POLYPEPTIDE CHAIN TERMINATION IN VITRO: ISOLATION OF A RELEASE FACTOR

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The growing polypeptide chain remains bound to the ribosome-messenger RNA complex through the sRNA carrying the last amino acid incorporated into the polypeptide chain.[1] On completion of the polypeptide chain a mechanism must exist for releasing it from the protein-synthesizing machinery. To date, most of the effort has been concentrated on establishing the coded information required for this event.[2-5] Very little is known about the biochemical mechanism of releasing the finished protein. The chain-terminating codon has to be read and the last peptidyl-sRNA bond must be cleaved. What components are required for this process remains to be determined.

It appears that a number of codons (UAA, UAG, UGA) can code for polypeptide chain termination.[6-9] If by genetic accident such a codon arises in the middle of a cistron, premature polypeptide chain termination occurs at the site of the mutation.[10] The RNA genome from the bacteriophage R17, carrying such a mutation, has been used to develop a specific assay for polypeptide chain termination. In this mutant, the first glutamine codon (CAG) in the R17 coat protein cistron has mutated to the chain-terminating codon UAG.[11] In a cell-free system, RNA from this mutant directs the synthesis of a small NH_2-terminal coat protein fragment, N-formyl-met-ala-ser-aspn-phe-thr, which is released into the supernatant.[12] We can stop the synthesis of this peptide before it reaches the UAG codon by starvation for a chosen amino acid. This allows us to control the very last step of protein synthesis, the release of the completed polypeptide chain. Exploiting this procedure a protein component required for polypeptide chain termination has been isolated.

Materials and Methods.—(a) *Peptide assay:* The quantitative assay employed to detect the synthesis of the NH_2-terminal coat protein fragment, N-formyl-met-ala-ser-aspn-phe-thr, is described in detail in a separate communication.[13] The assay exploits three properties of the peptide: (1) it is TCA soluble, (2) at acid pH's it is a neutral and hydrophobic peptide, and (3) at neutral pH's it is an acidic peptide. Because the peptide is TCA soluble, one can distinguish between the released coat protein fragment and the peptide still bound to the peptidyl sRNA (which is TCA precipitable). After removal of the released peptide, in the TCA phase, the bound peptide can be recovered by base hydrolysis of the peptidyl sRNA bond.

An example of the discrimination of the peptide assay is shown in Figure 1, which records the level of released peptide synthesized under the direction of amB_2 RNA as a function of the magnesium concentration. As controls, the same figure also shows the number of radioactive counts detected by the assay in (1) the absence of added mRNA and (2) the presence of wild-type R17 RNA, which should not direct the synthesis of the released coat protein fragment.

For the experiment recorded in Figure 1, the radioactive label was in threonine, the last amino acid in the polypeptide fragment. This position labels only the completed peptide. Any release observed could not have resulted from proteolytic hydrolysis anywhere in the polypeptide chain. Such a split would result in loss of the radioactive label, since the threonyl residue would then not be detected by the peptide assay. Separate labeling experiments have shown that the product detected by the peptide assay also contains formylmethionine, serine, and phenylalanine but does not contain lysine, arginine, valine, or isoleucine.

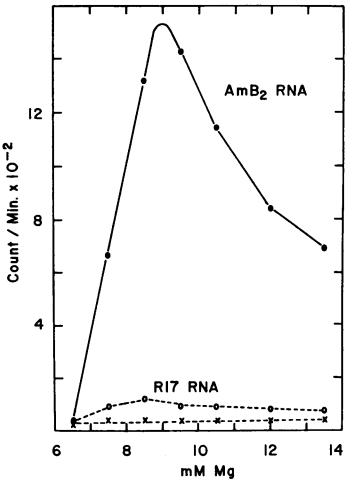

FIG. 1.—The magnesium dependence of C^{14}-threonine incorporation into released NH_2-terminal coat protein fragment in the presence of amB_2 RNA ●—●—●; R17 RNA O—O—O; and no RNA (×—×—×). The 100-μl reaction mixtures contained: 30 or 0 μg of mRNA; 100 μg of ribosomes; 5 μl of supernatant protein; 80 μg of sRNA; 10 μg of N^5-tetrahydrofolic acid; 3 μmoles NH_4; 0.3 μmole ATP; 0.02 μmole GTP; 0.05 μmole PEP; 2 μg pyruvate kinase; 1 μmole glutathione; 5 μmoles Tris, pH 7.8; 4 mμmoles of each amino acid; and 2.5×10^5 cpm of C^{14}-threonine (150 cpm/$\mu\mu$mole). After 12 min of incubation at 34°C each fraction was assayed for the released peptide, N-formyl-met-ala-ser-aspn-phe-C^{14}-thr.

(b) *Cell-free system:* For these experiments a cell-free protein synthesizing system was sought which was completely dependent on added sRNA and amino acids. Such a system was obtained by removal of the nucleic acid from a high-speed supernatant by liquid polymer partitioning between Dextran 500 and polyethylene glycol.[14] The remaining amino acids were removed by subsequent dialysis and ammonium sulfate precipitation (55%). When the resulting protein fraction was added to purified ribosomes, cell-free protein synthesis was observed to have a strong dependence on added messenger RNA, sRNA, a formyl donor, and amino acids, in addition to the components normally added to an S-30. For example, incorporation of C^{14}-threonine into TCA-precipitable product under the direction of R17 RNA goes from 1,100 to 52,000 cpm on addition of 1 mg/ml of purified total sRNA. Such 50-fold effects can also be observed by starvation for a single amino acid or by leaving out the messenger RNA template. Under optimal conditions the efficiency of the above cell-free amino acid-incorporating system, programed with R17 RNA, is comparable to a good S-30 preparation.

(c) *Ribosomes:* Ribosomes were purified by layering 8 ml of S-30 on top of 20 ml of 1 *M* sucrose (34%) buffered with 0.01 *M* $MgCl_2$, 0.10 *M* NH_4Cl, 0.05 *M* Tris, pH 7.6, 0.001 *M* EDTA, and 0.0005 *M* Cleland's reagent. The ribosomes were then pelleted through the sucrose by centrifugation at 30,000 rpm for 9.5 hr. The supernatant was drawn off and each tube washed three times with buffer. The crystal-clear ribosomal pellet was then resuspended in 50% glycerol, 0.01 *M* $MgCl_2$, 0.03 *M* NH_4Cl, 0.05 *M* Tris, pH 7.6, 0.001 *M* EDTA, and 0.0005 *M* Cleland's reagent. The ribosomal solution was then stored in the unfrozen state at −20°C.

Results.—(a) *Preparation of the substrate:* The ideal substrate for examining the mechanism of polypeptide chain termination is a polypeptide chain, of known sequence, caught just prior to being released from the ribosomal-messenger RNA-peptidyl sRNA complex. Such a substrate can be constructed.

Using the amino acid dependent protein synthesizing system described in *Materials and Methods*, starvation for a chosen amino acid can be employed to stop the synthesis of the coat protein fragment, formyl-met-ala-ser-aspn-phe-thr, at any prescribed point. The ribosome-mRNA-sRNA complex (carrying the incomplete coat protein fragment) is then isolated from the reaction mixture by sucrose gradient centrifugation. As shown in Figure 2*a*, the complex, sedimenting at 80*S*, is partially resolved from the 70*S* ribosomes. For this experiment the amB_2 RNA-directed protein synthesizing system was starved for threonine. The coat protein fragment was labeled with C^{14}-phenylalanine in the reaction mixture. After isolation of the complex on a sucrose gradient, each fraction was incubated at

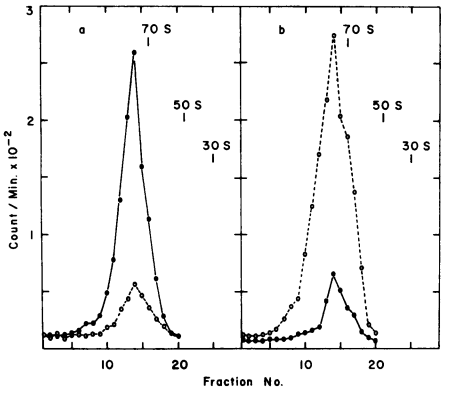

FIG. 2.—(*a*) Isolation of the ribosome-mRNA-peptidyl sRNA complex, carrying the incomplete coat protein fragment N-formyl-met-ala-ser-aspn-C^{14}-phe. (*b*) Release of the completed coat protein fragment from the complex. A 2.0-ml reaction mixture containing the same components described in Fig. 1 (except C^{14}-phenylalanine, 470 cpm/$\mu\mu$mole was substituted for C^{14}-threonine and no threonine was added) was incubated for 8 min at 34°C. 750 μl of this reaction mixture was layered on two 12-ml sucrose gradients (0.4–0.92 *M* exponential gradients).[19] After centrifugation at 40,000 rpm for 4.25 hr at 3.5°C, 350-μl fractions were collected. The positions of the 70*S* ribosomes and the 50*S* and 30*S* subunits were obtained by assaying a 40-μl aliquot from each fraction for absorbancy at 260 mμ. Each fraction for the gradient shown in (*a*) was incubated for 10 min at 34°C and then assayed for both bound (●——●——●) and released (O—O—O) NH_2-terminal coat protein fragment. For the gradient shown in (*b*), each fraction was incubated for 10 min at 34°C with 1 mμmole C^{12}-threonine; 50 μg sRNA; 0.2 μmole ATP; 0.05 GTP; and 5 μl supernatant enzymes, prior to assaying for bound (●——●——●) and released (O—O—O) counts.

37°C for 10 minutes and then assayed for released (*open circles*) and unreleased (*closed circles*) coat protein fragment. The assay is specific for the NH_2-terminal coat protein fragment (see *Materials and Methods*).

The experiment recorded in Figure 2*b* demonstrates that incubation of the substrate with threonine, ATP, GTP, sRNA, and supernatant enzymes released the coat protein fragment. The recovery of the released chain is excellent. In this experiment neither incubation with supernatant enzymes + GTP alone nor with charged threonyl sRNA + GTP alone alters the released and bound peptide profiles shown in Figure 2*a*. Both must be present. These experiments argue that in order to release the incomplete polypeptide fragment, formyl met-ala-ser-aspn-C^{14}-phe, the chain has to be completed (i.e., threonine must be added) and supernatant enzyme must be present.

Figure 3 shows that the process of adding the last amino acid to the coat protein fragment can be separated from the subsequent release of the finished polypeptide from the ribosomal-mRNA-sRNA complex. For this experiment the amB_2 RNA-directed amino acid incorporating system, starved for threonine, was not labeled with a radioactive amino acid. After isolating the complex on two parallel gra-

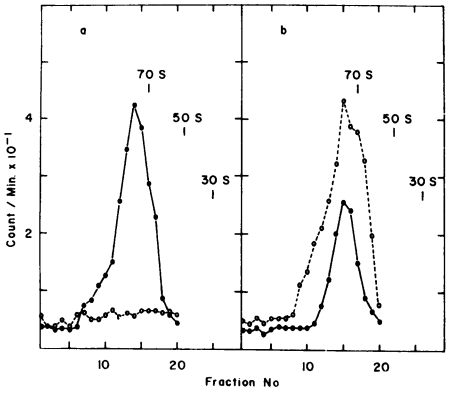

FIG. 3.—(*a*) Completion of the polypeptide chain F-met-ala-ser-aspn-phe without releasing the finished polypeptide chain from the complex. (*b*) Releasing the finished peptide on incubation with supernatant enzymes. The experimental details are identical with those described in Fig. 2 except C^{12}-phenylalanine was used in place of C^{14}-phenylalanine in the reaction mixture. Each fraction of the gradients shown was incubated with 45 μg H^3-threonyl sRNA (120 cpm/$\mu\mu$mole) + 0.05 μmole GTP and 45 μg H^3-threonyl sRNA + 0.05 μmole GTP + 5 μl supernatant enzymes, respectively. After incubation each fraction was assayed for the bound (●——●——●) and released (O—O—O) peptide.

dients, the fractions of the first gradient (Fig. 3*a*) and the second gradient (Fig. 3*b*) were incubated with H^3-threonyl sRNA + GTP and H^3-threonyl sRNA + GTP + supernatant enzymes, respectively. This experiment demonstrates that release of the finished coat protein fragment is dependent on added supernatant enzymes. Subsequent experiments will demonstrate that this dependence is not only on transfer enzymes (required to move the ribosome from the threonyl to the UAG codon) but also on a new supernatant factor which mediates polypeptide chain termination. Figure 3*a* also shows that the addition of a single amino acid to the polypeptide chain does not require added supernatant enzymes. This is in agreement with Monro, who found that the peptide bond-forming activity is an integral part of the ribosome.[15]

(b) *Isolation of the release factor:* The supernatant proteins were fractionated on a DEAE-Sephadex column with a linear salt gradient. Each fraction was assayed for transfer activity using the poly U-directed system (Fig. 4, *open circle*). This protein (from tube 14) was used to add C^{12}-phenylalanine and C^{14}-threonine to the sucrose gradient prepared substrate (carrying the incomplete protein fragment, F-met-ala-ser-aspn). The substrate was purposely made dependent on full transfer activity by requiring the addition of two amino acids to complete the chain. The addition of phenylalanine and C^{14}-threonine to the coat protein fragment proceeded with only a small fraction of the finished polypeptide chains being released (10%). Finally, we have the ideal substrate. Each fraction from the DEAE-Sephadex column was then assayed for the existence of a factor which would mediate polypeptide chain termination. The solid circles in Figure 4 designate the released peptide counts using the above substrate. A new factor has been isolated required for polypeptide chain termination.

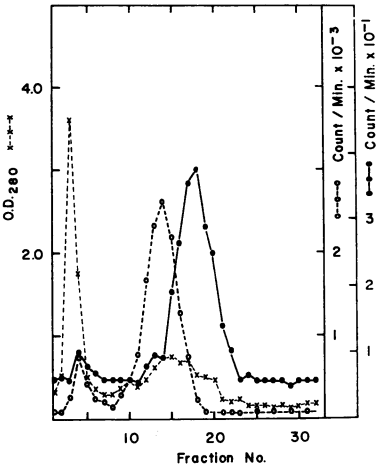

FIG. 4.—Isolation of the release factor activity (●——●——●). Approximately 40 mg of high-speed supernatant protein, devoid of nucleic acid, was fractionated on a 0.9- × 22-cm DEAE-Sephadex column. The protein was eluted with a linear (0.15–0.6 *M*) Tris gradient. Fractions of 3.0 ml were collected. 5 μl of each fraction was assayed for transfer enzyme activity using the poly U system (○—○—○). The ribosomes used for all of these experiments are saturated with G-factor, since on sucrose gradient washing only the T-factor is removed.[20] The open circles therefore represent T-factor activity. The transfer enzyme from tube 14 was used to add C^{12}-phenylalanine and C^{14}-threonine to the substrate carrying the peptide F-met-ala-ser-aspn. This proceeded to completion with only a small per cent of the finished chains being released. 10 μl of each fraction (1–32) was assayed for the ability to release the completed coat protein fragment F-met-ala-ser-aspn-phe-C^{14}-thr. After 10 min of incubation each fraction was assayed for the bound and released peptide counts. The sum of these counts was constant throughout the profile. Shown above are the released peptide counts (●——●——●).

(c) *Site of action for the release factor:* The release factor could mediate the polypeptide chain termination either directly at the ribosome or in the supernatant, by cleaving the peptidyl sRNA bond. In the experiment recorded in Figure 5, we show that the substrate for the R-factor is the ribosomal-mRNA-peptidyl sRNA complex (carrying the polypeptide F-met-ala-ser-aspn-phe-C^{14}-thr). This is

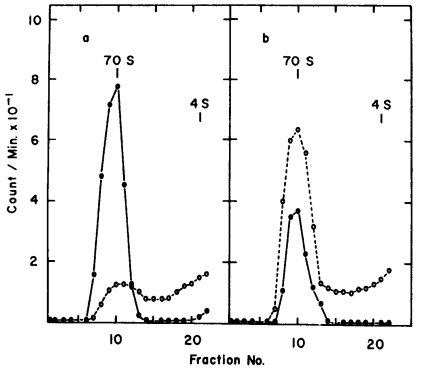

FIG. 5.—The release factor mediates polypeptide chain termination directly at the ribosome-mRNA-peptidyl sRNA complex. (*a*) After completion of the polypeptide chain by the addition of phenylalanine and C^{14}-threonine (using the transfer enzyme from tube 94, Fig. 4), the reaction mixture was rerun on a sucrose gradient to locate the peptidyl sRNA, carrying the completed coat protein fragment. Each fraction was assayed for the bound (●——●——●) and released (○—○—○) peptide, F-met-ala-ser-aspn-phe-C^{14}-thr. (*b*) Prior to assaying for the bound (●——●——●) and released (○—○—○) peptide, each fraction was incubated for 10 min at 34°C with 15 μl of R-factor from tube 18 (Fig. 4). The small amount of released peptide counts at the top of the gradient is due to contamination of the transfer enzyme, used to complete the chains, with R-factor.

shown in two steps. First, rerunning the substrate (after addition of phenylalanine and C^{14}-threonine) on a second sucrose gradient shows that the peptidyl sRNA is not ejected from the ribosome on completion of the coat protein fragment (Fig. 5*a*). Second, incubation of this substrate, after the second sucrose gradient fractionation, with just R-factor, releases a large proportion of the completed chains (Fig. 5*b*).

(d) *Size of the release factor:* An estimate of the molecular weight of the R-factor was obtained by sedimenting the protein from tube 18 (Fig. 4) on a linear sucrose gradient. The gradient was calibrated by running three protein markers on each of two parallel gradients. Figure 6 shows that the release factor activity sediments in the 4.0–3.5 region, consistent with a molecular weight of 40,000–50,000.

(e) *Sensitivity of the release factor to pancreatic RNase:* Since it is clear that polypeptide chain termination is triggered by certain codons, the release factor might be an RNA-protein complex. This was tested by determining if the release factor activity was sensitive to incubation with pancreatic RNase.

An aliquot of the release factor from tube 18 (Fig. 4) was incubated with 25 γ/ml of RNase for one hour at 0°C. The RNase was then removed from the R-factor by chromatography on a DEAE-Sephadex column (see Fig. 7). On a second identical DEAE-Sephadex column an equal aliquot of R-factor, not treated with pancreatic RNase, was subjected to the same elution procedure. Figure 7 shows that the 0.2 *M* wash successfully removed any detectable RNase and that incubation of the release factor with RNase had no effect on its activity (closed circles treated with RNase; open circles not treated with RNase).

Discussion.—In summary, a cell-free system has been developed for specifically examining the mechanism of polypeptide chain termination. Using this system a factor (the R-factor) required for polypeptide chain termination has been isolated. This release factor is judged to be a protein with a molecular weight of 40,000–50,000 by the following criteria: (1) the factor partitions as a protein on liquid polymer partitioning; (2) the factor behaves like a protein of DEAE-Sephadex; (3) the material which chromatographs with the factor activity contains less than 0.5 per cent nucleic acid as judged by optical density measurements; (4) the factor is resistant to incubation with pancreatic RNase. Isolating an enzymatic factor required for polypeptide chain termination is consistent with the observation of

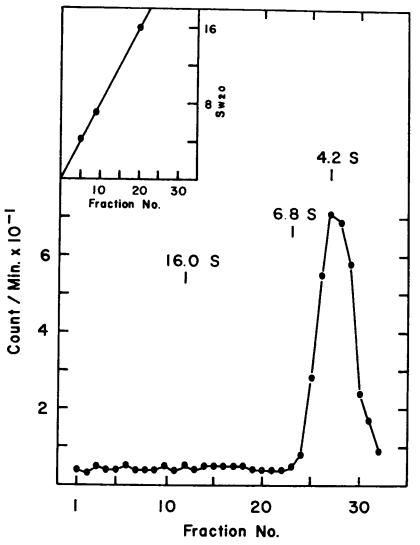

FIG. 6.—Size of the release factor. 200 μl of the protein from tube 18 (Fig. 4) was layered on a 5-ml linear sucrose gradient (0.40 *M*–0.92 *M*) and centrifuged at 64,000 rpm for 7.5 hr. 160-μl fractions were collected and assayed for release factor activity, using the substrate carrying the completed but unreleased polypeptide chain F-met-ala-ser-aspn-phe-C^{14}-thr. The closed circles indicate the released peptide counts. Each of the following protein markers were sedimented on two parallel gradients: β-galactosidase (16*S*), alcohol dehydrogenase (6.8*S*), and hemoglobin (4.2*S*). In the upper left-hand corner a plot of sedimentation constants of the markers versus fraction number is shown, indicating that the gradient was linear in the range tested.

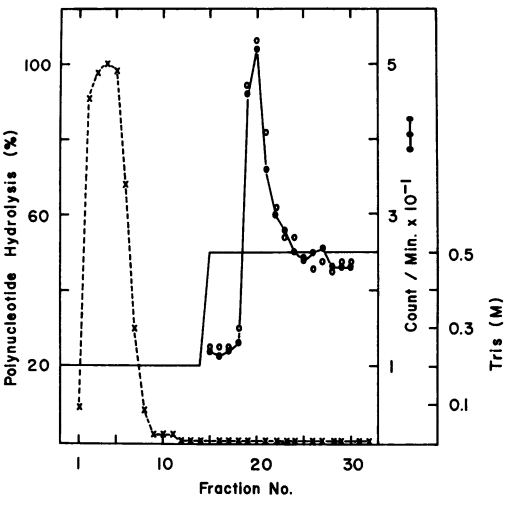

FIG. 7.—The release factor is resistant to digestion with pancreatic RNase. One ml of the protein from tube 18 (Fig. 4) was incubated with 25 μg of pancreatic RNase for 1 hr at 0°C. The protein was then applied to a 0.9- × 6-cm DEAE-Sephadex column. The RNase was eluted with 14 ml of 0.2 *M* Tris, pH 7.6. One-ml fractions were collected. The release factor was then eluted with 0.5 *M* Tris. On a parallel column, 1 ml of protein from tube 18, not incubated with pancreatic RNase, was subjected to the same elution procedure. 10 μl of each fraction was assayed for RNase activity (×—×—×) by incubation with 26,000 cpm of H^3-poly U for 3 min at 30°C. 40 μl of tubes 15–32 were assayed for release factor activity as described in Fig. 6 (●——●——● incubated with RNase; O O O not incubated with RNase).

Ganoza that her cell-free system, on purification, lost its capacity to release free polypeptide chains.[16]

The substrate for the R-factor has been shown to be the ribosome-mRNA-peptidyl sRNA complex. These studies have not determined whether the release factor works in conjunction with other supernatant or ribosomal factors. In particular, we have not been able to demonstrate whether or not the release factor works in conjunction with a hypothetical chain-terminating sRNA. Attempts to detect a chain-terminating sRNA in the total sRNA fraction have so far failed. For example, threonyl sRNA has been partially purified (approximately 10-fold) by reverse phase chromatography.[17] Using this sRNA to complete the coat protein fragment (see *Results*, section *a*) no hint has been obtained that an additional sRNA is required to release the finished polypeptide chain. That is, the initial rate of release of the coat protein fragment as a function of added threonyl sRNA appears to be linear.

Another approach has been to destroy, by periodate oxidation, all sRNA species

except those capable of accepting met, ala, ser, aspn, phe, and thr. After periodate oxidation, the above species retained greater than 90 per cent of their original accepting activity whereas the other tested amino acyl-sRNA's (lys, arg, leu, Ileu, val, and glu) were destroyed to 2 per cent or less of their original accepting activity. The amount of released coat protein fragment (F-met-ala-ser-aspn-phe-thr) as a function of added sRNA (both periodated and unperiodated) was found to be the same. The protein-synthesizing system used for these experiments is completely dependent on added sRNA (see *Materials and Methods*). These latter experiments simply argue that if a chain-terminating sRNA exists, it does not operate as a normal sRNA (i.e., exchange of a moiety at the 3′ terminal nucleotide revealing cis-hydroxyl groups).

These experiments have not been discussed as proof that a chain-terminating sRNA does not exist. One is always cautious of negative results; therefore, the search continues. They have been discussed merely to justify seriously entertaining models for polypeptide chain termination which do not involve a chain-terminating sRNA. Such models fall into two categories, those in which the nonsense codons (UAA, UAG, UGA) are read, and those in which the nonsense codons function because they cannot be read.

In the absence of a chain-terminating sRNA we are left with two candidates for the reading of the nonsense codons, the ribosome and the R-factor. The reading need not be highly specific. For example, the ability to recognize uracyl followed by two purines may be sufficient. That UGG does not lead to chain termination would be explained by a lopsided competition with the tryptophanyl sRNA. This is not unreasonable since the tyrosine suppressor sRNA (which is estimated to comprise less than 10% of the total tyrosyl sRNA)[18] competes effectively with the chain-terminating process.

The finding that codons containing UX (X = xanthylic acid) do not lead to chain termination[3] contradicts models in which any codon which cannot be read leads to polypeptide chain termination. These models could, however, be salvaged by restricting the allowable nonreadable codons which lead to chain termination. Such models are operationally equivalent to the ribosome reading the nonsense codon.

In conclusion, a system has been developed for examining just the last step of protein synthesis, the release of the finished polypeptide chain. In this system, one is free to manipulate all supernatant factors, nucleic acid, or protein. Hopefully, competition experiments between the R-factor, a suppressor sRNA (Su_I), and perhaps a chain-terminating sRNA will suggest which component reads the chain-terminating signal.

Note added in proof: Cuzin, Kretschmer, Greenberg, Hurwitz, and Chapeville have just reported on an N-acetylaminoacyl-sRNA hydrolase. Although the initial purification was similar, including liquid-polymer partitioning, the hydrolase is a different protein from the R-factor since the hydrolase washes through DEAE at low salt concentrations (0.01 *M*) whereas the R-factor is strongly retarded on DEAE-Sephadex (requiring 0.35–0.40 *M* salt for elution). The two proteins have different charge properties. Furthermore, addition of the fraction enriched for hydrolase (as judged by their purification) did not catalyze the release of the coat protein fragment.

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