytochrome *c*). Arrow *A* indicates dextran blue (void volume, 142.5 ml); *B*, pyruvate kinase (217 ml, MW 237,000); *C*, aldolase (252 ml, MW 158,000); *D*, ovalbumin (327 ml, MW 43,000), and *E*, cytochrome *c* (392 ml, MW 12,523).

facilitate R factor binding to ribosomes, the GTP hydrolysis probably occurs on ribosomes concomitant with, or after, the binding of R. The pmol of GTP hydrolyzed greatly exceeds the quantity of ribosomes added (34 pmol) and, in other studies where f[^{3}H]Met-tRNA·ribosome intermediates are used, the pmol of GTP hydrolyzed again exceed the pmol of f[^{3}H]Met released.

As shown in Table 5, GTP hydrolysis is not inhibited by sparsomycin, gougerotin, or anisomycin at concentrations that inhibit f[^{3}H]Met release. The GTPase activity does not appear coupled to the hydrolysis of nascent peptidyl-tRNA. While ribosomal-dependent GTPase activity is known to occur in conjunction with other factors in protein synthesis, the described activity appears to be specifically involved in peptide chain termination since: (*a*) the activity is stimulated by terminator codons, (*b*) the GTPase and R activities coincide in their elution from phosphocellulose and G-200, and (*c*) our preparations of R are devoid of transfer factors.

TABLE 3. *GTP requirement for in vitro peptide chain termination*

Guanine nucleotide added	Δpmol f[^{3}H]Met released		
	poly(A,G,U)	UAAA	20% ethanol
None	0.72	0.21	1.57
GTP	0.94	1.79	1.41
GDP	0.56	0.38	1.54
GDPCP	0.08	0.07	0.13

Reactions were incubated 10 min (oligonucleotides) or 20 min (ethanol) at 24°C and contained: 2.92 pmol of f[^{3}H]Met-tRNA$_f$·ribosome intermediates; 6.5 μg of reticulocyte R (Fraction V), and, as indicated, 0.2 A_{260} unit poly(A,G,U), 0.1 A_{260} unit UAAA, or 20% ethanol (v/v); 0.1 mM guanine nucleotide as indicated; and other components as described in *Methods*. The zero time value (0.24 pmol) is subtracted from all values.

The stimulatory effect of fusidic acid on R-dependent GTPase is further examined in Table 6. Fusidic acid stimulates GTPase associated with R 3-fold; at equivalent concentrations, it inhibits the GTPase activity of T_2. There is slight effect on ribosomes or R alone. Fusidic acid is a known inhibitor of translocation and the ribosomal-dependent GTPase activity associated with the translocase proteins T_2 (mammalian) (14) and G (*E. coli*) (15). Recently, it has been suggested that fusidic acid inhibits this event by stabilizing a G·GDP·ribosomal complex, thus inhibiting cyclic translocation events (ref. 16, and H. Weissbach, personal communication). While the mechanism by which fusidic acid stimulates R-dependent GTPase is not known, the antibiotic distinguishes R- and T_2-dependent ribosomal GTPase and, furthermore, may offer a means of probing the function(s) of GTP hydrolysis in peptide chain termination.

DISCUSSION

The RNA (mRNA) codon assignments for mammalian and bacterial cells are identical for peptide chain initiation (17–19), elongation (20, 21), and, as shown in these studies, termination. Since trinucleotides were ineffective as templates for *in vitro* peptide chain termination on reticulocyte ribosomes,

TABLE 4. *Ribosomal-dependent GTP hydrolysis with R*

Addition	Δpmol [^{32}P]GTP hydrolyzed
Expt. 1	
R	19.7
Ribosomes	11.6
R + ribosomes	108
R + ribosomes + UAAA	169
R + ribosomes + AAAA	115
R + UAAA	9.89
Ribosomes + UAAA	3.28
UAAA	0.79
Expt. 2	
R + ethanol	3.27
Ribosomes + ethanol	7.14
R + ribosomes	142
R + ribosomes + ethanol	210

Reactions were incubated 15 min, 24°C, and contained in 0.05 ml as indicated: 9 μM [γ-^{32}P]GTP, 9.1 μg of reticulocyte R (Fraction V), 1.5 A_{260} unit reticulocyte ribosomes, and 0.2 A_{260} unit UAAA or AAAA, 20% ethanol (v/v), and other components as described in *Methods*. The hydrolysis of [γ-^{32}P]GTP (1 pmol = 356 cpm, Expt. 1; 1 pmol = 129 cpm, Expt. 2) occurring in buffer alone (5.17 pmol) is subtracted from all values.

tetranucleotides that contained the terminator codons were used. The R-dependent hydrolysis of ribosomal-bound fMet-tRNA is directed by UAAA, UGAA, and UAGA, suggesting that UAA, UGA, and UAG are chain termination codons for mammalian cells. This extends the total number of codon assignments for mammalian mRNA to 59, all assignments being identical to those for bacterial mRNA. On the basis of this universality, it appears probable that no other terminator codons occur for the mammalian cell. The number of molecules involved in the recognition of UAAA, UGAA, and UAGA cannot be resolved from these studies. Since aminoacyl-tRNA species from bacterial and mammalian cells differ in the sets of codons they recognize (19), similar differences in sets of

TABLE 5. *Antibiotic effects on mammalian release and GTPase activity*

Antibiotic added	% of Control	
	f[^{3}H]Met hydrolyzed	[^{32}P]GTP hydrolyzed
None	100 (2.24 pmol)	100 (235 pmol)
Sparsomycin	3	102
Gougerotin	52	102
Anisomycin	44	100
Cycloheximide	98	95
Fusidic acid	88	130

Release reactions were incubated 7 min, 24°C, and contained in 0.05 ml: 3.05 pmol of f[^{3}H]Met-tRNA$_f$·ribosome intermediates, 6.5 μg of reticulocyte R (Fraction V), 0.1 mM GTP, 0.1 A_{260} unit UAAA, and antibiotics (1 mM, except sparsomycin, 0.1 mM) as indicated. The zero time value (0.25 pmol) is subtracted. GTP hydrolysis reactions were incubated 15 min, 24°C, and contained in 0.05 ml: 9 μM [γ-^{32}P]GTP, 6.5 μg of reticulocyte R (Fraction V), 1.5 A_{260} unit ribosomes, and antibiotics as indicated. The hydrolysis of [γ-^{32}P]GTP (1 pmol = 82 cpm) occurring in buffer alone (8.2 pmol) is subtracted.

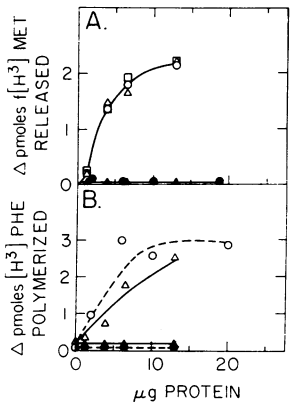

FIG. 3. Transfer Factor Content of R. *A*. Release reactions were incubated 7 min, 24°C, and contained: 2.92 pmol of f[^{3}H]-Met-tRNA$_f$·ribosome intermediates, 0.1 A_{260} unit UAAA; 0.1 mM GTP; R (○), T_1 (▲), or T_2 (●) as indicated; and other components as in *Methods*. High levels of transfer factors (13 μg of T_1 and 10 μg of T_2) were added to reactions containing variable amounts of R, indicated as R + T_1 (△) and R + T_2 (○). The zero time value (0.24 pmol) is substracted.

B. Phenylalanine polymerization was determined as described in *Methods* using 20 μg of T_2 for determination of T_1 activity with T_1 (△) or R (▲), and 13 μg of T_1 for determination of T_2 activity with T_2 (○) or R (●). The phenylalanine polymerized in the absence of T_1 + T_2 (0.03 pmol) is subtracted.

terminator codons recognized by mammalian and bacterial R species may occur. The preparation from reticulocytes has shown no evidence of separating into multiple R components when subjected to procedures that readily separate bacterial R1 and R2 (22).

Peptide chain termination with reticulocyte R requires GTP and gamma-phosphate hydrolysis. The larger size (MW ≈ 255,000) and associated GTPase activity of reticulocyte R suggest that it is a complex of proteins, or a multifunctional protein with characteristics of bacterial R and S factors. Since the ribosomal-dependent GTPase associated with reticulocyte R occurs in the absence of peptidyl-tRNA hydrolysis, the requirement for GTP hydrolysis appears to be essential to, but not directly coupled to, peptidyl-tRNA

TABLE 6. *Effect of fusidic acid on GTP hydrolysis with R and T_2*

Additions	Δpmol of [^{32}P]GTP hydrolyzed	
	R	T_2
Factor	16.5	22
Factor + fusidic acid	7.73	24.3
Factor + ribosomes	126	119
Factor + ribosomes + fusidic acid	333	52
Ribosomes	11.9	11.9
Ribosomes + fusidic acid	7.07	7.07

Reactions were incubated 15 min, 24°C, and contained in 0.05 ml: 9 μM [γ-^{32}P]GTP, other components as described in *Methods*, and, as indicated, 2 mM fusidic acid, 9.1 μg of reticulocyte R (Fraction V), 14 μg of T_2, and 1.5 A_{260} unit ribosomes. The hydrolysis of [γ-^{32}P]GTP (1 pmol = 356 cpm) occurring in buffer alone (5.17 pmol) is subtracted from all values.

hydrolysis. Furthermore, earlier studies with bacterial R factor indicate GTP is not required for peptidyl-tRNA hydrolysis (23). These studies suggest that GTP hydrolysis is required in an essential intermediate step in peptide chain termination. A similar requirement has been reported for fMet-tRNA participation in chain initiation (24) and aminoacyl-tRNA participation in chain elongation (25). It is attractive to speculate that the GTP hydrolysis essential for initiation, elongation, and termination is part of a single intermediate event, which is common to the three steps in peptide chain synthesis. Since GDPCP can substitute for GTP in the ribosomal binding of fMet-tRNA (24) and aminoacyl-tRNA (26), the hydrolysis of GTP is part of some other intermediate event. It should now be possible to assess the role of GTP in the analogous events in peptide chain termination.

Given the similarities in bacterial and mammalian peptide chain termination already outlined, the mechanism of this event is probably similar in the two types of organisms. Certain aspects of this mechanism have been more amenable to *in vitro* study in bacteria, e.g., separation of individual protein components (R1, R2, and S) involved, while other aspects are more easily studied in mammalian cells, e.g., the role of GTP hydrolysis. The study of both cell types has proved useful in delineating the mechanism of codon-directed peptide chain termination, and should lead to a more accurate understanding of *in vivo* events.

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