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Studies on termination of protein synthesis in wheat germ

Estudios sobre terminación de la síntesis de proteínas en germen de trigo

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Oligophenylalanines are soluble in m-cresol, but oligophenylalanyl-tRNAs are not. This diferential solubility can be used to assay oligophenylalanines released from tRNA during their synthesis by wheat germ extracts. When poly U is the message, virtually no free product appears. When poly AU (A < U) is used, a considerable amount of oligophenylalanines are released. The fraction of product released is aproximately constant with time, implying that a steady-state is not achieved between initiation and release. The dependence of release on various reaction variables and the effects of several inhibitors on release indicate that the reaction is probably catalyzed by peptidyl transferase, in accord with the mechanisms described for mammals and prokaryotes.

INTRODUCTION

As the nascent protein elongates, the ribosomes advances along the message. Eventually, a termination triplet is reached and the completed protein is released (Figure 1A). In *E. coli*, one of two soluble proteins is required for codon recognition. RF1 recognizes UAA or UAG, and RF2, UAA or UGA. Catalytic recycling of these proteins requires a third, RF3, which may link termination to GTP hydrolysis (29). RF1 and RF2 both require an intact 16S rRNA 3' terminus for their activity (5) and compete with tRNA for A-site binding (12, 27). Hydrolysis (i.e., release) of the peptidyl-tRNA is catalyzed by the peptidyl transferase center of the ribosome (4, 33, 36). Codon-dependent release, like most of the other partial reactions of protein synthesis, is not obligatorily linked to GTP hydrolysis. Instead, hydrolysis of GTP appears to act primarily as an allosteric effector, increasing the velocity of various intermediate reactions (19, 29, 30). A number of additional (and for the most part, uncharacterized) activities are required for normal *in vivo* termination (10).

A similar sequence of events is found in mammals (Figure 1B). Major differences are the use of a single factor, RF, as the combined functional equivalent of RF1, RF2, and RF3, and a GTP requirement for RF binding to the ribosome (29). As in bacteria, hydrolysis of the GTP is not obligatory for peptide release but is necessary for maximum reaction velocity and

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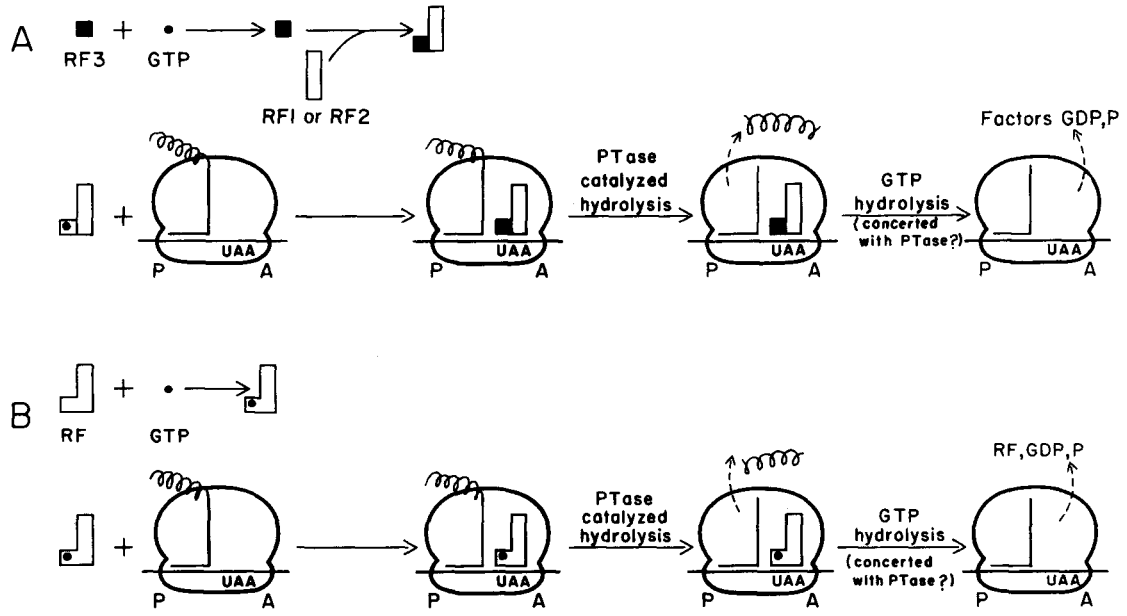

Fig. 1. Termination and release during protein synthesis in prokaryotes (a) and eukaryotes (b).

factor recycling (29, 30). Several additional stimulatory activities have been detected in mammalian extracts (6, 14, 17). The details of termination in mammals are less well understood than in bacteria, which are very much easier to manipulate genetically In the absence of such genetic flexibility, it is considerably more difficult to relate results *in vitro* to the physiologically real process of termination *in vivo*.

There are several ways in which termination may function as a control point during protein synthesis. Reinitiation within polycistronic mRNA, for instance, is related to the state of the post-termination complex (25). Ribosome release following termination stimulates both DNA and RNA synthesis (18). The ribosome itself may be sensitive to termination information on the message and may undergo a reprogramming process, depending on the nature of its interaction with this region (10).

Ribosome-mRNA association shields a portion of the message from nuclease attack (19). Thus, the formation of densely packed polyribosomes as a consequence of rate limiting release may indirectly influence mRNA longevity. The regulator of such a phenomenon could be a hormone or protein interacting with the 3' untranslated region. A similar regulatory event could also alter the availability of active ribosomes to other messages. Release is the rate limiting step in the synthesis of a class of mammalian liver proteins (6, 9). A cAMP-binding protein has been shown to stimulate specifically the release of these proteins, causing a six-fold increase in their synthesis.

Prior to our investigations, termination and release in plants received little attention, despite the recent popularity of wheat germ as a model of eukaryote protein synthesis. Even though higher plants and animals diverged from each other long ago, we nevertheless expect the basic mechanism of protein synthesis to be conserved in both groups. On other hand, regulation of gene expression in the two groups differs in a number of respects and we assume that this is true even at the translational level. Although termination events are not normally considered in discussions of regulation, the inherent physiological complexity of the multicellular eukaryotes has, we suspect, made control at this level a necessity under certain circumstances. The previously cited work on stimulation of liver protein synthesis by a spe-

cific release factor would seem to be an example.

In this paper, we describe a method for assaying elongation-dependent release in wheat germ extracts. We have found this assay useful in the initial characterization of termination of protein synthesis by higher plants and we are currently using variations of it (including the elongation-independent release of N-acetylphenylalanine from N-acetylphenylalanine tRNA) to study the process in greater detail. We plan to coordinate these results with genetic studies using plant tissue culture techniques. Such an approach, we feel, should be quite productive in the analysis of translational regulation in wheat.

METHODS

Wheat germ ribosomes and S 100. 200 g of wheat germ were added to 150 m1 Buffer H (10 mM HEPES, pH 7.5; 10 mM $MgCl_2$; 25 mM KCl; 6.75 mM 2-mercaptoethanol) and homogenized (Virtis 45) at high speed for 30". The slurry was centrifuged 16,000 × g for 10 min, and the supernatant was filtered through glass wool and centrifuged 30,000 × g for 30 min. Crude ribosomes were isolated from the postmitochondrial supernatant by centrifugation at 100,000 × g for 4 hours. The central two-thirds of the supernatant from each tube was pooled, recentrifuged 100,000 × g for 4 hours, and passed over Sephadex G25 (33 cm × 2 cm; 100 packed ml) preequilibrated in buffer H. The first 30 ml of this S100 following the void volume were collected and stored at -80° as 0.5 ml aliquots. The ribosomal pellets were resuspended with a Potter-Elvehjem tissue homogenizer in 36 ml buffer H and cleared of aggregates by centrifugation at 30,000 × g for 30". The supernatant was diluted by addition of an equal volume of buffer H containing 1 M KCl, layered carefully over 5 ml cushions of buffer H containing 0.5 M KCl plus 0.25 M sucrose, and centrifuged at 200,000 × g for 2 hours in the Beckman T-50 rotor. The pellets were washed by resuspending and recentrifuging a second time in the same manner but without added KCl. The pellets were resuspended in Buffer H plus 0.25 M sucrose, aggregates spun out, and the concentration was adjusted to give and A_{260} of 200. The final high-salt washed ribosomes were stored as 0.5 ml aliquots at -80°. The yield was approximately 4,000 A_{260} units per preparation with an A_{260}/A_{280} ratio of 1.9.

Wheat germ tRNA. tRNA was prepared from wheat germ as previosly described for black-eyes peas (13).

Synthetic polynucleotides. Poly UA of variable base ratio was synthesized by a modification of the procedure of Thach and Doty (32, 31). Reactions contained in 2.5 ml 200 mM Tris-HCl (pH 8.5), 1 mM $MgCl_2$, 400 mM NaCl, 0.1 mM $CuCO_4$, 20 mM total UDP + ADP, 0.1 mM ApA as primer, and 100 μg polynucleotide phosphorylase *(M. lysodeikticus;* Sigma). A drop of toluene was added to inhibit bacterial growth, and the mixture incubated 24 hr at 37°. Monomer incorporation was followed by thin layer chromatography on PEI plates with added fluorescent indicator (CEL-300, Machery-Nagel & Co.) developed with 2 M sodium phosphate (pH 3.4). Polynucleotides remain at the origin in this system. Reactions were stopped by heating at 100° for 2 min, and precipitated overnight at -20° by addition of 0.2 vol. 1 M potassium acetate (pH 4.7) plus 2.5 vol. chilled 95% EtOH. Precipitates were collected by centrifugation, dried *in vacuo* over P_2O_5, and dissolved in double distilled H_2O to give an A_{260} of 100. The final preparation was stored at −20°.

Cell-free protein synthesis. Incubations contained 50 mM HEPES (pH 7.2), 7 mM $MgCl_2$, 100 mM KCl, 1 mM ATP, 0.3 mM GTP, 10 mM phosphocreatine (Sigma), 50 μg/ml creatine kinase (Sigma), 5 mM 2-mercaptoethanol, 50 μM (^{14}C) phenylalanine or (^{14}C) lysine (50 μCi/ μmol), 50 μM each of unlabelled remaining 19 amino acids, 20A_{260} units/ml ribosomes, 1 mg ml S100 enzymes, 5 A_{260} units/ml polynucleotide, and 2 A_{260} units/ml tRNA. Reactions were incubated at 35° for 45' min or as indicated. Aliquots (50 μl) were assayed for oligophenylalanine synthesis by hot TCA precipitation, or for oligolysine synthesis by the P-81 method (2). Reported results represent the average of duplicate determinations of 50 μl samples.

MATERIALS

Nucleotides and most antibiotics were purchases from Sigma, with the following exceptions: poly U and poly A were from Miles; negamicin was the generous gift of Dr. Yoshimara Uehara (Institute of Microbial Chemistry, Tokyo); and sparsomycin was the gift of the National Cancer Institute, NIH (USA). Other chemicals and reagents were reagent grade whenever possible.

RESULTS

If poly AU (A > U) is used as a message during protein synthesis *in vitro*, then incorporation will be predominantly of lysine (codon AAA). Codon UAA will also occur and, under the correct conditions, will stimulate release of the growing oligopeptide. One of us (J.R.M.) was codeveloper of a method for measuring such poly AU-dependent release by the differential binding characteristics of lysine-rich peptides and peptidyl-tRNA on Whatman P81-cellulose paper (2, 21). Initially, we attempted to apply this methodology to a study of termination in wheat germ and other higher plant tissue. Figure 2 shows the results of an experiment in which poly AU (5:1) and poly A were used as messages. Total incorporation of lysine was linear for 2 hr and nearly the same regardless of message. Poly AU promoted the release of

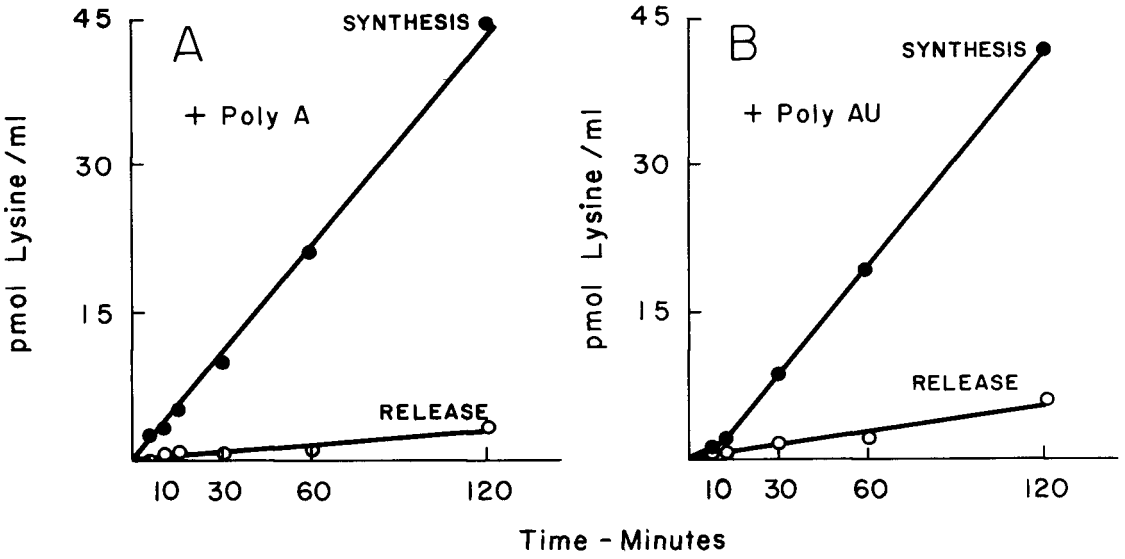

Fig. 2. Lysine incorporation and release of lysine rich peptide were measured as described in Methods. (A) poly A as message: (B) poly AU (5:1) as message. Each point represesents the average of duplicate 50 μl samples. The straight lines represent least squares best fits to the data.

about 14% of total incorporated material, versus 7% for poly A.

Although these results are qualitatively similar to those for *E. coli,* they were disappointing for several reasons. First, incorporation of lysine was very low. We have also found this true in cell-free extracts of the Jack-bean *(Canavalia ensiformis)* (16) and rat liver (unpublished results). We suspect that poor translation of adenosine-rich polynucleotides may represent a general property of eukaryotes. We have also discovered a proteolytic activity in wheat germ which attacks oligolysine and may further complicate the apparent levels of incorporation (15). Second, poly AU-stimulated release was low in comparison with *E. coli,* in which release can approach 40% or more under identical circumstances (23). Finally, there was a high background of release in the presence of the poly A control message. This may be the consequence of dissociation of oligolysyl-tRNA from the elongation complex followed by hydrolysis catalyzed by peptidyl-tRNA hydrolase. Enhanced dissociation of oligolysyl-tRNA as compared to other peptidyl-tRNA's has been found in *E. coli* cell free extracts (11).

We have used a similar experimental approach to detect phenylalanine-rich oligopeptide ("phe-rich peptide") release stimulated by poly AU (A < U). Again, we assume that in the course of polymerization an ochre (UAA) triplet will eventually reach the A-site, and that its decoding will result in hydrolysis of the phe-rich peptidyl-tRNA. The greatest problem with this approach lay in distinguishing between tRNA-bound and free phe-rich peptide. We found that a modification of the m-cresol solubility assay of Maden *et al.* (20) for detection of oligophenylalanyl-puromycin was suitable for this purpose.

Figure 3 shows the results of two such experiments. Paired aliquots of incubations containing either poly AU (1:4) or poly U were sampled and precipitated with cold TCA on glass fiber filters. One of the samples was assayed for total hot TCA-insoluble product. The other was treated first with m-cresol to eliminate free phe-rich peptide, followed by hot TCA (to eliminate any contaminating phe-tRNA). The difference between the two samples was taken as net release. »Percent release« is this value expressed as a percent of total hot TCA-

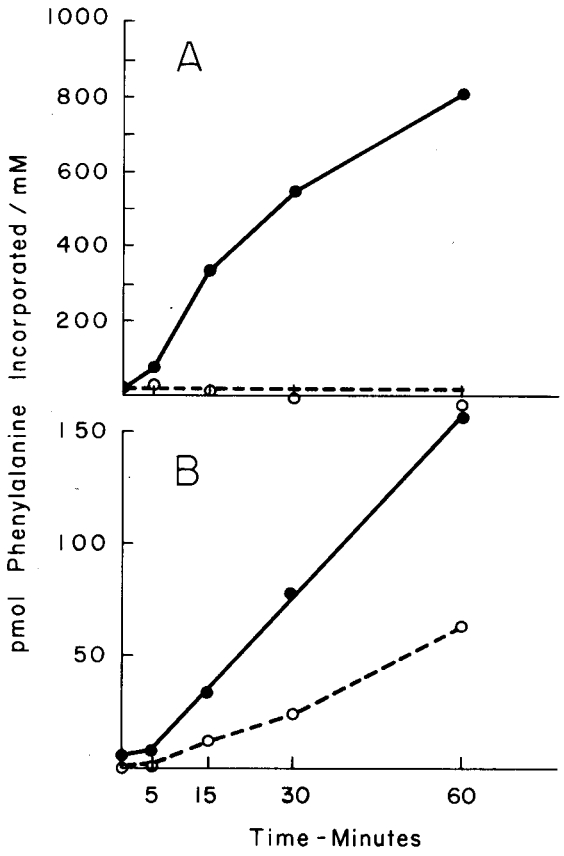

Fig. 3. Phenylalanine incorporation and phe-rich peptide release were measured as described in Methods. (A) 20 amino acids present, plus poly U; (B) 20 amino acids present, plus poly AU (1:4); (C) phenylalanine only, plus poly U; (D) phenulalanine only, plus poly AU (1:4). Each point represents the average of duplicate 50 µl samples.

insoluble product. In the first experiment (A, B) all 20 amino acids were present with only phenylalanine labelled. Poly AU promoted close to 40% released during most of the experiment. When only phenylalanine was present (C, D)., about 30% of product was released but total incorporation more than doubled. The latter phenomenon is the result of miscoding, which is frequently observed during poly U-directed incorporation (37). In neither of the two experiments was appreciable release observed in the presence of poly U. These results compare very favorably with peptide release in *E. coli* assayed by the »P-81« technique, and are clearly free of the problems associated with the detection of oligolysine release in wheat germ.

Synthesis and release of phe-rich peptide were both affected in a parallel manner by changes in levels of Mg^{++}, K^{+}, tRNA, and ribosomes. An example is the $MgCl_2$ dependency presented in Figure 4. For this reason, conditions were generally chosen which increased incorporation to the greatest extent possible.

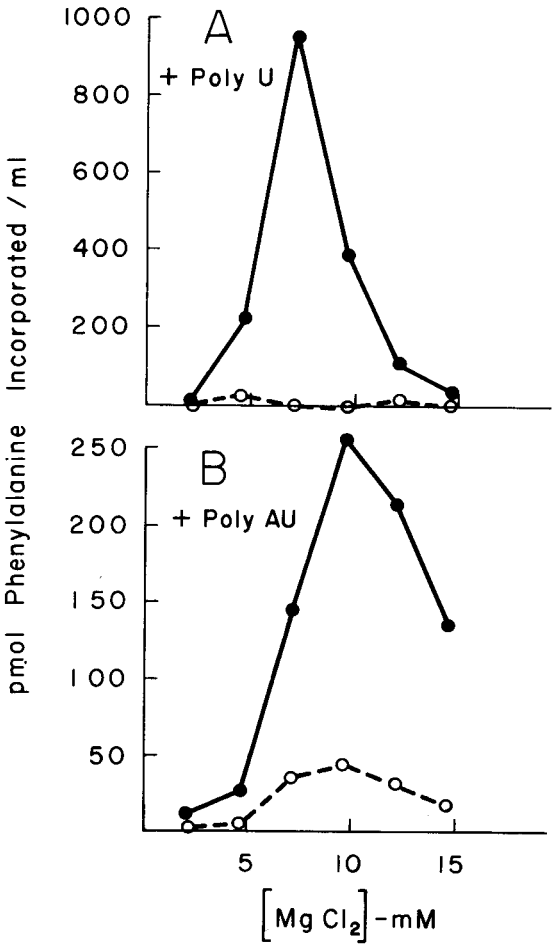

Fig. 4. Phenylalanine incorporation and phe-rich peptide release were measured as describd in Methods, with the $MgCl_2$ concentration varied as indicated in the figure. (^{14}C) phanylalanine was the only amionoacid present.

A variety of inhibitors perturbed synthesis and release of phe-rich peptide in a predictable manner (Table 1). These results will be discussed in detail in the following section.

TABLE I

Effects of Various Inhibitors on Phe-rich Peptide Synthesis and Release

Inhibitor	*Message*	*Synthesis*	*Release*	
		(pmol phe/ sample)	*(pmol phe/ sample)*	*(% of synthesis)*
Control	poly U	37.9	1.8	5
	poly AU	9.4	4.9	52
Sparsomycin	poly U	12.5	0.3	2
	poly AU	2.4	0.9	38
Puromycin	poly U	13.0	7.6	58
	poly AU	2.2	1.2	55
Methanol (10%)	poly U	21.3	6.2	29
	poly AU	23.3	6.9	30
Fusidic acid	poly U	19.5	1.1	6
	poly AU	4.1	1.5	36
GDPCP	poly U	3.4	1.2	35
	poly AU	1.0	0.3	30
Cycloheximide	poly U	22.1	2.4	11
	poly AU	4.5	1.3	29

Table I
Phenylalanine incorporation and phe-rich peptide release were measured as described in Methods. (^{14}C) phenylalanine was the only amino acid present. Ribosomes and inhibitors were premixed prior to the introduction of the remaining components of the reaction mixture. Each value represents the average of duplicate 50 μl samples. The A:U ratio of poly AU was 1:4. Unless otherwise indicated, inhibitors were present in a final concentration of 0.1 mM.

DISCUSSION

No studies have been published to date on the termination events of plant protein synthesis. However, reports on multiple-copy translation of messages with known 3' sequences show that all three nonsense triplets are terminators in wheat germ (26, 7). Therefore, we conclude that genetic punctuation —like the rest of the code— is universal.

We first began studying plant termination by characterizing coupled elongation and release reactions. There were several reasons for this. First, such reactions are physiologically closer to their *in vivo* counterparts than highly defined assays, and are therefore less difficult to interpret. They yield immediate (although qualitative) information about many aspects of release. They should also prove useful-perhaps even necessary— in the investigation of additional factor requirements and the function of any sequence information following the terminator triplet. Finally, correctly chosen coupled reactions can be uncoupled by appropriate modifications. The measurement of oligolysine synthesis and release by the "assay" for instance, was successfully modified to measure only release (21). In this vein, we plan to isolate termination intermediates prepared with purified wheat germ EF2 plus high Mg^{++} as substitute for S100 enzymes. We have also begun to look at ribosomal hydrolysis of N-acetyl-aminoacyl-tRNA as a model of natural termination.

In 1965, Bretscher and his colleagues were the first to show that phe-rich peptide was released when poly AU (A < U) was used as message (2). In the same paper, they reported the P81-assayed release of lysine-rich peptide with poly AU (A > U). Simultaneously, Gano-

za and Nakamoto published similar data on oligolysine release using an ECTEOLA cellulose assay, which could be adapted to study the release of most non-acidic soluble peptides (11). In the absence of well-defined synthetic oligonucleotides or highly purified native mRNA the ECTEOLA assay —like the P81 assay— is confined mainly to the measurement of lysine-rich peptides. Both methods are of little use in plant systems, which translate adenine-rich oligonucleotides poorly. Bretscher's technique of sucrose-gradient sedimentation for the assay of phe-rich peptides is expensive and slow.

Our method of determining phe-rich peptide is an adaptation of the oligophenylalanyl-puromycin assay of Maden *et al.* (20), who first reported the solubility of this compound in m-cresol. The solvent interacts with the stacked phenyl rings, destroying the rigid-rod structure which makes oligophenylalanine insoluble in virtually all other events. In addition, acyl-tRNA derivatives are neither hydrolyzed nor dissolved by m-cresol.

The time-course results of peptide release (Figure 3) are very similar to those reported for synthesis and release of oligophenylalanine and oligolysine in *E. coli* (2, 23, 11). It is clear that poly AU stimulates release of the growing peptide chains, and that poly U does not. The inference is that this is due to ochre codons in the synthetic message, although this cannot be proven until defined polynucleotides are used. The kinetics of release show a relatively constant percent of product released, independent of time with the exception of a brief initial lag. If translation were extremely efficient and each message sequence were read and terminated several times, this would not be the case. Rather we would expect to see the release curve running parallel to the synthesis curve, but displaced by the time of synthesis of the average chain. This is very nicely illustrated for hemoglobin synthesis in the data of Schreier and Staehelin (28). Therefore, we conclude that in all such coupled synthesis and release systems using synthetic messages, a steady-state between initiation and release is not achieved before some rate-limiting component is exhausted. This could be the result of slow intrinsic release rates, or of improper or abortive ribosome dissociation following termination, etc.

The inhibitor results (Table 1) indicate that the overall mechanism of termination and release described for *E. coli* and mammals is probably common to wheat germ. The sparsomycin, puromycin, and methanol data are consistent with catalysis of release by the peptidyl transferase (PTase) activity of the ribosome.

Sparsomycin is a potent and specific inhibitor of PTase in both 70S and 80S ribosomes. It inhibited both synthesis and release in our assay to about the same extent, as would be expected if peptide bond formation and chain release were catalytically similar events.

Puromycin is an aminoacyl-tRNA analogue. It accepts nascent peptides through a peptide linkage catalyzed by PTase. In our experiments, it promoted considerable abortive "release" of the elongating chains through the formation of m-cresol soluble oligophenylalanyl-puromycin. The puromycin release product cannot participate further in protein synthesis. The amount of product was a constant fraction of total synthesis, regardless of synthetic message. This is consistent with the known codon insensitivity of puromycin.The lack of any additivity between normal codon-dependent release and the effects of the antibiotic may indicate competition by puromycin for a site normally occupied by a release factor. This, in fact, is probably the case, since aminoacyl-tRNA has been shown in *E. coli* to compete directly with RF1 for the A-site, a portion of which also defines the puromycin binding site (12).

Methanol is known to uncouple elongation from codon dependency in prokaryotes and eukaryotes (24, 35). Ethanol has a similar effect on termination, although release under these conditions is still dependent on the appropriate release factors (33). Our results indicate that 10% methanol uncouples both synthesis and release from message translation, in agreement with both of these observations.

Fusidic acid stabilizes the post-translocation EF2-GDP-ribosomes complex (34). When EF2 exceeds the number of actively translating ribosomes (presumably the case in our experiments), peptidyl-tRNA is stabilized in the P-site. Our data show that both elongation and release are affected similarly by the inhibitor, as expected if EF1, EF2, and the hypothesized wheat germ release factor

all occupy a common site. The rate limiting step for peptidyl-transferase function would then be diffusion of the inhibitor-stabilized EF2 from the ribosome.

Cycloheximide and β γ-methylene-guanosine-5'triphosphate (GDPCP) both inhibit translocation by stabilizing the pretranslocation association of EF2 with the ribosome (3, 38). The GDPCP complex is very tight, whereas the cycloheximide effect is easily reversed. Thus, the progress of peptidyl-tRNA through the A-site is slowed by cycloheximide, and essentially halted by GDPCP. Peptidyl-tRNA binds rather poorly to this site (8, 22). The longer it remains, the greater the probability of its diffusion away from the ribosome and attack by peptidyl-tRNA hydrolase. Our results agree well with this analysis. GDPCP is a much more pronounced inhibitor of protein synthesis in wheat germ than is cycloheximide. On the other hand, the percent of product released in poly U-containing controls doubles when cycloheximide is present, and becomes indistinguishable from poly AU-"stimulated" released when GDPCP is present. GDPCP is a good inhibitor of RF-dependent release in mammals (29, 30). Therefore, the apparent high percent release in our data is almost certainly due to peptidyl-tRNA hydrolase.

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