PEPTIDE CHAIN TERMINATION, V. THE ROLE OF RELEASE FACTORS IN mRNA TERMINATOR CODON RECOGNITION

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Abstract.—The protein release factors, R1 and R2, bind to ribosomes in response to specific terminator codons (R1 to UAA or UAG, R2 to UAA or UGA). In reactions containing ribosomes, the tritiated oligonucleotide UA[^{3}H](A)$_2$ is retained on nitrocellulose filters in response to either R1 or R2, and UA[^{3}H]G in response to R1. These results indicate that an R·terminator codon·70*S* ribosome intermediate occurs during terminator codon recognition and suggest that protein release factors R1 and R2 recognize terminator codons.

Introduction.—Peptide chain termination can be studied *in vitro* by sequential use of initiator (AUG) and terminator trinucleotides (UAA, UAG, or UGA) to first bind f[^{3}H]Met-tRNA^f to ribosomes, and subsequently to release f[^{3}H]-methionine.[1] The release of f[^{3}H]methionine requires one of two codon specific protein release factors: R1 active with UAA or UAG, R2 with UAA or UGA.[2] In these studies, we report a means of directly assessing terminator codon recognition which requires R factors, tritiated oligonucleotides, and ribosomes. A release factor·terminator codon·ribosome intermediate is formed which is stabilized with 10 per cent ethanol, and detected by the retention of tritiated terminator codons on nitrocellulose filters. The results suggest that protein release factors recognize mRNA terminator codons on ribosomes.

Methods.—Assay of ribosomal bound [^{3}H]-oligonucleotide: Each 0.050-ml reaction mixture contains 0.10 *M* Tris-acetate, pH 7.2; 0.02 *M* magnesium acetate; 0.10 *M* ammonium acetate; either 15.0 pmoles of UA[^{3}H]G 1.0 Ci/mmole; or 7.5 pmoles of UA-[^{3}H](A)$_2$, 24.2 Ci/mmole and 550 pmoles of AAA; 10% ethanol (v/v); *E. coli* MRE 600 ribosomes[1] and *E. coli* B release factors[3] as indicated. Reactions are initiated with ethanol, incubated at 4°C for the indicated times, diluted with 3.0 ml of the above buffer containing 10% ethanol at 4°C, and washed under gentle suction onto a nitrocellulose filter (HA Millipore filter, 25 mm diameter, 0.45 μ pore size, lot no. 8568) as in the aa-tRNA codon recognition assay of Nirenberg and Leder.[4] All measurements of binding, unless otherwise noted, are of extent. Incubations and Millipore plating must be performed at 4°C. Filters are dried and counted in a liquid scintillation counter in toluene-PPO-POPOP at 15–18% efficiency.

Release assay: The fMet-tRNA^f, release factors, ribosomes, and fMet-tRNA^f·AUG·ribosome complex are prepared as previously described.[3] Release of f[^{3}H]methionine is assayed by the method of Caskey *et al.*[1] R1 and R2 used in these experiments correspond to fraction VI and fraction VII, respectively.[3]

Materials: The [^{3}H]GDP, [^{3}H]ADP, and [^{3}H]methyl-methionine (5.1 Ci/mmole) were purchased from Schwarz BioResearch. Sparsomycin and amicetin were the gift of Dr. P. Leder. Tetracycline and streptomycin were obtained from Lederle. Antibiotics were buffered in 0.01 *M* Tris-HCl, pH 7.2. Both the UA(^{3}H)G and UA[^{3}H](A)$_2$ were prepared as previously described except that the concentration of UpA primer was equal to or in excess of the concentration [^{3}H]GDP or [^{3}H]ADP.[5]

Analysis of UA[^{3}H]G in two paper chromatography systems (H_2O:*n*-propanol:NH_3, 35:55:10 by volume; 40 gm ammonium sulfate dissolved in 100 ml 0.1 *M* potassium phosphate, pH 7.0) indicated co-chromatography with authentic UpApG[6] with no detectable contaminating radioactivity. The tetranucleotide consists of a mixture of UA[^{3}H](A)$_2$ and [^{3}H] AAA and, therefore, nonradioactive AAA was added in 75-fold excess of the [^{3}H]AAA to inhibit recognition of the [^{3}H]AAA, and the —A[^{3}H]A[^{3}H]A translational frame. The AAA did not inhibit R1 or R2 directed binding of UA[^{3}H]A-[^{3}H]A and only UA[^{3}H]A[^{3}H]A was found in an analysis of tritiated oligonucleotide retained on the filter in response to R factors.

Results.—The retention of UA[^{3}H](A)$_2$ by nitrocellulose filters in the presence of ribosomes and either R1 or R2 is shown in Figure 1. There is little binding in the absence of R factors. For both R1 and R2, the binding of UA[^{3}H](A)$_2$ is proportional to the amount of release factor. Since neither R-factor preparation is homogeneous and further since contaminating proteins do interfere with UA[^{3}H](A)$_2$ binding, it is not yet possible to relate the stoichiometry of R factor content and UA[^{3}H](A)$_2$ bound.

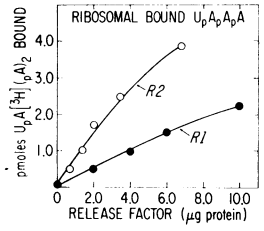

FIG. 1.—Ribosomal binding of UA[^{3}H] (A)$_2$. Each reaction mixture is incubated for 15 min. and contains in 0.050 ml: 2.8 A^{260} ribosomes, R1 or R2 as indicated, and additional components as described in *Methods*.

The specificity for R factor-dependent retention of UA[^{3}H]G and UA[^{3}H](A)$_2$ on nitrocellulose filters is shown in Table 1. R1 directs the binding of both UA[^{3}H]G and UA[^{3}H](A)$_2$; R_2 directs the binding of UA[^{3}H](A)$_2$ but not of UA[^{3}H]G (values enclosed in boxes). Although tritiated UGA was not available, the full terminator codon specificity for each R factor was investigated by competition studies between tritiated and nonradioactive terminator codons. The addition of nonradioactive UAA in 100-fold excess of radioactive oligonucleotides abolishes the R1 directed binding of both UA[^{3}H]G (competition) or UA[^{3}H]H(A)$_2$ (isotope dilution); addition of nonradioactive UAG abolishes the R1 directed binding of UA[^{3}H]G (isotope dilution) and UA(^{3}H](A)$_2$ (competition). Addition of UGA has no effect on R1-directed binding of UA[^{3}H]G or UA[^{3}H](A)$_2$. Similarly, addition of UAA or UGA but not UAG abolishes the

TABLE 1. *Specificity for terminator codon binding to ribosomes.*

Addition		ΔpMoles Ribosomal Bound	
R Factor	Nonradioactive codon	UA[^{3}H]G	UA[^{3}H](A)$_2$
R1	None	[2.93]	[1.17]
	UAA	0.05	0.02
	UAG	0.00	0.02
	UGA	2.80	1.20
R2	None	[0.00]	[1.15]
	UAA	—	0.02
	UAG	—	0.92
	UGA	—	0.01
None	None	(0.31)	(0.04)

Each reaction mixture is incubated for 15 min and contains in 0.05 ml:2.8 A^{260} ribosomes; 3.0 μg R1 or 1.0 μg R2; where indicated 0.04 A^{260} (1200 pmoles) UAA, UAG, or UGA; and additional components as indicated in *Methods*.

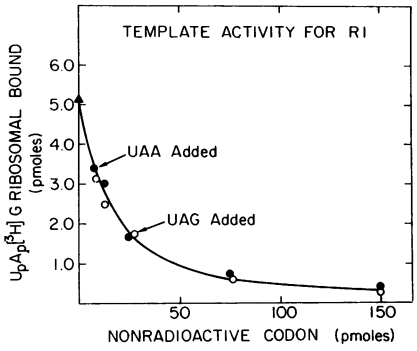

FIG. 2.—Template activity of UAA and UAG. Each reaction mixture is incubated for 15 min at 0°C and contains in 0.050 ml: 8.0 μg R1, 2.1 A^{260} ribosomes, UAA and UAG as indicated and additional components as described in *Methods*. UA[^{3}H]G bound (0.25 pmoles) in the absence of R factor has been subtracted from all values. ●——●, UAA added; ○——○, UAG added.

R2-directed binding of UA[^{3}H](A)$_2$. The results suggest R1 directs the binding of UAA or UAG, and R2 directs the binding of UAA or UGA, and agrees with earlier determinations of R factor codon specificity.[2]

The relative template activity of UAA and UAG for R1 is examined in Figure 2. The R1-directed UA[^{3}H]G binding is inhibited equally by increasing amounts of UAA or UAG. The results indicate that R1 has equal affinity for UAA or UAG, and suggest that the 3-fold difference in the UA[^{3}H]G and UA[^{3}H](A)$_2$ binding (Table 1) with R1 reflects differences in the oligonucleotide preparations. In other studies not shown, a similar isotope dilution study with UAA and UGA for R2 UA[^{3}H](A)$_2$ binding suggests that R2 has slightly greater affinity (1.2×) for UGA than UAA.

The requirements for retention of tritiated terminator codons on nitrocellulose filters are shown in Table 2. Maximum binding requires R factor, ethanol, ribosomes, and magnesium. The small amount of binding observed in the absence of ethanol is not observed if ethanol is omitted from the wash buffer. The requirements suggest that retention of tritiated terminator codon on the filter is a measurement of the formation of an R·terminator codon·ribosome complex which is stabilized by ethanol. In other studies, the requirements for 70*S* ribosomes could not be replaced by 30*S* or 50*S* ribosomal subunits alone but required both 30*S* and 50*S* subunits. These ribosomal subunits requirements are similar to those for enzymatic binding of aa-tRNA to ribosomes.[7]

The effect of ethanol, monovalent cation concentration, time, and pH on the R1-directed binding of UA[^{3}H]G to ribosomes is shown in Figure 3. Maximal binding is observed after 5–15 minutes, with 10 per cent ethanol, at 0.10 *M* ammonium ion, and over a broad pH range of 7.0 to 8.0 (Fig. 3A–D). The tritiated

TABLE 2. *Requirements for UA[^{3}H]G binding with R1.*

Components	pMoles UA[^{3}H]G bound
Complete	8.32
—R1	0.14
—Ribosomes	0.17
—ETOH	0.28
—R1, Ribosomes, ETOH	0.10
—Mg^{++}	1.35

Each reaction mixture is incubated for 15 min and contains in 0.05 ml: 2.8 A^{260} ribosomes, 8.0 μg R1, and additional components as described in *Methods*.

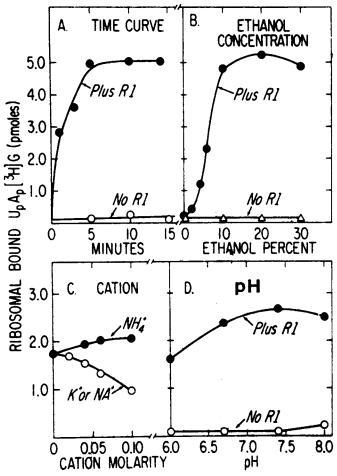

FIG. 3.—Conditions for UA[^{3}H]G ribosomal binding.

(*A*) Each reaction mixture is incubated for the times indicated and contains in 0.050 ml: 2.46 A^{260} ribosomes, 6.0 μg R1, and additional components as described in *Methods*.

(*B*) Reactions are incubated for 15 min and contain components as described in (*A*) except for ethanol as indicated. All reactions are washed onto nitrocellulose filters with 10% ethanol containing buffer.

(*C*) Reactions are incubated for 15 min and contains components as described in (*A*) except for 0.5 A^{260} of ribosomes and monovalent cation as indicated. UA[^{3}H]G bound (0.25 pmoles) in the absence of R1 is subtracted from all values.

(*D*) Each reaction mixture is incubated for 15 min and contains in 0.050 ml: 2.05 A^{260} ribosomes; 4.0 μg R1; 0.05 *M* ammonium acetate; 0.03 *M* magnesium acetate; 0.10 *M* imidazole buffer, pH as indicated; 15 pmoles of UA[^{3}H]G; and 10% ethanol.

trinucleotide binding is inhibited by Na+ or K+. Inhibition by Na+ is similar to the effect on aa-tRNA codon recognition; inhibition by K+ is not observed for aa-tRNA codon recognition.[8]

Although it is possible to elute ribosomes and intact tritiated terminator codons from the nitrocellulose filter after performing the binding reaction, it has not been possible to elute the active R factor. Therefore, a direct demonstration of an R · terminator codon · ribosome complex using this technique has not been possible. However, the binding of R factors to ribosomes in response to terminator codons is shown in Table 3. Reactions containing ribosomes, 10 per cent ethanol, and

TABLE 3. *Codon specific R1 and R2 binding to ribosomes.*

R binding reaction additions		UAA Dependent R Activity pmole f[^{3}H]Methionine Fraction	
R Factor	Codon	Supernatant	Ribosomal
R1	None	1.45	0.55
	UAG	0.07	1.34
	UAA	0.12	1.50
	UGA	1.40	0.62
R2	None	1.54	0.49
	UAA	0.12	1.24
	UGA	0.45	1.02
	UAG	1.52	0.47

Each reaction is incubated for 5 min at 4°C and contains in 0.10 ml: 6.0 A^{260} ribosomes; 0.25 *M* ammonium acetate; 0.03 *M* magnesium acetate; 0.05 *M* Tris-acetate, pH 7.4; 0.006 *M* β-mercaptoethanol; 10% ethanol; 10.0 μg R1 or 10.0 μg R2; and 2.5 nmole of the indicated trinucleotide codon. Ribosomal and supernatant fractions of each reaction are separated by 60 min 200 K × *g* centrifugation in a Spinco No. 50 rotor with tube adaptors. The supernatant is aspirated and the pellet suspended in 0.10 ml of buffer containing 0.25 *M* ammonium acetqte; 0.05 *M* Tris-acetate, pH 7.2; and 0.006 *M* β-mercaptoethanol.

Assay of supernatant and ribosomal-bound fraction for release activity: Each release assay (1) is incubated for 15 min at 24°C and contains in 0.05 ml: 4.5 pmoles f[^{3}H]Met-tRNA · AUG · ribosome; 2.5 nmole UAA; 0.05 *M* ammonium acetate, 0.05 *M* Tris-acetate, pH 7.2; 0.03 *M* magnesium acetate, and 0.005 ml of the indicated ribosomal or supernatant fractions. The f[^{3}H]methionine released (0.22 pmole) in the absence of added fractions is subtracted from each value.

R1 or R2 were incubated at 0°C in the presence of each nonradioactive terminator codon, and separated into ribosomal and supernatant fractions by centrifugation. The release activity of each fraction was determined in the presence of UAA. The codons UAA and UAG but not UGA direct the binding of R1 to ribosomes; UAA and UGA but not UAG direct the binding of R2 to ribosomes. In other studies, the simultaneous demonstration of R1 and UA[^{3}H]G in the ribosome pellet has also been possible. Therefore, the ribosomal binding of R factors occurs in response to terminator codons with the same specificity as previously described. Furthermore, since R1 bound with UAG and R2 bound with UGA are active in the presence of UAA, a single R factor is active with two terminator codons.

Earlier studies had suggested that R factors compete for ribosomal binding sites.[2] In Table 4, the R1-directed binding of UA[^{3}H]G to ribosomes is inhibited by R2 and UGA. The results suggest that the ribosomal site of attachment for both R1 and R2 is at least partially the same.

Prior studies have shown that antibiotics which inhibit either tRNA codon recognition or the peptidyl transferase reaction inhibit the R1, UAA-directed release of f[^{3}H]methionine.[2] Table 5 compares the effect of such antibiotics on the R1-directed ribosomal binding of UA[^{3}H]G and the R1, UAG-directed release of f[^{3}H]methionine. Tetracycline and streptomycin inhibit appreciably (70–90%) both binding and the codon-directed release. Sparsomycin and amicetin have

TABLE 4. *Apparent competition of R factor binding.*

Components	Δ pmoles UA[^{3}H]G bound	% Inhibition
R1	4.36	0
R1 + UGA	4.38	0
R1 + R2	4.01	8
R1 + R2 + UGA	2.61	41
(—R1)	(0.25)	

Each reaction mixture is incubated for 10 min at 0°C and contains in 0.05 ml: 0.41 A^{260} ribosomes 6.0 μg R1, 1.5 μg R2, and 2.5 nmoles of UGA where indicated, and additional components as described in *Methods*. These quantities of R1 and R2 bind equal amounts of UA[^{3}H](A)$_2$.

TABLE 5. *Antibiotic inhibition of codon recognition or codon-directed release.*

Components	Δ pMoles UA[^{3}H]G bound	Δ pMoles f[^{3}H]Methionine released
Complete	3.91	1.73
+ Sparsomycin	3.75	0.02
+ Amicetin	3.52	0.48
+ Tetracycline	0.61	0.27
+ Streptomycin	1.08	0.19
(−R Factor)	(0.14)	(0.21)

Each binding reaction mixture is incubated for 15 min and contains in 0.05 ml: 0.33. A^{260} ribosomes, 6.0 μg R1, and either 1×10^{-5} *M* sparsomycin, 1×10^{-4} *M* amicetin, 2×10^{-4} *M* tetracycline, 1×10^{-4} *M* streptomycin, and additional components as described in *Methods*. Antibiotics have no effect on the binding of UA[^{3}H]G to the filter in the absence of R factor.

Each release reaction is incubated for 10 min at 0°C (rate determination) and contains in 0.05 ml; 4.88 pmoles f[^{3}H]Met-tRNA·AUG·ribosome complex; (0.33 A^{260} ribosomes; 4.98 pmoles fMet-tRNA^f; 2.5 nmoles AUG) 0.10 *M* ammonium acetate; 0.03 *M* magnesium acetate; 0.10 *M* Tris-acetate, pH 7.2; 2.5 nmoles and 3.2 μg R1. Sparsomycin and amicetin also inhibit release in the presence of 10% ethanol.

little effect on the extent (or in other studies on the rate) of R1 codon recognition, (4 and 10%, respectively) but significantly inhibit the rate of f[^{3}H]methionine release (96 and 70%, respectively).

Discussion.—The results of these and other *in vitro* studies support the model for peptide chain termination shown below:

$$\text{R factor} + \text{terminator codon} + \text{70S ribosome}\cdot\text{AUG}\cdot\text{fMet–tRNA}^f$$
$$\downarrow$$
$$\text{R}\cdot\text{terminator codon}\cdot\text{70S}\cdot\text{AUG}\cdot\text{fMet–tRNA}^f$$
$$\downarrow$$
$$\text{fmethionine}$$

Both R1 and R2 bind to ribosomes with the same codon specificity as previously reported for R factor directed fmethionine release: R1, UAA, or UAG; R2, UAA, or UGA. A release factor·terminator codon·ribosome intermediate is formed and is detected by the retention of tritiated terminator codons on nitrocellulose filters. The formation of this intermediate is analogous to the formation of [^{14}C]aa-tRNA·[^{3}H]codon·ribosome intermediates which occur upon recognition of aa-tRNA codons as described by Hatfield and Nirenberg *et al.*[9, 10] The simplest interpretation of the reported data is that R1 and R2 are the recognition molecules for terminator codons.

Since R factors have characteristics of proteins[1] and are unaffected by RNase A and T1 RNase,[1, 11] terminator codons apparently are recognized by a protein nucleic acid interaction. The characteristics of terminator codon recognition and aa-tRNA codon recognition have many similarities as determined by codon specificity,[1] and template activity of analog codons.[12] Protein recognition of nucleotides has been described for ribonucleases,[13] and protein recognition of nucleic acids in the cases of aa-tRNA synthetases, DNA and RNA polymerases, repressors, and antibodies.[13] Release factors are apparently an example of trinucleotide recognition. Although further study is required to define the R factor sites essential for terminator codon recognition, alkylation of R factors with *N*-ethylmaleimide has been found to inhibit R factor directed binding of radioactive codons.

The requirement of terminator codon for both the binding of R factor to ribosomes and the release of fmethionine suggests release occurs subsequent to the formation of an R·terminator codon·70S·AUG·fMet-tRNA^f intermediate. Sparsomycin and amicetin inhibit release without inhibiting terminator codon recognition. Whether or not these antibiotics, known to inhibit the peptidyl transferase reaction,[14] effect an event common to peptide bond formation and peptide release is unresolved at this time.

This report focuses on the recognition of terminator codons independent of release; recently, methods have been developed for the study of release independent of codon recognition.[15] These *in vitro* approaches to the study of peptide chain termination dissect codon recognition and peptide release. Such approaches should be useful in defining the role of S protein[3, 16] and other possible intermediate events in peptide chain termination.

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