PROKARYOTIC TRANSLATIONAL TERMINATION

STUDIES ON THE MECHANISM OF PROKARYOTIC TRANSLATIONAL TERMINATION

By

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ABSTRACT

Using an *in vitro* prokaryotic termination assay, it was demonstrated that sequences neighbouring UA are recognized by RF-1 and stimulate cleavage of ribosome-bound f-met-$tRNA_f^{met}$. The ability of UA to signal release depends upon the nature of nucleotides adjacent both 3′ and 5′ to this sequence. RF-1 exhibits different specificity when potential termination sequences are covalently linked to AUG within the same polynucleotide, as in mRNA. Under these circumstances, within certain base context, (1) UA functions as a termination signal, (2) UA-containing terminator signals can be read out of the AUG-aligned reading frame and (3) RF-1 competes with aminoacyl-tRNA for sequence UUA.

Another factor has been discovered, which partially corrects the specificity of RF-1. This factor (designated Specificity Factor) appears to be a protein, or a protein-containing component, and enhances RF-1-mediated termination caused by UAA but inhibits termination caused by UA. The factors known to participate in protein synthesis are not responsible for conferring specificity to the RF-1-mediated termination reaction. For this reason, it is believed that the Specificity Factor may be a new protein.

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ABBREVIATIONS

A	adenine
C	cytosine
DEAE	diethylaminoethyl
DNase	deoxyribonuclease
DTT	dithiothreitol
E. coli	Escherichia coli
EDTA	ethylenediaminetetraacetic acid
EF-G	elongation factor-G
EF-T	elongation factor-T
EF-P	elongation factor-P
5-FU	5-fluorouracil
f-[^{35}S] met-tRNA$_f^{met}$	N-formyl-[^{35}S]methionyl-transfer RNA$_f^{met}$
G	guanine
g	gram
GTP	guanosine triphosphate
HCl	hydrochloric acid
IF	initiation factor
KCl	potassium chloride
leu-tRNA^{leu}	leucyl-transfer RNA^{leu}
lys-tRNA^{lys}	lysly-transfer RNA^{lys}
met-tRNA^{met}	methionyl-transfer RNA^{met}
mg	milligram
$MgCl_2$	magnesium chloride
min	minute
M	molar

mRNA	messenger RNA
N-acetyl-[^{3}H]met-$tRNA_f^{met}$	N-acetyl-[^{3}H]methionyl-transfer RNA_f^{met}
NH_4Cl	ammonium chloride
nmol	nanomole
phe-$tRNA^{phe}$	phenylalanyl-transfer RNA^{phe}
pmol	picomole
POPOP	p-bis-[2-(5-phenyloxazolyl)]-benzene
PPO	2,5-diphenyloxazole
RF	release factor
RNA	ribonucleic acid
RR	ribosome-releasing factor
S	stimulatory factor
SDS	sodium dodecyl sulphate
sec	second
sup-$tRNA^{tyr}$	suppressor-transfer RNA^{tyr}
Tris	Tris(hydroxymethyl)aminomethane
U	uracil
µg	microgram
V	volt
val-$tRNA^{val}$	valyl-transfer RNA^{val}

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INTRODUCTION

Proteins are synthesized in three discrete steps, namely initiation, elongation and termination. Each of these stages may be studied using model systems where the requirements per step are easier to determine. The results obtained from such model reactions may then be verified using a natural system.

1.1 The Initiation Process.

Ribosomes consist of two different subunits. In Escherichia coli, the 30S subunit consists of one 16S RNA molecule and 21 proteins. The larger subunit (50S) is composed of two RNA molecules, namely 5S and 23S RNAs, and 34 proteins (Pongs et al., 1974). The 70S ribosome is believed to contain two sites, the peptidyl (or P) site as well as the aminoacyl (or A) site.

The ultimate result of the prokaryotic initiation process is formation of a 30S ribosomal subunit (P site)• messenger RNA• N-formyl-methionyl-$tRNA_f^{met}$ complex. The nonribosomal initiation proteins IF-1, IF-2 and IF-3 are implicated in this process which requires the hydrolysis of GTP (Ganoza, 1977).

The two initiation factors IF-1 and IF-2 are required for optimum formation of initiation complexes with synthetic oligoribonucleotides. However, addition of IF-3 appears to be essential for chain initiation with natural mRNAs, suggesting that IF-3 is involved in the recognition of specific nucleotide sequences (Suttle et al., 1973). The apparent role of IF-3 is to bind to and stabilize the mRNA• 16S RNA initiation complex. As illustrated in Figure 1, one molecule of f-[^{35}S]met-tRNA$_f^{met}$ is bound to IF-2 and one GTP molecule. The resulting complex, whose formation is stimulated by IF-1 is then bound to the 30S subunit such that the anticodon of the tRNA pairs in a complementary fashion with the initiation codon of the message. The most common initiation codon is AUG, however GUG and UUG are more infrequently used. The 50S ribosomal subunit binds to form the 70S initiation complex, releasing the initiation factors and GDP (Gold et al., 1980).

In the simplest model assay of initiation, f-[^{35}S]met-tRNA$_f^{met}$ is bound to 70S ribosomes with AUG, requiring magnesium and ammonium cations (optimally 10 mM Mg^{2+} and 50 mM NH_4^+) (Ganoza et al., 1982).

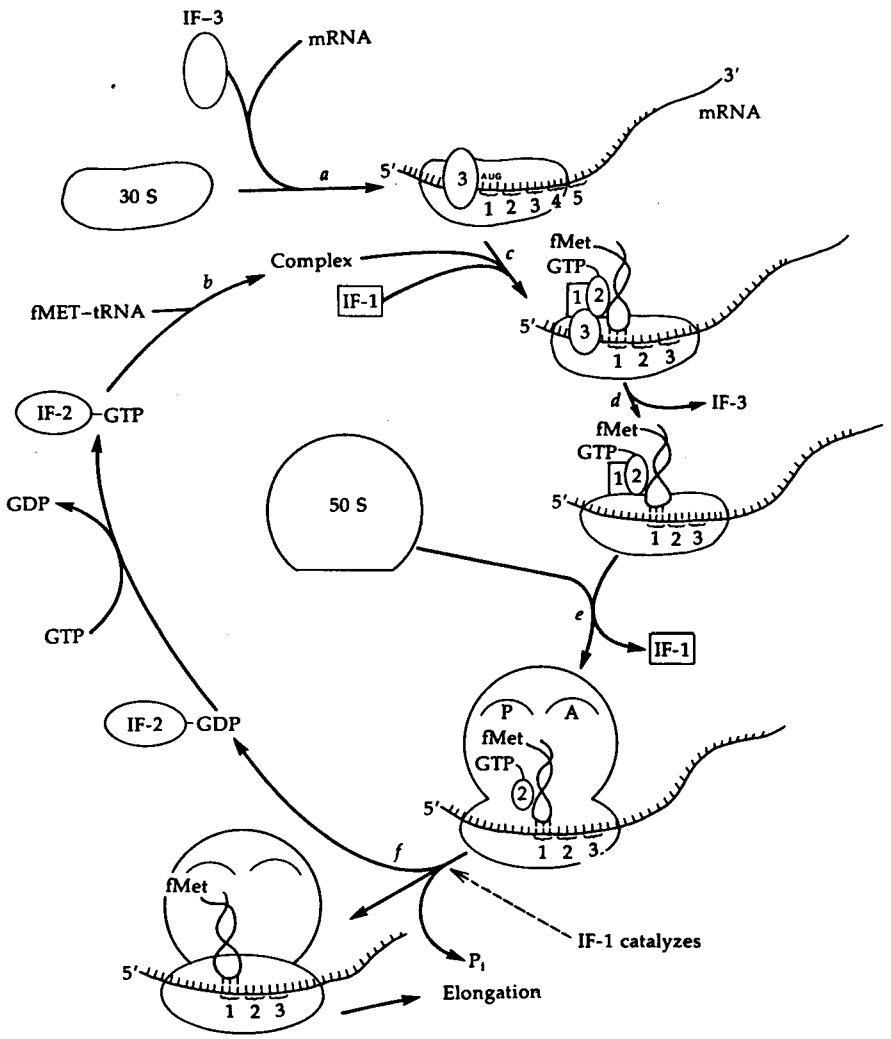


Figure 1. Prokaryotic Initiation (from Metzler, 1977).

1.1.1 The Initiation Sequence May Be Longer Than A Triplet.

There is increasing evidence which suggests that the initiation sequence is actually larger than a single codon. The triplet GUG is decoded by f-[^{35}S]met-tRNA$_f^{met}$ during initiation but by valyl-tRNA^{val} when it occurs internally in a protein. Similarly, as methionine is specified by only one codon, AUG may also be recognized by either f-[^{35}S]met-tRNA$_f^{met}$ or met-tRNA^{met} depending on its location in mRNA. Therefore, other features of the mRNA in addition to the presence of a single triplet are required to begin translation (Salser et al., 1969).

Initially, proximity to the beginning of the mRNA molecule was believed to be responsible for determining which amino acid, methionine or N-formyl-methionine, would be incorporated in response to AUG. However, sequencing data revealed that AUG codes for N-formyl-methionine in positions far from the 5′-terminus of some mRNAs. In fact, in several cases the triplet coding for initiation of protein synthesis is not the first such codon in the mRNA (Salser et al., 1969).

Secondary or tertiary structure could be responsible for initiation codon designation and efficiency. For

example, the proposed secondary structure of phage MS2 coat mRNA contains double-stranded portions and more exposed regions, without hydrogen-bonding, referred to as loops. The AUG coding for initiation of the coat protein occurs on a loop and is exposed, while the triplet which starts the A protein and replicase are buried by hydrogen-bonding. In intact phage, only the initiation signal for the coat protein is available for ribosome binding *in vitro*, which may explain why ribosomes must translate part of the coat protein before translation of the replicase or A protein mRNA can commence (Lodish, 1976).

Further studies revealed that all three initiation sites become available in fragmented mRNA, where the small degree of fragmentation is unlikely to disrupt the secondary structure. Also, in all models of the secondary structure of MS2 RNA, other AUG, GUG or UUG triplets occur in regions more exposed than the initiator itself. Therefore, correct initiation must rely on some property of the mRNA other than its secondary or tertiary structure (Lodish, 1976).

However, if the secondary structure of the RNA is completely melted, the synthesis of all three proteins is begun. In addition, many other formyl-methionine-containing peptides are made, suggesting that many internal AUG, GUG or UUG codons are capable of specifying initiation unless masked by secondary and/or tertiary folding (Lodish, 1976). It seems reasonable therefore, that such secondary and

tertiary structures of RNA contribute to the frequency with which genes are translated (Ganoza, 1977).

The fact that untranslated stretches of nucleotides exist at the 5′-termini of many mRNAs, and are inevitably conserved, suggests that these regions function in initiation. As ribosomes bind only to correct initiation codons in RNAs which contain out-of-phase AUGs, it would seem that the specificity of initiation lies in the sequence of the initiator region rather than in overall secondary or tertiary structure of the RNA. Initial sequencing data of ribosome binding sites of various mRNAs implies that all intercistronic regions contain an in-phase or out-of-phase termination codon (Steitz et al., 1975). These termination triplets, either UAA or UGA but not UAG, are separated from the initiation codon by a variable range of twenty or fewer nucleotide residues (Atkins, 1979). It is proposed that the termination codons actually serve a dual purpose by terminating translation as well as preparing the apparatus for initiation. Using a model assay system, oligoribonucleotide AUGUAA, bound to ribosomes, over seventy percent of f-[^{35}S]met-tRNA$_f^{met}$ added, however no such binding was observed with UAAAUG. The UAAAUG hexamer did not inhibit formation of the initiation complex with AUG which implies that although the termination and initiation processes may be linked, spacing may be required between the stop and start codons (Ganoza, 1977). However, as

sequencing data accumulates, it is easy to find mRNAs whose precistronic region does not contain a nonsense codon (Neilson et al., 1980). Thus, the presence of a nonsense codon 5′ to the start triplet may contribute, but is not essential for the initiation process.

By studying the sequence of mRNA regions protected by ribosomes from nuclease attack, an adenosine and guanosine-rich region (the Shine-Dalgarno region) 5′ to initiator codons was discovered. The region was complementary to the 3′-end of the 16S ribosomal RNA. Since recognition of mRNA initiation signals in prokaryotes appears to be primarily due to the 30S subunit of ribosomes, it appears significant that precistronic regions of the message, which are called ribosome binding sites, exhibit this complementary sequence (Shine et al., 1974).

This hypothesis is also based on several observations including the formation of a 16S rRNA•mRNA double helical complex using mRNA of phage R17 A protein which contains eight nucleotide residues complementary to 3′-fragments of 16S rRNA (Neilson et al., 1980). Also, since the R17 A protein binding site exhibits the most possible base-pairs with 16S rRNA in comparison to coat and replicase precistronic regions, it is expected that the RNA of the A protein would be more efficiently initiated. It was indeed shown that the isolated A protein initiator fragment of R17 RNA could interact with E. coli ribosomes forty and eleven

times more efficiently than with the coat and replicase fragments respectively. However, the highly efficient binding, caused by the RNA of the A protein initiator fragments, was not exhibited using intact R17 RNA *in vitro*, where twenty mol of coat protein and five mol of replicase are synthesized per mol of A protein. Presumably it is the secondary structure of the intact RNA which causes this inconsistency, impeding binding of the 30S subunit to the A protein initiation region (Shine *et al.*, 1974).

Genetic data also support the hypothesis that the Shine-Dalgarno region is important for initiation. Mutants which have this region altered produce significantly reduced levels of protein (Neilson *et al.*, 1980).

Examination of known precistronic regions reveals however, that half contain three to five bases complementary to the 3′-end of 16S rRNA. Although complexes can form between eight mRNA bases complementary to the 16S rRNA, whether only three or four nucleotide residues suffice to anchor the messenger to the ribosome has not yet been demonstrated. Hydrogen-bonding of oligoribonucleotides, which contain three bases complementary, requires conditions inconsistent with physiological protein synthesis (Neilson *et al.*, 1980), which questions the absolute necessity of the "Shine-Dalgarno interaction" in initiation. Also, many internal AUG and GUG codons are preceded by sequences which actually display more complementarity to the 16S rRNA than

the initiator itself (Ganoza et al., 1978). In lambda phage, translation of a cI-repressor mRNA lacking a precistronic region occurs. Furthermore, in the cro mRNA of lambda phage, base changes at a distance 5′ and far from the Shine-Dalgarno region revealed a dramatic difference in cro mRNA translation. Therefore, the Shine-Dalgarno region is not sufficient in itself to define an initiation signal (Ganoza et al., 1982).

Recently it has been proposed that the base context around the AUG codon modulates the initiation reaction. In model assays, the presence of one pyrimidine 5′ to the initiation codon is more effective than a purine in enhancing f-[^{35}S]met-tRNA$_f^{met}$ binding to 70S ribosomes. The initiation factors had no effect on the observed binding at 10 mM Mg^{2+} but were required at 5 mM Mg^{2+} however, the purine/pyrimidine effect 5′ to AUG was still maintained. Therefore the initiation factors do not appear to affect the ability of ribosomes to recognize the base content of mRNA (Ganoza et al., 1978).

In a study using Qβ RNA mutants, differential binding of f-met-tRNA$_f^{met}$ was observed when the nucleotide 3′ to the initiation codon was altered. More specifically, Qβ RNA mutated from AUGG to AUGA bound more tightly to ribosomes. The initiator tRNA$_f^{met}$ is unusual because the bases 3′ to the anticodon are not modified, and it also has two bases 5′ to the anticodon all of which

together with the anticodon have the potential to base pair with four or five bases of mRNA (Ganoza et al., 1982). The strength and extent of such an interaction may play a vital role in selecting the initiation codon from various internal AUG and GUG codons.

Any of the above properties of mRNA and its proposed interactions with 16S rRNA or $tRNA_f^{met}$ does not totally explain the mechanism whereby a start codon is chosen from other internal AUGs and GUGs to begin initiation. It is very possible that another property of mRNA or yet another interaction with a piece of the translational apparatus accounts for the specificity of the initiation process. However, the specificity and efficiency of initiation may be just the sum of several, or all, of these possible interactions.

1.2 The Elongation Cycle.

Figure 2 illustrates polypeptide propagation which begins when the non-ribosomal elongation protein EF-T, in concert with GTP, binds aminoacyl-tRNAs onto ribosomes in a site believed adjacent to the bound f-met-$tRNA_f^{met}$ (A site). After this positioning, which is accompanied by hydrolysis of GTP, peptide bond formation is catalyzed by peptidyl transferase which is an integral part of the 50S

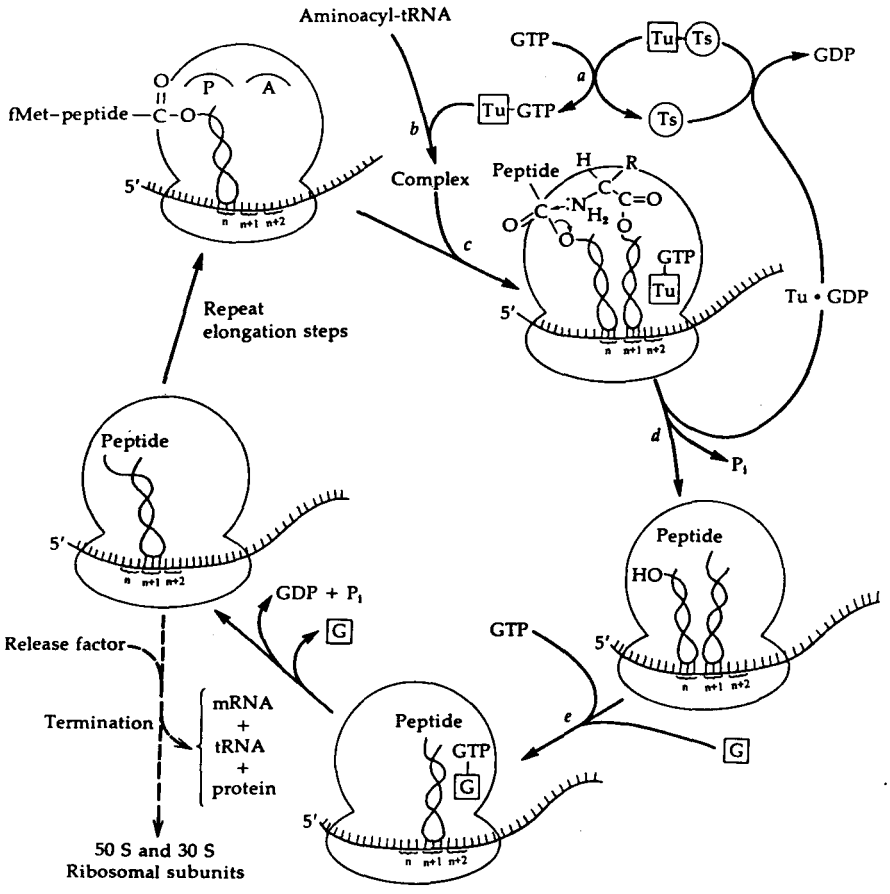


Figure 2. Prokaryotic Elongation (from Metzler, 1977).

subunit (Lucas-Lenard et al., 1971). More specifically, the amino group of the incoming aminoacyl-tRNA attacks the ester linkage between the carboxyl end of formyl-methionine and the 3'-hydroxyl group of the ribose moiety of the terminal adenosine of the tRNA (Ganoza et al., 1975). Peptide bond formation is thermodynamically feasible therefore not requiring the hydrolysis of GTP (Lucas-Lenard et al., 1971).

The elongation factor EF-G, stimulates the ejection of deacylated tRNA from the peptidyl site as well as causes the translocation of the peptidyl-tRNA•mRNA complex from the A site to the P site. This process requires GTP hydrolysis, and is cyclical in nature (Ganoza, 1977 and Glick et al., 1979).

Elongation factor T is actually a mixed dimer of Ts•Tu. Ts is stable and has a molecular weight of approximately 28,000 (Hachmann et al., 1971). Factor Tu (unstable) has a molecular weight of 42,000, and is present in amounts greatly exceeding those of Ts. Protein Tu•Ts interacts with GTP, resulting in the release of Ts and formation of a GTP•Tu complex, which then combines with aminoacyl-tRNA and the ribosome. These reactions occur at the peptidyl transferase centre of the 50S subunit, which also contains the ribosomal proteins L7 and L12 (Lucas-Lenard et al., 1971 and Metzler, 1977).

The peptide chain is then transferred to the amino group of the aminoacyl-tRNA occupying the A site. Although

this reaction does not require energy, the bound GTP is nevertheless hydrolyzed. This energy may be used for alignment of aminoacyl-tRNAs, ejection of elongation factors or release of the codon-anticodon interaction after peptide-bond formation (Ganoza, 1977). The Tu•GDP complex is released from ribosomes, and dissociates, allowing Tu to react with Ts, reforming the Tu•Ts dimer (Lucas-Lenard et al., 1971).

The aminoacyl-tRNA resides in the A site while these two steps occur. During the first stage it is combined with EF-Tu and GTP, and in the second, the aminoacyl-tRNA resides in the A site alone. During both steps, its anticodon is bound to an mRNA triplet thus if the wrong aminoacyl-tRNA is bound, there are two successive opportunities for the mistake to be corrected. Perhaps the role of EF-T is actually to provide a proof-reading role, allowing ribosomes a second opportunity to reject mispaired aminoacyl-tRNA molecules (Watson, 1976).

The movement of peptidyl-tRNA from the A site to the P site requires EF-G. First, an EF-G•GTP•ribosomal complex forms. Translocation, resulting in hydrolysis of GTP, occurs and EF-G is then released. The GTP hydrolysis is necessary for this movement, with one GTP molecule hydrolyzed per translocation step (Watson, 1976). Factor G also stimulates ejection of the deacylated tRNA from the ribosome (Glick et al., 1979).

There is substantial evidence that EF-G also binds near ribosomal proteins L7 and L12. Competition experiments reveal EF-G and EF-T bind to the same location on the ribosome (Metzler, 1977).

The mRNA template must be advanced three nucleotides during the translocation process. The simplest explanation states that the movement of a codon is a consequence of its binding to the anticodon of a tRNA. As the tRNA is translocated into the P site, it drags the mRNA along. There is evidence for this hypothesis from frameshift suppressor-tRNAs, which have a four-nucleotide anticodon and bind four nucleotides of mRNA. When such a tRNA is translocated, the mRNA template is also advanced by four bases, suggesting that mRNA movement is actually due to tRNA movement (Watson, 1976).

The entire process of elongation is cyclical in nature, and does not halt until the entire cistron has been translated. Thus the end product of chain extension is a completed protein bound to the 3′-hydroxyl of the ribose of the terminal adenosine of tRNA (Ganoza, 1977).

1.2.1 Other Proteins Implicated In Elongation.

Several antibiotics have been useful in defining the steps of protein synthesis. For example, puromycin, which

resembles the 3′-end of a charged tRNA, interrupts chain elongation by binding in the A site of the ribosome (see Figure 3). This process is very efficient and competitively inhibits the entry of normal aminoacyl-tRNAs. More importantly, peptidyl transferase substitutes puromycin into the nascent peptide. Since puromycin residues bind very weakly to the A site, these peptides fall off of the ribosomes, producing incomplete chains of varying lengths (Watson, 1976). Experiments using only the 50S subunit of ribosomes and phenylalanine imply that inhibition by puromycin does not involve EF-G, EF-Tu, nor hydrolysis of GTP (Maden et al., 1967). The antibiotic sparsomycin, is also believed to exert its inhibitory effect on elongation by its interaction with peptidyl transferase (Menninger, 1971).

Using puromycin, E. coli K12 70S ribosomes, $tRNA^{phe}$ and its derivatives namely, N-acetyl-phe-$tRNA^{phe}$ and phe-$tRNA^{phe}$, Rheinberger and collegues are believed to have found three sites on ribosomes, each of which can bind tRNA molecules. In addition to the established A and P sites, a third binding site may exist to codon-dependently bind deacylated-tRNA. This "third" tRNA binding site is called the entry (or E) site. The sequence for filling the sites is P, E and A (Rheinberger et al., 1981).

Although protein synthesis appears very well

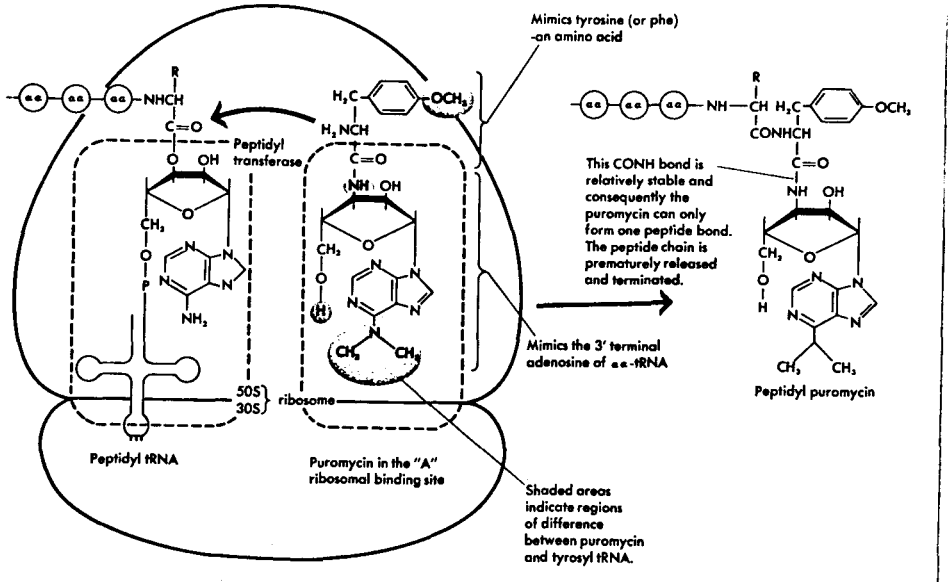


Figure 3. Structure of Puromycin (from Watson, 1976).

documented, efforts to "reconstruct" translation using pure proteins IF-1, IF-2, IF-3, EF-Tu, EF-Ts and EF-G with phage f2 RNA fails. These experiments indicate that the proteins which are necessary to catalyze the polymerization of polyphenylalanyl-$tRNA^{phe}$ require some additional factor(s) not yet discovered, in order to decode natural mRNA, as such synthesis could be restored with small amounts of crude soluble (S-100) extract obtained from E. coli cells. By using a natural message, another factor (W) was found to be required for translation which has a molecular weight of 50,000. Inclusion of this factor stimulates protein synthesis past the dipeptide stage, however further data is required before its exact site of action is determined (Ganoza, 1977 and Ganoza et al., unpublished).

Stereochemical studies of peptide-bond formation reveal that aromatic phenylalanine aids the formation of a peptide linkage by permitting the stacking of hydrophobic rings of the aromatic amino acid with the terminal adenine and adjacent cytosine of the 3′-end of tRNA. Puromycin also contains a modified adenosine moiety which, when altered, abolishes its ability to form a peptide-bond. This seems to indicate that different amino acids may not be stereochemically equivalent in peptide-bond synthesis (Ganoza, 1977). The protein, elongation factor-P, was discovered to markedly stimulate dipeptide sythesis with aminoacyl-tRNAs which are inefficient in the peptidyl

transferase reaction (Glick et al., 1979). Until this time, the formation of peptide bonds was believed to be spontaneous, not requiring other soluble factors nor energy sources (Glick et al., 1975).

Evidence suggests that EF-P is not a ribosomal protein. The molecular weight of EF-P is 21,000 and although all ribosomal proteins, with the exception of S1, have molecular weights of 28,000 or less, more than ninety percent of the EF-P activity is found in the S-100 fraction (Glick et al., 1975 and Glick et al., 1979).

Since purified EF-P and EF-G are unable to substitute for each other, and EF-G has a molecular weight of 83,000 (Lucas-Lenard et al., 1971), EF-P is believed to be different from EF-G. Also, EF-P does not catalyze the exchange of [^{3}H]GDP while EF-T does. Similarly, EF-T does not stimulate f-met-puromycin synthesis, thus EF-P is believed to be a new elongation factor (Glick et al., 1975).

Factor P does not affect the binding of f-met-$tRNA_f^{met}$ to ribosomes, therefore limiting the activity of EF-P to elongation (Glick et al., 1975). It has been estimated that EF-P is present in approximately 4000-6000 copies per cell. EF-P has a distinct elongated shape, and although the substrate catalyzed by EF-P resides on the 30S particle, while peptidyl transferase is part of the 50S subunit, EF-P appears long enough to span both subunits (Glick et al., 1979).

It has been proposed that the role of EF-P is to enhance chain growth by increasing the activity of the ribosomal peptidyl transferase for certain aminoacyl-tRNAs which are poor substrates in this reaction (Glick *et al.*, 1979).

Reconstruction studies reveal that both *E. coli* factors W and P are required along with purified *E. coli* initiation (IF-1, IF-2 and IF-3) and elongation factors (EF-T and EF-G), to translate phage f2 RNA. However, this translational activity is not one hundred percent of that obtained when S-100 is added (Ganoza, 1977). It is obvious that further studies are required before translation is completely understood.

1.3 Hydrolases And Their Roles In Translation.

Crude extracts of *E. coli* have been shown to hydrolyze synthesized oligolysyl-tRNAlys. The protein responsible for this activity was isolated and designated peptidyl-tRNA hydrolase (hydrolase I) with a molecular weight of 13,000-20,000. Further studies revealed that peptidyl-tRNA hydrolase does not attack N-acetyl-met-$tRNA_f^{met}$, but only uses peptidyl-tRNAs as substrates (Menninger *et al.*, 1970).

A mutant strain of *E. coli*, temperature-sensitive for growth, is deficient in this hydrolase, however its

translation processes were identical to the parental strain. Thus peptidyl-tRNA hydrolase does not appear to have a role associated per se with translation (Menninger et al., 1973). How does defective hydrolase cause protein synthesis to cease?

By switching the mutant to the non-translational-permissive temperature, it was proven that protein synthesis stops only after a lag of six to eight minutes. This fact implies that it is the accumulation of a poison, which when it reaches a critical level, halts protein synthesis. The substrate specificity of this hydrolase suggests that this poison is a peptidyl-tRNA. It is believed that this peptidyl-tRNA has been ejected from the ribosome during protein biosynthesis (Menninger et al., 1973).

Further studies using this mutant reveal that any tRNA can form a peptidyl-tRNA which dissociates from the ribosome and it seems clear that the metabolic role of hydrolase is to scavenge peptidyl-tRNAs which have been ejected from ribosomes (Menninger, 1976). Studies suggest that synthesis ceases in mutants of this hydrolase because a population of tRNAs, whose concentration is limiting, is occupied in the form of ribosome-ejected peptidyl-tRNA, and is therefore, not available for protein synthesis (Menninger, 1978).

It has been proposed that peptidyl-tRNA falls off of

ribosomes because the erroneous tRNA can not form a stable interaction with the triplet of mRNA. The ribosome could either edit this erroneous peptidyl-tRNA, resulting in its ejection into the cytoplasm, or propagate the error into a complete protein. Some evidence for this hypothesis exists as "rel" strains, which accumulate peptidyl-tRNA slowly, have substantially more errors in completed proteins (Menninger, 1976).

Ganoza's group discovered another hydrolase activity, with a totally different substrate specificity. This hydrolase uses only f-met-$tRNA_f^{met}$ or N-acetyl-met-$tRNA_f^{met}$ as its substrate. Purification reveals a high molecular weight complex (200,000 g/mol) with low hydrolytic activity, which, when either further purified or digested with ribonuclease, yields a high activity f-met-$tRNA_f^{met}$ hydrolase, with a molecular weight of 43,000 (hydrolase II). These data suggest that an RNA species blocks hydrolase II activity, however this RNA has not been studied further. The high molecular weight hydrolytic activity is ribosome-dependent, whereas the low molecular weight hydrolytic activity is ribosome-independent (Ganoza et al., 1976).

Purified hydrolase II is not inhibited by antibiotics which inhibit translation, suggesting that hydrolase II is not directly coupled to translation. Perhaps the role of

hydrolase II, in the 200,000 molecular weight complex, is to prevent translational mistakes during initiation of protein synthesis, by hydrolyzing f-met-$tRNA_f^{met}$ if a sterically inert complex forms prior to initiation. In agreement with this hypothesis is the observation, in reticulocytes, of an interaction of a hydrolase with initiation factors (Ganoza et al., 1976). Perhaps the RNA of the complex is required to decrease the activity of hydrolase II so that normal initiation complexes are not excessively hydrolyzed. It is also possible that this masked hydrolase only has activity towards such sterically inert initiation complexes.

1.4 Translational Termination.

The final product of translation is free polypeptide. Chain termination takes place when the completed peptide, esterified to the 3'-hydroxyl of the ribose moiety of the terminal adenosine of tRNA, is hydrolyzed in response to one of the nonsense codons UAA, UAG or UGA of mRNA (see Figure 4) (Ganoza, 1977). The nonsense codons may be identified genetically because, if by mutation, such a codon appears in phase within a cistron, premature chain termination occurs. This codon assignment was independently shown by cell-free studies with random U, A and U, A, G polymers which direct

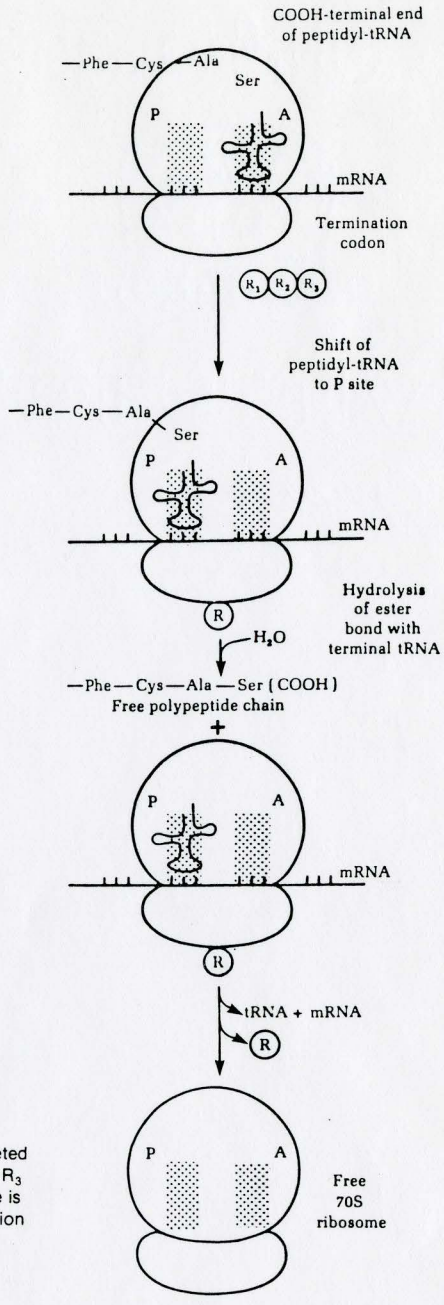


Figure 4
Steps in release of the completed polypeptide chain. R_1, R_2 and R_3 are release factors. Which one is used depends on the termination codon.

Figure 4. Prokaryotic Termination (from Lehninger, 1975).

protein synthesis as well as peptide release from tRNA (Capecchi, 1967, Capecchi et al., 1969 and Ganoza et al., 1966). A more direct confirmation of UAA as a nonsense codon was demonstrated with polyribonucleotide $AUGUUUUAA_n$ which directed the synthesis of the released dipeptide N-formyl-methionyl-phenylalanine (Ganoza et al., 1969).

Another assay system was developed which used mutant phage MS2 RNA, which has its seventh codon of the coat protein cistron mutated from CAG to UAG. In a cell-free system, the hexapeptide F-met-Ala-Ser-Asn-Phe-Thr is synthesized and then released (Capecchi, 1967). Using these assay systems, an E. coli supernatant was searched for the nature of the factors which cause the termination reaction.

Surprisingly, these studies revealed that a protein was responsible for termination rather than a tRNA with an anticodon capable of decoding nonsense triplets. This protein has been designated release factor (RF) (Capecchi, 1967, Ganoza et al., 1966 and Ganoza et al., 1969).

Caskey and associates devised a model assay system far more simplified than that of Capecchi, since this assay system uses only codons. In the first step, N-formyl-methionyl-$tRNA_f^{met}$ binds with AUG to E. coli ribosomes. In the second step of the assay, a termination codon and release factor are added, which cause the release of f-met (see Figure 4) (Caskey et al., 1968 and

Caskey et al., 1969).

This release reaction is believed to be analogous to the termination of nascent peptides during natural protein chain termination, because omission of ribosomes, Mg^{2+}, NH_4^+ or AUG prevents release suggesting that termination occurs from a f-met-$tRNA_f^{met}$ • AUG • ribosomal intermediate rather than from dissociated components. Also, other trinucleotides were tested, using this assay, for their ability to stimulate release of f-met, but only UAA, UAG and UGA are able to serve this function. These results indicate that the trinucleotide assay is indeed analogous to natural translational termination. The final product, f-met, is quantitated by first acidifying the reaction mixture (pH 1) then extracting f-met into ethyl acetate (Caskey et al., 1968).

Addition of aminoacyl-tRNAs, IFs or GTP (with a GTP-generating system) did not alter the rate nor extent of nonsense codon-dependent release of f-met. Therefore, these components were not implicated in translational termination (Caskey et al., 1968).

Release factor has been isolated and partially purified from E. coli cells, using both model assay systems, namely, that of Capecchi (hexapeptide) and that of Caskey (trinucleotide). Two proteins have been implicated in the termination assay, namely RF-1 and RF-2. RF-1 activity is not stimulated by RF-2 and vice versa. The small inhibition

observed when RF-1 and RF-2 are assayed together is believed to be due to competition between release factors for binding sites on ribosomes. The results suggest that termination is dependent upon either release factor, but not on both simultaneously. The release factors respond to a different set of termination codons. RF-1 responds to UAA and UAG whereas RF-2 responds to UAA and UGA only (Scolnick *et al.*, 1968).

There are two ways in which the mechanism of termination could be activated by a nonsense codon. In one mechanism release is triggered passively and occurs because these codons can not be translated. In the other mechanism of termination, release is due to an active recognition of these triplets. However, when the ribosome is confronted with oligonucleotide sequences with defined structure in certain mRNAs, which do not contain nonsense codons, synthesis occurs up to the end of the mRNA but the product remains attached to tRNA. Therefore, at least *in vitro*, reaching the end of a messenger does not result in termination, which implies that an active mechanism may be required to stimulate chain release. Also, using the trinucleotide assay of Caskey, f-met was not released to any significant degree unless a nonsense codon was included. On the basis of such data, RF-dependent termination appears to be a consequence of codon translation rather than merely the absence of translation (Scolnick *et al.*, 1968).

The pattern of codon degeneracy exhibited by RF-1 resembles that found with some species of aminoacyl-tRNA. For example, the molecule which interacts with an adenosine residue at the third base position of mRNA codons may also interact with guanosine (UAA, UAG). Alternate recognition of guanosine and adenosine in the first position is also found with initiator codons (AUG, GUG) translated by f-met-$tRNA_f^{met}$. However, the degeneracy pattern found with RF-2 differs from patterns found with aminoacyl-tRNAs, because an equivalence of adenosine and guanosine at the second base position of codons is observed (UAA, UGA) whereas only recognition of adenosine at the third position is tolerated. Such a pattern is not found with aminoacyl-tRNAs (Scolnick et al., 1968).

Inactivation studies reveal that release factors are acidic proteins with free sulfhydryl groups. Incubation with trypsin, T1 RNase and RNase A show that release factors are proteins not complexed with active RNA species (Scolnick et al., 1968). The more stringent criterion of phosphorous content was applied to purified RF-1, revealing less than one atom of phosphorus per molecule of protein, completely negating the possibility of a nucleic acid component (Capecchi et al., 1969).

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis revealed that the release factors each consist of only one species. The molecular weight of RF-1

was 44,000 whereas that of RF-2 was 47,000. The behaviour of the factors on calibrated sucrose gradients and on Sephadex G-100 columns is consistent with each factor being composed of one single peptide chain (Capecchi et al., 1969).

The purification schemes of RF-1 are lengthy and do not result in pure fractions (Capecchi et al., 1969, Caskey et al., 1969 and Ganoza et al., 1970). On the basis of these purification schemes, the cellular amounts of RF-1 and RF-2 have been estimated. Caskey's group estimate 100 molecules each of RF-1 and RF-2, whereas Capecchi and co-workers estimate 600 RF-1 molecules per cell. These results are in good agreement with the fact that EF-G (which participates in each amino acid addition) exists in an abundance far in excess of the number of ribosomes, whereas release factor occurs in amounts far less than the number of ribosomes, which is consistent with the idea that termination occurs only once during the synthesis of a polypeptide chain (Capecchi et al., 1969).

There are several possibilities for the role of release factors in termination. The factors may be involved in the recognition of the termination codons or hydrolysis of peptidyl-tRNAs or in the conversion of 70S ribosomes to 30S and 50S subunits. Since antibiotics, which inhibit the peptidyl transferase reaction, also inhibit the release reaction, peptidyl transferase is believed to hydrolyze the

peptidyl-tRNA linkage (Scolnick et al., 1968). Other studies, using the trinucleotide assay, indicate that the nonsense codons, in the presence of ten percent ethanol, actually cause the ribosomal binding of release factors. Thus the role of RF-1 and RF-2 appears to be recognition of the codons for peptide chain termination (Caskey et al., 1969). Efforts by Capecchi and co-workers to duplicate these results reveal that tetranucleotides must be used in the absence of ethanol, in order to detect such binding. Contrary to expectation, some irregular binding was seen with RF-1 and sequence ACAA or CUGA.

RNA of the form $(AUG)_n$ has three reading frames $(AUG)_n$, $(UGA)_n$ and $(GAU)_n$ which code for polymethionine, nonsense and polyaspartic acid respectively. Addition of RF-2 could only block polymethionine and polyaspartic acid synthesis extensively when allowed to first interact with the mRNA before addition of tRNAs, which implies that RF-2 can actually bind to UGA of mRNA (Ganoza and Ghosh, unpublished).

Another line of evidence exists which suggest that release factors actually recognize termination codons. When the stop codon UAG appears by mutation within a gene, premature chain termination ensues (Ganoza et al., 1970). Suppressing tRNAs (sup-tRNAs), which have a mutation at the anticodon (or somewhere else, as in sup-$tRNA^{trp}$ which has guanosine replaced by adenosine at position 24 (Watson,

1976)) are able to decode nonsense triplets thereby adding an amino acid to the peptide rather than causing termination. If release factor recognizes UAG, addition of sup-tRNAs should compete with termination. Using a cell-free hexapeptide assay (where the seventh triplet in the coat protein cistron of phage f2 is mutated from sense to a nonsense codon (UAG)), hexapeptide was released. Addition of sup-tRNA^{tyr} resulted in a substantial amount of insertion of tyrosine in response to UAG proving that termination and suppression are competing events. Therefore release factors compete with sup-tRNAs for nonsense codons (Ganoza et al., 1970).

A study with modified trinucleotides was undertaken by Smrt and co-workers in an effort to determine which portions of the nonsense codon are required for termination. The data suggest that the N-3 proton and the C-4 carbonyl groups of uridine are required for termination. The C-6 amino and N-1 moieties of adenosine are also necessary for termination (Smrt et al., 1970).

The specificity exhibited by the release factor termination codon interaction closely resembles that of Watson-Crick base pairing (U pairs with A and C pairs with G) and wobble pairing (G is equivalent to A); 1) RF-1, UAA or UAG (wobble pairing, A is equivalent to G and in positions 1 and 2, only uridine and adenosine are tolerated) and 2) RF-2, UAA or UGA (wobble pairing, A is equivalent to

G, and in positions 1 and 3, only uridine and adenosine are tolerated, as in normal Watson-Crick base pairing) (Smrt et al., 1970).

The idea that proteins interact with nucleic acids (as in release factor•nonsense triplet complexes) has been documented often with, for example, nucleases, aminoacyl-tRNA synthetases, repressors and polymerases. More specifically, RNase A recognizes uridine or cytidine residues and RNase T1 recognizes guanosine or inosine (Smrt et al., 1970).

If release factor participates in recognition of nonsense codons what component is responsible for hydrolysis of peptidyl-tRNA? Several lines of evidence suggest that the peptidyl transferase of the 50S particle participates in the cleavage; 1) antibiotics which inhibit peptidyl transferase also inhibit release activity (Capecchi et al., 1969) and 2) peptidyl transferase catalyzes the formation of ester bonds in the presence of a suitable acceptor (such as puromycin) and the carboxyl group of the nascent peptide (Watson, 1976). Consequently, peptidyl transferase may catalyze the reverse reaction, namely hydrolysis of peptidyl-tRNA.

Thus release factors appear to function in chain termination by recognizing nonsense codons and subsequently triggering peptidyl transferase, which catalyzes the transfer of the peptide from tRNA to water (Capecchi et al.,

1969).

The antibiotic fusidic acid, which inhibits EF-G (the translocation step) does not affect the trinucleotide termination assay. This result suggests that translocation of the peptide from the A site to the P site of ribosomes is not required for release of f-met (Scolnick et al., 1968).

Sequencing analysis reveals that quite frequently a nonsense codon is either immediately followed or separated by several codons from another nonsense triplet. It is suggested that the second termination codon is present to ensure that release of the peptide will occur even if the first nonsense codon is mistakenly translated as a sense triplet. However, at most, only thirteen percent of termination signals are tandem (Lu et al., 1971). Also, as the suppressor tyrosine-$tRNA^{tyr}$ is able to propagate across a repeated UAG termination sequence forming polytyrosine, it would not seem as if a double stop codon, or even a nonsense codon separated by several triplets from another nonsense codon, is of much help in ensuring termination (Ghosh et al., 1972).

1.4.1 Other Components May Function In Termination.

The 3′-terminus of 16S RNA of the 30S subunit of E. coli ribosomes is 5′-$ACCUCCUUA_{OH}$-3′. It has been

proposed that the UUA_{OH} portion can recognize the terminator codon UAA by normal Watson-Crick base-pairing, and the triplets UGA and UAG could also base-pair to this sequence of 16S RNA with wobble pairing in the second or third positions respectively (Shine et al., 1974).

This hypothesis receives some support from competition studies between sup-tRNAs and release factors. The likely stability of interactions of the three nonsense codons with UUA_{OH} should vary inversely with their levels of suppression. This relationship appears to be true, as UAA, which forms three A•U base pairs with UUA_{OH} can be suppressed only weakly (one to fifteen percent). Similarly, UAG forms Watson-Crick A•U pairs in the first and second positions, with wobble pairing in the third and amber suppressors have an efficiency of thirty to sixty percent. Triplet UGA can form Watson- Crick base pairs with UUA_{OH} in only the first and third positions. Wobble pairing in the second position would disrupt the helix somewhat more than if the wobble pairing occurs at the helix end. This decreases the stability of the UGA•UUA interaction (Romaniuk et al., 1979). This decreased stability is reflected in the observation that UGA is a "leaky" terminator since even normal (non-mutated) $tRNA^{trp}$ read UGA as tryptophan with low efficiency. These data substantiate the notion that the competition between suppression and termination is caused by sup-tRNA

and the 3′-end of 16S rRNA for the nonsense triplet. Thus, it is possible that the interaction of UUA_{OH} and the nonsense codon is actually a signal to bind or activate release factors (Shine et al., 1974).

This hypothesis, although plausible, can not explain why purified release factors can bind radioactive oligonucleotides during equilibrium dialysis studies, in which ribosomes are not included (Capecchi et al., 1969). Also, in studies with ribosomes and nonsense codons, addition of release factor enhances the binding of the termination triplet to ribosomes. Thus release factors have an active role in codon recognition (Goldstein et al., 1970a).

Sequence analysis of other 16S rRNAs reveal that the 3′-end is not a conserved region, consequently other species can not use the sequence $5'\text{-}UUA_{OH}\text{-}3'$ during termination. The codon recognition properties of RF-1 and RF-2 from E. coli and B. subtilis on the ribosomes of each species did not reveal qualitative differences in ribosome specificity. Therefore, it would seem likely that the sequence $5'\text{-}UUA_{OH}\text{-}3'$ of 16S rRNA does not play a vital role in codon recognition (Caskey et al., 1977).

Purification of the release factors uncovered another component (stimulatory factor S or RF-3) in an ammonium sulphate precipitation of E. coli S-100, which increases the rate of f-met release in the trinucleotide assay. S

stimulates both release factors but does not alter their codon specificity nor extent of f-met released. The amount of f-met-$tRNA_f^{met}$ bound to ribosomes is not affected by S. It would appear that S stimulates a rate-limiting step of *in vitro* termination. Since other studies suggest that the rate of release is limited by nonsense triplet concentration, S may stimulate termination codon recognition in a similar fashion as EF-T which stimulates the recognition of aminoacyl-tRNA for codons (Milman *et al.*, 1969).

Further studies reveal that addition of S to a mixture of ribosomes, release factor and termination codons, increases the binding of both nonsense triplet and release factor to ribosomes. This enhanced reaction retains its specificity since S stimulates the ribosomal binding of UAG with RF-1 but not with RF-2 (Goldstein *et al.*, 1970b).

The addition of GTP to the trinucleotide assay does not have an effect unless S is included. In the presence of S, addition of GTP inhibits ribosomal binding of nonsense codons and release factors, when the concentration of codon is low (1×10^{-6} M). However, in the presence of NH_4^+ (but not K^+) and a high level of nonsense triplet (1×10^{-4} M), S decreases the response of the trinucleotide release reaction. Subsequent addition of GTP actually enhances this release. This paradox has been explained by a model. Protein S is believed to confer

stability to the postulated release factor•termination codon•ribosome intermediate, whereas GTP causes its dissociation. Thus, in experimental mixtures with high trinucleotide concentration, S decreases termination by forcing release factor into the RF• stop codon•ribosome complex, but addition of GTP dissociates the intermediate, increasing release factor turnover and thus termination (Goldstein et al., 1970a).

Whether protein S is a new factor or just one which has already been discovered is disputed. According to Caskey's group, purification of S (or RF-3) results in its separation from IFs, EF-Ts, EF-G and the majority of EF-T. This group is able to purify EF-Tu and show that this fraction does not contain S activity. Also, two highly purified preparations of Tu have no effect on f-met release either in the presence or absence of S (Goldstein et al., 1970b).

Another group has encountered factor S while studying termination but contributes this activity to another protein. Capecchi and co-workers isolated factor alpha which was proven pure by SDS polyacrylamide gel electrophoresis. Addition of this protein fraction to the trinucleotide assay stimulated the rate of RF-1- and RF-2-mediated f-met release, and this activity was abolished by GTP. However, addition to a polyphenylalanine synthesizing system whose mRNA was poly U, revealed that Tu

activity could be replaced by alpha. In conclusion, since Capecchi's alpha factor shares the properties of Caskey's S factor as well as the activity of Tu, Capecchi believes that alpha, S and Tu are the same protein (Capecchi *et al.*, 1969).

The study of termination could be simplified by the use of mutants. With this idea in mind, a search was undertaken and *E. coli* mutant N4316 was discovered. Strain N4316 exhibits a specific pattern of suppression as it dies at 43°C (temperature-sensitive growth) and at 36°C suppresses the termination codons UGA and UAA but not UAG (temperature-sensitive suppression). This temperature sensitive protein synthesis occurs both *in vivo* and *in vitro* during translation of phage f2, QβI, MS2, R17 and T4 mRNAs (Ganoza, 1977).

Mapping studies reveal that the temperature-sensitivity and suppression properties are not caused by the same mutation however, suppression of both UAA and UGA is due to the same mutation.

The region of the chromosome coding for the temperature-sensitive growth phenotype, appears to code for a new protein since its purified preparation was free of known initiation, elongation and release factors. This new factor, the rescue protein, has a molecular weight of 100,000, and analysis with SDS-polyacrylamide gel electrophoresis reveals two proteins, with molecular weights

of 51,000 and 53,000 which may be subunits. Although reconstruction of full activity has not been obtained with the subunits, preliminary data suggest that rescue is a dimer of these subunits. Seventy percent of rescue occurs in the soluble cell fraction and the remaining portion is associated with 70S ribosomes. However, rescue is not found on dissociated ribosomal subunits (Ganoza, 1977).

At high temperatures, extracts of strain N4316 accumulate complete, or nearly complete, proteins attached to tRNAs. Isolation of these nascent peptides and subsequent exposure to rescue and release factors, allows a small stimulation of cleavage of nascent chains, which suggested that the lesion in N4316 affects termination (Ganoza, 1977). However, addition of rescue did not affect the trinucleotide assay. In a similar assay, using poly A_3U as message, rescue did not affect the release of polypeptides from ribosomal-bound peptidyl-tRNA (Ganoza *et al.*, 1973). Therefore, rescue was proposed to participate at the level of chain termination when a natural mRNA was synthesized.

In an experiment using a high-temperature extract of N4316, addition of rescue obtained from the wild-type parent of N4316, namely D10, immediately stimulated translation of phage f2 RNA. In fact, within thirty seconds to one minute, addition of wild-type rescue restored synthesis of coat protein even though the time required to complete the

synthesis of one coat protein molecule was four minutes (Ganoza, 1977).

Since the early effect of rescue did not appear to be related to protein chain termination, but rather initiation, the possibility that rescue is actually an initiation factor was examined. However, the AUG-directed binding of f-met-$tRNA_f^{met}$ to ribosomes was not stimulated by rescue, revealing that rescue did not have initiation factor activity. Also, with extracts of N4316 obtained at high temperatures, the interaction of f-met-$tRNA_f^{met}$ with f2 RNA was no different than at lower permissive temperatures, and subsequent addition of wild-type rescue did not affect the formation of the f-met-$tRNA_f^{met}$ • ribosome•f2 RNA complex. Thus, rescue did not appear to play a role in initiation of natural nor artificial mRNAs (Van der Meer et al., 1975). Model assays for propagation and ribosome recycling fail to respond to a highly purified preparation of rescue (Ganoza, 1977).

The specificity of rescue for natural mRNAs only and its inability to affect model assays for initiation, propagation, termination and ribosome recycling raises the question of the role of rescue in protein translation. Ganoza and co-workers suggest that rescue may be involved in recognition of punctuation signals found in extra-cistronic regions of natural mRNAs. Since nonsense codons often precede as well as follow cistrons, the postulated

specificity of rescue for these sequences would explain both its early effects on translation as well as the effect on termination (Atkins, 1979, Ganoza, 1977 and Knauber et al., manuscript in preparation).

1.4.2 Fate Of Ribosomes After Termination Of Protein Synthesis.

After peptidyl transferase catalyzes the transfer of the peptide from tRNA to water (in response to a nonsense codon) (Capecchi et al., 1969), the tRNA and mRNA are still attached to ribosomes. Also, before initiation may begin, the ribosomes must dissociate into 30S and 50S subunits. Two proteins, EF-G and ribosome-releasing factor (RR), are believed to be implicated in this process (Hirashima et al., 1972).

Factor RR has been purified and its molecular weight is 18,000. Approximately equimolar amounts of EF-G and RR participate in the release of ribosomes from mRNA and during this release, ribosome-bound tRNAs are also liberated. The size of mRNA does not change, indicating that EF-G and RR do not hydrolyze mRNA during this process (Hirashima et al., 1972).

The release of ribosomes from mRNA by EF-G and RR depends on puromycin, which indicates that ribosomes with

nascent peptidyl-tRNA are not released from mRNA. The data are consistent with the notion that ribosomes, released by factors EF-G and RR, are in an intact 70S form which would then be dissociated into their subunits by IF-3 (Hirashima et al., 1972).

The exact role of ribosome release factor has been questioned. It has been reported that release factors by themselves, are sufficient to cause breakdown of the ribosome•mRNA•tRNA complex during termination with coat protein of phage R17 (Kung et al., 1977).

1.4.3 The Termination Signal May Be Longer Than A Triplet.

Translation of mRNA is assumed to proceed independent of the sequence neighbouring each triplet. This is the assumption on which model assays were built. However, recent data suggest that the translation apparatus responds to a sequence larger than a triplet during initiation of protein synthesis. There is another situation in which the protein biosynthetic machinery is strongly influenced by context, namely suppression of nonsense codons. Since suppression and termination are competing events, if neighbouring sequences influence suppression, it therefore follows that context also affects termination (Salser et al., 1969).

It was discovered that different nonsense codons in

the same gene vary widely in their response to different suppressor-tRNAs. However, this observation was not believed to be significant, because the amino acid inserted at the nonsense triplet, by the suppressor-tRNA probably differs from the wild-type amino acid, which could result in altered biological activity. Since these first studies were concluded, data have been accumulated from many other experimental systems which imply that this interpretation may be incorrect because the efficiency of suppression largely depends on the nature of the neighbouring bases which surround a nonsense codon (Salser et al., 1969).

Three lines of genetic evidence support this conclusion. The first concerns the fact that UAA can be obtained via transition mutagenesis only from CAA (which codes for glutamine), UAG or UGA. Ochre mutants, coding for premature chain termination, which were obtained in a single step from wild-type, must therefore be derived from the glutamine codon, since the other two possibilities themselves would also have coded for premature release rather than wild-type. 5-Fluorouracil (5FU) may then be added and is incorporated into mRNA instead of uracil, however 5-fluorouracil is misread as cytosine, causing 5FUAA to be translated as CAA. Thus 5-fluorouracil suppression of transition ochre mutants gives rise to the wild-type gene product. Since the same amino acid was replaced, the objection that the variability of suppression arises from

insertion of a different amino acid into the protein was eliminated. Experiments of this sort reveal an enormous difference in the efficiency of suppression of different ochre mutants using the rIIB region of phage T4B (Salser et al., 1969).

The second experimental system, which leads to the conclusion that suppression efficiency depends on the reading frame, involves amber or ochre mutants in the B_1 region of the rIIB cistron. Since this whole region is non-essential, replacement of one amino acid by suppression is unlikely to affect the activity of the rIIB gene. The third line of evidence depends upon the fact that the essential region of the rIIB gene is connected to the rIIA cistron by the deletion r1589. The resultant protein has B activity but not A activity. Using deletion mutant r1589, amino acid substitutions in the A fragment are very unlikely to affect the function of B. In this case also, suppression of nonsense mutants in a region which is non-essential to the B-gene protein, affect B function in a manner which varies greatly. These results confirm the suggestion that the efficiency of suppression differs depending on the position of the nonsense mutation in the mRNA (Salser et al., 1969).

In these experiments, there was no simple correlation between the distance of the nonsense mutation from the end of the gene, and the efficiency of suppression. Obviously,

the nature of the signal specifying suppression is much more complicated than previously thought (Salser et al., 1969).

The termination signal must vary because efficiency of suppression varies. Since suppression and termination events compete, it seems reasonable to assume that whenever nonsense codons are used as natural chain terminators, they must be part of a termination signal which favors termination over suppression, to such an extent that even very strong suppressor-tRNAs do not have an effect (Salser et al., 1969).

Although context has been demonstrated many times to affect suppression, the molecular nature of this mechanism remains unknown. As suppressor-tRNAs compete with release factors during nonsense codon suppression , it follows that context affects either; 1) the reaction catalyzed by the release factors or 2) the function of the suppressor-tRNA. If context only affects the reaction catalyzed by the release factors, than different suppressor-tRNAs should behave the same with respect to context. In general, the results presented are consistent with this prediction (Bossi, 1983 and Salser et al., 1969).

The experimental data previously cited clearly indicate that during termination, the translational machinery responds to a nucleotide sequence longer than merely a nonsense codon. Since phase need not be maintained after translation of a cistron, there is no reason for the

assumption that the termination signal is as short as a triplet or even an integral multiple of a triplet (Capecchi et al., 1969). That release factors may respond to a longer sequence is not surprising. Since these factors are proteins, and not protein-nucleic acid complexes, release factor•mRNA recognition need not depend on the same triplet recognition exhibited during tRNA•mRNA interactions (Salser et al., 1969). In fact, equilibrium dialysis studies did not reveal any interaction between the release factors and oligoribonucleotides unless oligomers at least four nucleotides in length were used (Capecchi et al., 1969).

The idea that the prokaryotic termination signal is longer than three nucleotides seems plausible when compared to eukaryotic translation where, although translation also obeys the triplet coding theory (one codon, one amino acid), the termination signal is in fact a tetranucleotide instead of a trimer (Beaudet et al., 1971).

1.4.4 The Nature Of The Termination Signal.

If the termination signal is longer than a triplet, it is probable that there is a special termination site on the ribosome. When the mRNA termination sequence is in the termination site (T site) of the ribosome then two types of codons can be in the A site namely, a sense or nonsense codon. If the triplet in the A site was a sense codon, the

ribosome could either obey the termination signal, resulting in release of the peptide, or it could decode the sense codon in the A site, thereby adding another amino acid to the growing peptide chain. This latter possibility would result in translocation of mRNA, shifting the termination signal three nucleotides and this signal would not reside in the T site and termination is no longer possible (Salser et al., 1969).

However, if normal terminator signals were constructed such that a nonsense codon was in the A site when the termination signal was in the T site, only termination could occur. It has been postulated that this is the true role of nonsense codons (Salser et al., 1969).

The case of nonsense mutants is somewhat more complicated as each mutationally derived nonsense triplet will have a different context, and the chance that this context resembles the termination signal is quite small. In strains of bacteria, which do not contain suppressor-tRNAs, it is easy to imagine that the ribosome could take longer to terminate at a mutational nonsense codon which does not have a termination signal in its surrounding nucleotide sequence. There is experimental evidence that this is the case, as 5-fluorouracil is misread as cytosine more often at nonsense codons. This fact implies that ribosomes are delayed at a nonsense codon, allowing 5-fluorouracil more time in which to undergo a tautomeric shift to its cytosine-like structure

(which results in insertion of glutamine rather than termination) (Salser et al., 1969).

The most interesting aspect of this model is that it suggests that suppression is the result of a competition between weak reactions namely, termination, which occurs in the absence of a complete terminating sequence, or elongation. Studies indicate that termination and suppression actually do compete (Ganoza et al., 1970 and Ganoza, unpublished).

It is possible that reading context varies the efficiency of suppression in a manner which depends on how closely the nucleotide sequence, neighbouring the mutational nonsense codon, resembles the natural termination signal (Salser et al., 1969).

Such a model would predict that different suppressors should be affected similarly, that is, mutants strongly suppressed by one suppressor-tRNA should tend to be strongly suppressed by others. The data suggest that this is so, and it is believed that context affects the termination reaction (Bossi, 1983 and Salser et al., 1969).

The location of the termination signal of mRNA may be located on either side of, or even overlap, the nonsense codon. It seems unlikely however, that this signal is 5′ to the nonsense triplet because it would than be expected that the identity of one, or several of the 3′-terminal amino acids of peptides would have to be conserved in all

proteins. Such a preferrence has not yet been found. Thus, the termination signal of mRNA is believed to reside somewhere 3′ to the nonsense codon (Lu *et al.*, 1971 and Salser *et al.*, 1969).

Some evidence for this hypothesis exists: While studying readthrough of UGA codons by normal tryptophan-$tRNA^{trp}$ in an *E. coli* system, it was found that the presence of an adenosine residue 3′ to UGA, specified suppression of UGA by insertion of tryptophan (Engelberg-Kulka *et al.*, 1981).

In a collaborative study, using a lac I-Z fusion system in *E. coli*, the efficiency of suppression of nonsense codons was measured with different suppressor-tRNAs. Again, different suppressor-tRNAs behaved similarly with respect to context. The results reinforce the idea that the efficiency of suppression is determined at each site by the specific reading context of the nonsense codon. Only the triplet 3′ and 5′ to the nonsense codon was studied however, and context effects were shown to be due to the nucleotides 3′ to the nonsense codon. The base immediately adjacent to the nonsense codon appears to have the most important role. When this base is a purine (adenine or guanine), the nonsense codons are suppressed well however, those codons followed by uracil or cytosine residues are poorly suppressed. There are exceptions to this rule as nonsense codons, followed by the sequence CUG or CUC are well

suppressed (Bossi, 1983 and Miller et al., 1983).

Several theories have been devised to explain these results however, none fully explain these phenomena. The most plausible hypothesis invokes the fact that all known tRNAs have a uridine residue 5′ to the anticodon which makes it conceivable that this uridine residue may form a base-pair with the adenosine or guanosine residue 3′ to the codon of the mRNA, increasing the mRNA•tRNA interaction. This would result in an increase in suppression of nonsense codons. This theory cannot explain the results when the nonsense codons are followed by CUC or CUG because suppression is very efficient in these cases (Bossi, 1983 and Miller et al., 1983). However, most of the suppression data to date are consistent with the hypothesis that context affects the termination reaction, rather than the function of suppressor-tRNA (Bossi, 1983).

Another possibility is that secondary structure affects suppression efficiency. It is possible that certain structures inhibit the action of release factor by, for instance, including some portion of the nonsense codon in internal hydrogen-bonding. In such a case, the nonsense codon is not very accessible to participate in termination (Miller et al., 1983).

However, studies with phage MS2 RNA suggest that the termination codons of the A-protein, coat protein and replicase are located in the loop of a hairpin. Analysis of

the sequence 3′ to these nonsense codons reveals that regardless of whether the nonsense triplet is followed by a guanosine or uridine residue, the secondary structure does not appear to have an adverse effect (Iserentant et al., 1980). Thus, the difference in efficiency of suppression can not be explained by secondary structure.

Context appears to affect the termination reaction however, the molecular mechanism is still unknown. The areas requiring further investigation include; 1) confirming the existence of the termination signal hypothesized as being longer than a mere triplet, 2) whether it is located 5′ or 3′ to the nonsense codon and 3) its actual position. The simple trinucleotide termination assay of Caskey would appear to be the assay best qualified for this study, as other termination assays rely on the addition of initiation and elongation factors. The results thus obtained could be due to one of these other factors (or initiation or elongation reactions) rather than the release factors (or the termination reaction). Using this assay system, I have attempted to study these areas.

EXPERIMENTAL

2.1 MATERIALS

All oligoribonucleotides were synthesized and kindly donated by Dr. Thomas Neilson and co-workers of McMaster University, Hamilton, Ontario. Oligomers AUG and UAA were also obtained from Sigma Chemicals.

[^{35}S] Methionine (1000 Ci/mmol typically) and [^{3}H] methionine (typically 100 Ci/mmol) were purchased from the New England Nuclear Corporation. [^{35}S] Methionine (typically 1000 Ci/mmol) was also obtained from the Amersham Corporation. A purified mixture of tRNAs (containing all members), isolated from E. coli B cells, was purchased from Boehringer-Mannheim. Pure $tRNA_f^{met}$ was kindly donated by Dr. David Novelli, Oak Ridge Laboratories, Tennessee.

E. coli K12 and E. coli B cells were obtained from Miles Laboratories and E. coli Q13 or MRE-600 cells were grown to mid-log phase (Ganoza et al., 1976).

Column materials DEAE-cellulose and P-11 cellulose phosphate were purchased from Whatman Incorporated. DEAE-Sephadex A-50 came from Pharmacia.

The antibiotic sparsomycin was a generous gift of Dr.

Sidney Pestka of the La Roche Institute, Nutley, New Jersey, U.S.A.

2.2 METHODS

2.2.1 Isolation Of 70S Ribosomes.

Mid-log phase *E. coli* Q13 cells were broken by grinding with an equal weight of alumina. Deoxyribonuclease (1 mg/ml) was added to the paste (1 µl DNase/2 g cells). The paste was suspended in a pH 7.4 buffer containing 0.01 M Tris-HCl, 0.01 M $MgCl_2$, 0.03 M NH_4Cl and 0.001 M dithiothreitol (DTT). This slurry was centrifuged at 12,000 x g for 10 min, then at 15,000 x g for 10 min and, in both cases, the resultant pellet was discarded. The supernatant was centrifuged at 30,000 x g for 15 min, and subsequently, this supernatant was spun at 100,000 x g, to sediment 70S ribosomes. (The resultant S-100 was used as a source of initiation factors) (Ganoza *et al.*, 1975).

The ribosomes were washed twice by using the following procedure twice; ribosomes were suspended in a pH 7.4 buffer (high magnesium buffer) containing 0.01 M Tris-HCl, 0.01 M $MgCl_2$ and 0.50 M NH_4Cl, then

centrifuged at 30,000 x g for 10 min. This supernatant was centrifuged at 100,000 x g to pellet ribosomes (Ganoza et al.,1975).

Ribosomes were washed a third time, after suspension to a concentration of 10 mg/ml in a pH 7.4 buffer (low magnesium buffer) containing 0.01 M Tris-HCl, 0.001 M $MgCl_2$ and 0.50 M NH_4Cl (Ganoza et al., 1982). The low concentration of magnesium dissociates 70S ribosomes into 30S and 50S subunits (Spirin, 1974). The supernatant, containing the dissociated 70S fraction, was spun 10 min at 30,000 x g. The resultant supernatant was then spun at 100,000 x g to pellet the thrice-washed ribosomes (Ganoza et al., 1982).

The ribosomes were suspended to a concentration of 10 mg/ml in the high magnesium pH 7.4 buffer (which would have allowed the subunits to associate and reform 70S particles) and washed for the fourth time by centrifuging for 10 min at 30,000 x g. This supernatant was spun at 100,000 x g, and the resultant ribosomal pellet was suspended in the high magnesium pH 7.4 buffer at a concentration of 100-200 mg/ml and stored at $0^{o}C$. Ribosomes, stored in liquid nitrogen, were suspended in a high magnesium buffer, pH 7.4, which contained 0.01 M Tris-HCl, 0.01 M $MgCl_2$ and 0.05 M NH_4Cl, (instead of 0.50 M NH_4Cl), as ribosomes stored at $0^{o}C$ required a higher salt concentration to prevent bacterial contamination (Ganoza et al., 1982).

2.2.2 Preparation of N-acetyl-[^{3}H]met-tRNA$_f^{met}$ and f-[^{35}S]met-tRNA$_f^{met}$.

The source of activating enzymes, used to aminoacylate and formylate tRNA$_f^{met}$ (whose source is *E. coli* B), was an S-100 of *E. coli* MRE-600 cells. The tRNA was aminoacylated, (using a mixture containing nineteen unlabelled amino acids and [^{35}S] methionine) and subsequently [^{35}S]met-tRNA$_f^{met}$ was formylated. This reaction also contained 0.008 M Tris-HCl, pH 7.4, and 0.008 M $MgCl_2$, an ATP-generating system (which consisted of a phosphate donor (phosphoenolpyruvate), phosphokinase (which catalyzed phosphate transfer) and a phosphate acceptor (ATP-ADP)), and a source of formyl groups (calcium leucovorin) (Ganoza *et al.*, 1976).

The reaction mixture was incubated at 35°C for 15 min to ensure aminoacylation of tRNA and formylation of [^{35}S]met-tRNA$_f^{met}$. The charged tRNA was extracted twice into water-saturated phenol and six times into ether (to ensure elimination of all phenol). The tRNA was precipitated by absolute ethanol and suspended and dialyzed with a pH 7.4 buffer containing 0.002 M DTT and 0.0005 M EDTA (Ganoza *et al.*, 1976 and Ghosh *et al.*, 1972).

The specific activity of the preparations of

f-[^{35}S]met-tRNA$_f^{met}$ was determined by isotope dilution and varied with each preparation. For this reason, amounts of radiolabelled f-met-tRNA$_f^{met}$ were expressed in pmol (Ganoza et al., 1976).

The extent of formylation was analyzed on aliquots of f-[^{35}S]met-tRNA$_f^{met}$ after 10 min of hydrolysis in 0.33 N KOH at 37°C (which deacylated aminoacyl-tRNA). The reaction mixture was adjusted to pH 1 by addition of HCl. An organic layer (ethyl acetate) was added and the sample was vortexed 15 sec to ensure mixing. A brief centrifugation followed to separate completely the two phases from denatured protein (which is mainly sandwiched between the two phases). An aliquot of the organic layer was extracted and added to Bray's scintillation fluid. This assay was specific for formyl-methionine extraction only as neither [^{35}S]met-tRNA$_f^{met}$ nor [^{35}S]methionine nor f-[^{35}S]met-tRNA$_f^{met}$ were soluble in the organic layer (Caskey et al., 1968 and Ganoza et al., 1976). In general, 90-100 percent of the [^{35}S]met-tRNA$_f^{met}$ was formylated.

Pure tRNA$_f^{met}$ was aminoacylated (using the same reaction conditions as those above) with [^{3}H]methionine using activating enzymes from an S-100 of E. coli MRE-600 cells, which had been partially purified. Partial purification consisted of passage, of the S-100,

through a DEAE-cellulose-32 column (Ghosh et al., 1972).

The pure [^{3}H]met-tRNA$_f^{met}$ was purified from protein by addition of sodium dodecyl sulphate (SDS) and two extractions with water-saturated phenol. Six ether extractions were used to ensure that any traces of phenol were eliminated. The labelled met-tRNA$_f^{met}$ was precipitated by absolute ethanol, then the pellet was suspended in 0.002 M DTT (Ghosh et al., 1972).

Acetic anhydride was added, over a one hour period, to this mixture maintained at 0°C. The pure N-acetyl-[^{3}H]met-tRNA$_f^{met}$ was then precipitated by addition of absolute ethanol. This method has been reported to result in 100 percent acetylation of aminoacyl-tRNA (Haenni et al., 1966).

2.2.3 Ribosome·Oligoribonucleotide Complex Formation.

Unless otherwise stated, ribosome·oligoribonucleotide complexes were formed in an incubation volume of 0.045 ml containing 100 µg of four-times washed E. coli Q13 ribosomes, 300 pmol of oligoribonucleotide and approximately 2.5 pmol of labelled N-blocked- (either by acetylation or formylation) met-tRNA$_f^{met}$. These mixtures were buffered to pH 7.4 by 0.022 M Tris-HCl, 0.056 M NH_4Cl and were 0.011 M in $MgCl_2$. The reaction mixtures were

incubated at 24°C for 15 min and the reactions were halted by plunging tubes into ice baths. The following procedure was used to measure the binding of N-acetyl-[^{3}H]met-tRNA$_f^{met}$ and N-formyl-[^{35}S] met-tRNA$_f^{met}$; approximately 5 ml of a pH 7.4 high magnesium binding buffer, containing 0.010 M $MgCl_2$, 0.050 M Tris-HCl and 0.100 M NH_4Cl were added to each tube and reaction mixtures were subsequently filtered on Millipore filters (0.045 μ thick) and washed with more pH 7.4 high magnesium binding buffer (Ganoza et al., 1975 and Ganoza et al., 1982). The filters were then dried and counted in a scintillation fluid which contained toluene, PPO and POPOP (Nirenberg et al., 1964).

2.2.4 Isolation Of Factors For Peptide Synthesis And Termination Studies.

Initiation factors were isolated from E. coli K12 cells (Suttle et al., 1973) by Ganoza and co-workers. EF-T was prepared from E. coli K12 cells by the method of Wurmbach and Nierhaus (1979) or Arai and co-workers (1972), by Ganoza and colleagues. Rescue was made from E. coli Q13 cells and was donated by B. Murphy of Ganoza's laboratory. Rescue was made by the method of Van der Meer and co-workers (1975), with the following modification; ribosomes isolated

by centrifugation for 2.5 hours at 154,000 x g were suspended in the pH 7.4 buffer described however, it contained 0.001 M $MgCl_2$ rather than 0.010 M $MgCl_2$.

EF-G was prepared from E. coli K12 cells according to Ravel and co-workers (1971) only to the hydroxylapatite step, by Ganoza and co-workers. EF-P was also prepared by Ganoza and co-workers through Steps 1, 2 and 3 of the method of Glick and co-workers (1979). Factor W was a generous donation of Christine Cunningham of Ganoza's laboratory.

For dipeptide synthesis, reaction mixtures contained partially purified initiation factors and EF-T. These mixtures contained an approximate 70-fold excess of hexaribonucleotide (AUGUUA) relative to ribosomes. Oligomer AUGUUA was used in the dipeptide synthesis assay in the absence and presence of RF-1 in an effort to determine the effect of release factor on the extent of formation of the dipeptide, formyl-methionine-leucine (f-met-leu). Incubation mixtures (0.045 ml) contained 5 µg of AUGUUA, 2.6 µg IF-1, 2.5 µg IF-2, 1.2 µg EF-T, 0.010 M $MgCl_2$ and approximately 4 pmol each of f-[^{35}S]met-$tRNA_f^{met}$ and unlabelled leu-$tRNA^{leu}$ and 100 µg of 70 S E. coli Q13 ribosomes (Ganoza et al., 1982). When indicated, 14 µg of release factor (purified by DEAE-cellulose chromatography) were added (Ganoza et al., 1970). After 20 min incubation at 24°C, an aliquot (0.03 ml) was withdrawn and the ester linkage between the tRNA and the

dipeptide was hydrolyzed in 0.5 M NH_4OH for 30 min at 37°C. About 0.05 ml of the reaction mixture was spotted onto Whatman 3 mm filter paper and dried under nitrogen (Ganoza et al., 1982).

Electrophoresis was carried out at 125 mA for 60 min (200 V/cm) in pyridine:glacial acetic acid:water (1:10:189, v/v/v). Formylated dipeptide standards (including f-met-leu) were stained by spraying the paper with a solution of 0.1 N sodium dichromate in glacial acetic acid (1:1, v/v), then subsequently sprayed with 0.1 N silver nitrate. Each strip of paper (upon which a reaction mixture was spotted and then electrophoresed) was further subdivided into one cm strips which were counted using toluene, PPO and POPOP as scintillation fluid (Ganoza et al., 1982).

2.2.5 The Trinucleotide Assay For Termination.

After ribosome·f-[^{35}S]met-$tRNA_f^{met}$·oligoribonucleotide complex formation (as described in Section 2.2.3 of Methods) the reactions were halted by plunging tubes into an ice bath and 0.03 ml aliquots, which contained release factor, were added. Nonsense codon UAA was added (as indicated), only to AUG·ribosome·f-[^{35}S]met-$tRNA_f^{met}$ complexes. Unless otherwise specified, the termination reaction proceeded at 24°C

for thirty minutes and was halted by the addition of 0.25 ml of 0.1 M HCl and 1.6 ml of ethyl acetate. This mixture was vortexed 15 sec to ensure mixing, then centrifuged at low speed for 5 min to separate phases. The amount of f-[^{35}S]met or N-acetyl-[^{3}H]met released was analyzed after extraction of 1 ml aliquots from the ethyl acetate layer and counted (Caskey et al., 1968).

Both f-[^{35}S]met-tRNA$_f^{met}$ and N-acetyl-[^{3}H]met-tRNA$_f^{met}$ behaved identically in the termination reaction and were used interchangeably, depending on whether only pure blocked met-tRNA$_f^{met}$ was desired. N-acetyl-[^{3}H]met-tRNA$_f^{met}$ preparations contained only one aminoacyl-tRNA and were used in experiments where competition from other aminoacyl-tRNAs had to be avoided.

2.2.6 Purification And Characterization of Release Factor-1.

DEAE-cellulose purified release factor-1 was prepared from either E. coli K12 or E. coli B cells using steps 1, 2 and 4 of the purification scheme of Ganoza and co-workers (Ganoza et al., 1970). E. coli cells were broken, using a French Press, and then centrifuged at 8,000 x g for 10 min. The supernatant was then spun at 12,000 x g for 10 min, then

the soluble portion was extracted and centrifuged again at 12,000 x g for 10 min. This supernatant was spun at 30,000 x g for 15 min and the pellet discarded, then re-spun at 100,000 x g and the resultant pellet discarded. The protein of the supernatant S-100, was precipitated by the addition of ammonium sulphate (80 percent by weight) and centrifuged. This pellet was dissolved and dialyzed in a pH 7.4 buffer containing 0.010 M Tris-HCl and 0.002 M DTT, to eliminate the ammonium sulphate. This fraction is reported to contain all RF-1 activity (Caskey et al., 1969).

The protein fraction was then applied to DEAE-cellulose 32 or 23 columns pre-equilibrated with 0.100 M KCl (loaded according to 2 g protein/112 x 2.8 cm column) (Caskey et al., 1969 and Ganoza et al., 1970). The protein was eluted using a linear potassium chloride gradient (0.1 M - 0.4 M KCl), buffered to pH 7.4 with 0.010 M Tris-HCl and 0.002 M DTT, followed by a 0.4 M KCl wash buffered as above. The fractions were assayed using the trinucleotide assay (see Section 2.2.5 of Methods). Release factor (RF-1 and RF-2) activity was found in the 0.4 M KCl wash (Ganoza et al., 1970).

During subsequent preparations of release factor, the purification step using DEAE-cellulose 32 or 23 was replaced by DEAE-Sephadex A-50 columns for better separation of RF-1 and RF-2. The release factor obtained by DEAE-Sephadex chromatography, was first partially purified by ammonium

sulphate precipitation of the S-100 fraction (as discussed above) (Ganoza et al., 1970). This dialyzed extract was then loaded onto a DEAE-Sephadex A-50 column (loaded according to 2.2 g protein/112 x 2.8 cm column) which was pre-equilibrated with a pH 7.4 buffer containing 0.010 M Tris-HCl, 0.002 M DTT and 0.100 M KCl. The protein was eluted using a linear salt gradient (0.20 M - 0.60 M KCl), buffered to pH 7.4 with 0.010 M Tris-HCl and 0.002 M DTT, and the resulting fractions were assayed for release factor activity using the trinucleotide assay (Caskey et al., 1968). The release factor activity was found in two fractions; RF-1 activity in the 0.35 M KCl fraction and RF-2 in the 0.40 M KCl fraction (Caskey et al., 1969).

For further purification, the DEAE-cellulose fraction (or DEAE-Sephadex fraction) of release factor was then adsorbed on Alumina Cγ gel (5 mg of gel per mg of protein) (step 3 of Ganoza et al., 1970). The factor was extracted from the gel with a pH 7.4 buffer containing 0.025 M potassium phosphate, and 0.002 M DTT. Subsequently, the alumina Cδ step was replaced by a P-11 cellulose phosphate column, as Alumina Cδ became impossible to obtain (Alumina Cδ can be made but the amount of aging necessary for reproducability is somewhat questionable). The DEAE-cellulose (or DEAE-Sephadex) fraction, containing release factor, was loaded on a P-11 column (5 mg protein/ml of packed P-11 cellulose phosphate) pre-equilibrated with a

pH 6.0 buffer containing 0.010 M imidazole-HCl and 0.002 M DTT. The protein was eluted batch-wise first with 0.100 M KCl, followed by 0.200 M KCl, thirdly with 0.400 M KCl, fourthly with 0.600 M KCl and lastly with 0.800 M KCl solutions (each of these buffers was at pH 6.0 and contained 0.010 M imidazole-HCl and 0.002 M DTT). The bulk of the protein eluted with 0.1 M salt with release factor activity in the 0.1 M KCl and 0.2 M KCl fractions (as determined by the trinucleotide release assay).

The fraction of release factor obtained from the P-11 phosphocellulose column was mixed with bromophenol blue (as a marker) and sucrose (20 percent by weight), and loaded onto a discontinuous polyacrylamide gel (3 percent acrylamide in stacking gel and 7 percent in separating gel), and electrophoresed overnight at 0°C, and 5 mA (200 V) in a pH 7.4 buffer containing 0.010 M Tris-HCl, 2.88 percent glycine and 0.002 M DTT (Davis, 1964 and Ornstein, 1964).

The first time the release factor was purified using this electrophoretic procedure, the entire separating gel was horizontally subdivided into eight equal-sized pieces. Each piece was rolled into a ball and gently pushed into the bottom of a plastic ten ml pipette (previously prepared with a small ball of glass wool inside the pipette and a dialysis tube attached outside to the tip of the pipette). Glass wool was gently placed on top of the gel piece. During this procedure, the pipette and dialysis tube must remain

submerged under the buffer, and free of air bubbles.

The ten ml pipettes (each containing one strip of the gel and each with dialysis tubing knotted and affixed to the pipette tip) were placed inside a horizontal gel apparatus, and the samples were electro-eluted into the dialysis tubing at 0°C and 125 V overnight in a pH 7.4 buffer containing 0.010 M Tris-HCl and 0.002 M DTT (Cleveland et al., 1977).

The resulting samples were extracted from the dialysis tubing and assayed for activity using the trinucleotide assay (Caskey et al., 1968). The fraction containing release factor was stored in liquid nitrogen.

Subsequent purifications of release factor-1 by electrophoresis yielded samples which were purer than the sample obtained when the procedure was carried out the first time (when the whole separating gel was cut into eight pieces and electro-eluted). Using this procedure, it was determined, by Rf values, which gross portion of the gel contained RF-1 (Rf value=distance of gel portion containing release factor (from top of separating gel)/length of separating gel). In subsequent preparations, the gross region of the gel, known to contain RF-1, was subdivided horizontally into eight strips, electro-eluted and assayed (Caskey et al., 1968). Using this refined procedure, the RF-1 was determined to be in that region of the gel with Rf between 0.592 and 0.711 (see Figure 5).

The specific activity of release factor was

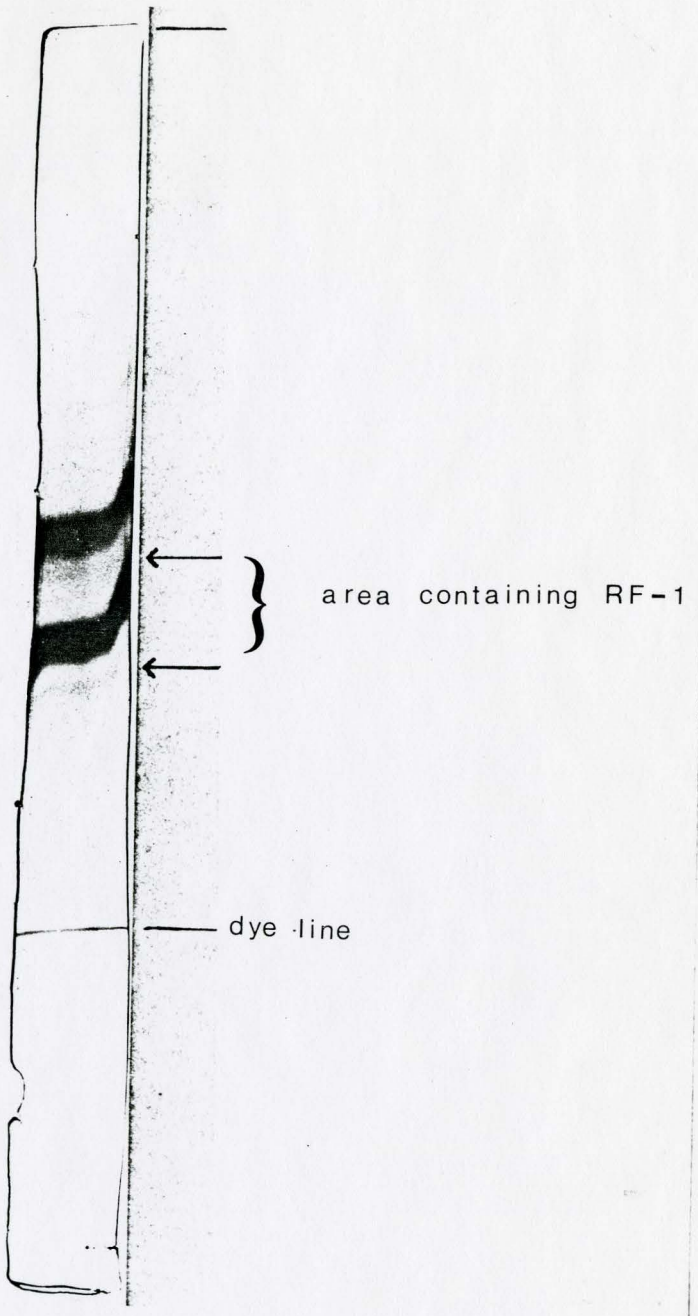


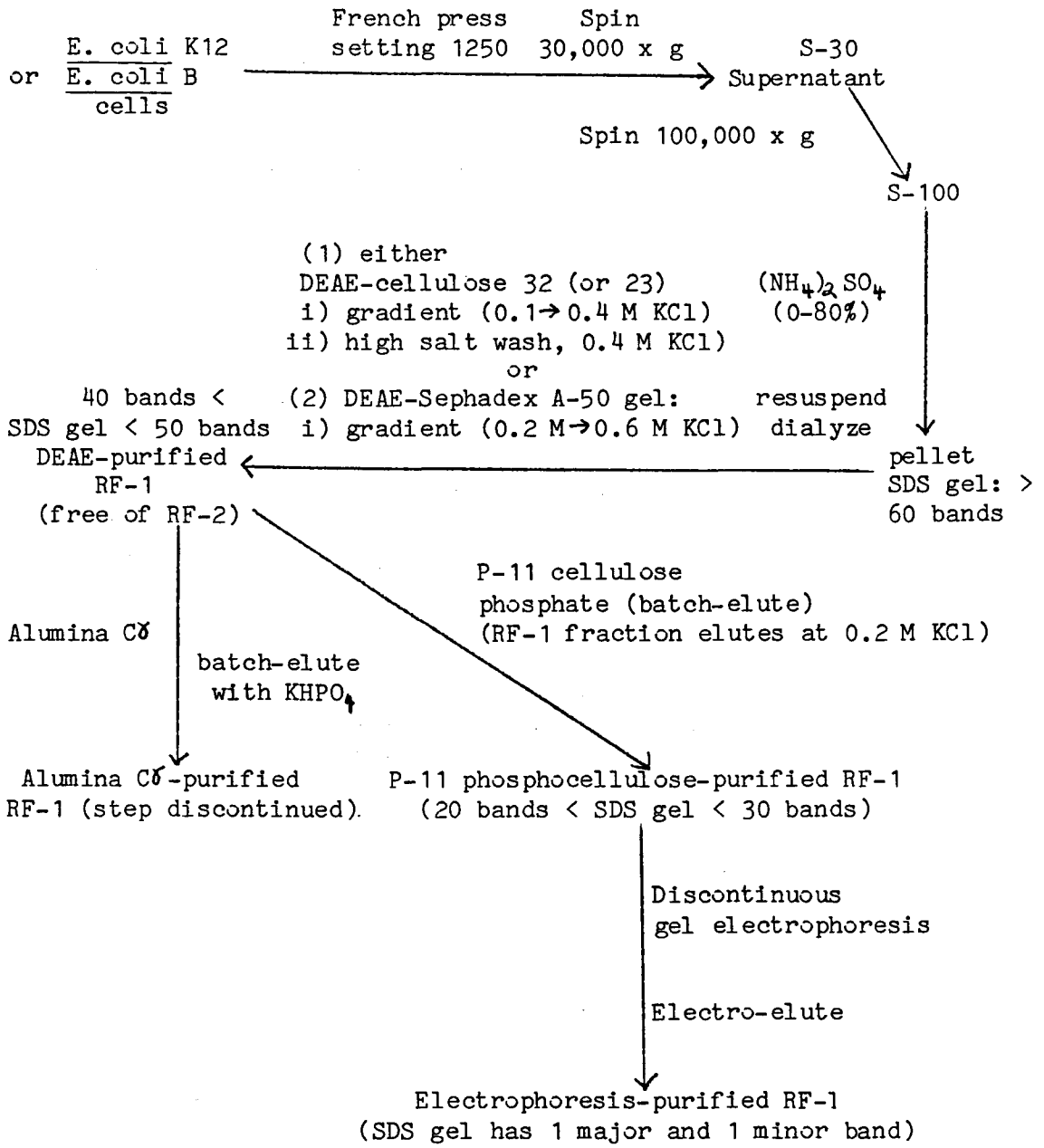
Figure 5. Typical Discontinuous Polyacrylamide Gel for Preparing RF-1 (See Experimental, Section 2.2.6).

determined on RF-1 fractions purified by; 1) ammonium sulphate precipitation, 2) ammonium sulphate precipitation followed by DEAE-cellulose (or DEAE-Sephadex A-50) chromatography, 3) same as in 2) with added step of P-11 phosphocellulose chromatography and 4) same as in 3) with added step of electrophoresis. The specific activity was assayed according to Milman and co-workers (1969).

This assay system was very similar to the trinucleotide assay (Caskey et al., 1968) and had the following modifications 1) 7.6 nmol of triplet UAA was only added to AUG•f-[^{35}S]met-tRNA$_f^{met}$•ribosome complexes, 2) the second 24°C incubation proceeded for fifteen minutes, 3) the amount of RF-1-containing aliquot added to f-[^{35}S]met-tRNA$_f^{met}$•ribosome• oligoribonucleotide complexes was approximately 10 µg and 4) specific activity was also calculated with oligomer AUGUUA (and in this case, triplet UAA was not added during the second incubation). Specific activity of RF-1 is expressed as pmol of formyl-methionine released/min of incubation/mg of RF-1-containing aliquot (Milman et al., 1969). The purification scheme used to purify release factor is shown in Figure 6.

The purified fractions of release factor were electrophoresed using the SDS gel system described by Laemmli (1970) (5 percent acrylamide in the stacking gel and 15 percent acrylamide in the separating gel). The samples

Figure 6: Purification Scheme for RF-1.



are mixed with SDS (which dissociates proteins into their individual polypeptide chains, and equalizes charge so that proteins are separated according to their molecular weights only), bromophenol blue (as tracking dye) and sucrose (20 percent by weight), and loaded onto the gel. The gel was run overnight at 0°C and 90 V in a buffer containing 3.02 percent Tris-HCl, 14.4 percent glycine and 0.1 percent SDS (by weight). Several proteins were also loaded onto the gel to serve as markers. These include bovine serum albumin (molecular weight of 68,000), ovalbumin (molecular weight of 45,000), aldolase (molecular weight of 39,000) and lysozyme (molecular weight of 14,000). The gel is shown in Figure 7.

The gel was stained with a solution containing 0.125 percent Comassie Blue stain (by weight), 50 percent methanol and 4.6 percent acetic acid (v/v) and destained with 5 percent methanol and 7.5 percent acetic acid (v/v).

Figure 7. SDS Gel of RF-1 Fractions. Tracks 1 and 2 contain BSA, 3 and 11 contain ovalbumin and aldolase and tracks 12 and 13 contain lysozyme as molecular weight markers. The ammonium sulphate fraction of RF-1 (10 µg) is shown in track 4. Track 5 contains 49 µg of RF-1 obtained by purification on a DEAE-cellulose 32 column. RF-1, further purified by P-11 phosphocellulose chromatography was loaded on tracks 6 and 7 in amounts of 17 and 8.5 µg respectively. Tracks 8, 9 and 10 contain 18, 72 and 36 µg of RF-1 subsequently purified by non-denaturing gel electrophoresis.

RESULTS AND DISCUSSION

3.1 Investigation Of The Sequence 3′ To UAA.

It has been suggested that the efficiency of termination *in vivo* may involve a signal longer than a nonsense triplet (Fluck *et al.*, 1977 and Salser *et al.*, 1969). Since all amino acids are found (without bias) at the carboxy-termini of *E. coli* proteins, if a longer termination signal exists, it is not expected to reside 5′ to the nonsense codon (Lu *et al.*, 1971). For this reason, the nucleotide sequence 3′ to the termination codon was examined using the tetraribonucleotides UAAA, UAAG, UAAC or UAAU in the trinucleotide assay (Caskey *et al.*, 1968). These oligomers were added to f-[^{35}S]met-tRNA$_f^{met}$•AUG•ribosome complexes, then incubated at 24°C for thirty minutes and the ability of these tetranucleotides to stimulate RF-1-mediated release of f-[^{35}S]met was tested (see Figure 8). The RF-1 used in this particular assay was purified by DEAE-cellulose chromatography (see Figures 6 and 7). Figure 8 shows that each tetranucleotide promotes termination to almost the same extent as the UAA reference codon.

However, the tetramers do not stimulate release as well as triplet UAA, and this effect is probably due to

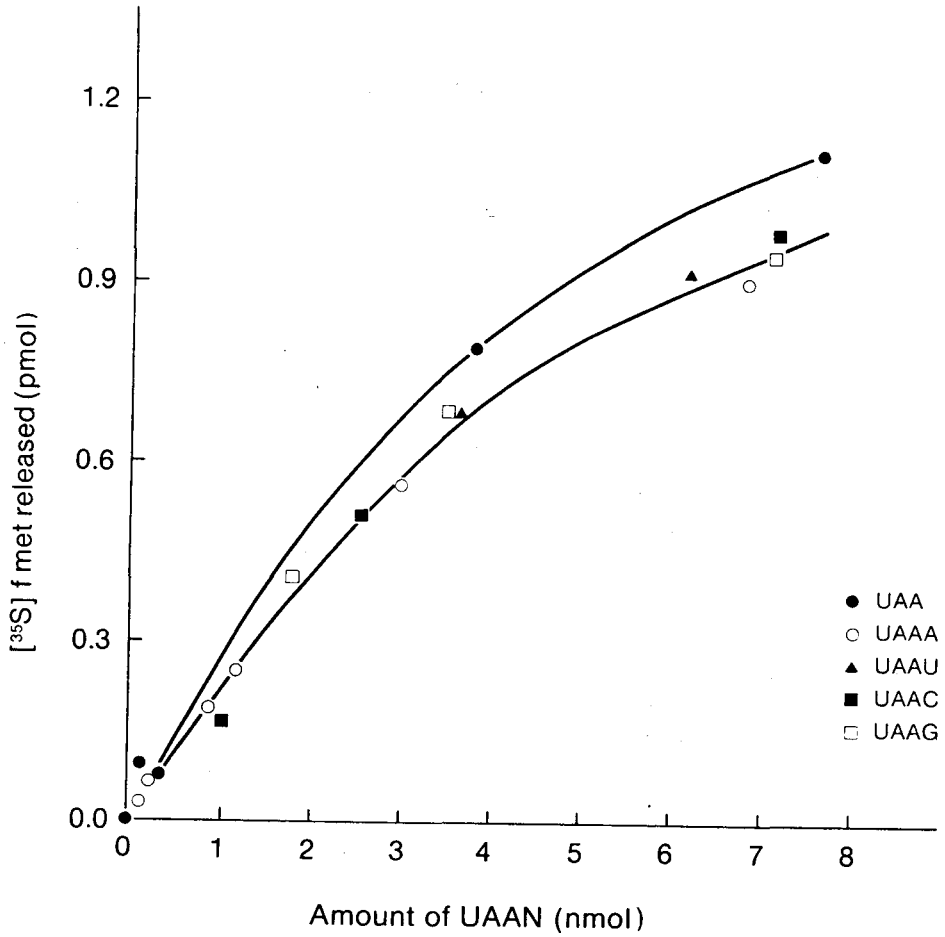


Figure 8. Stimulation of the Termination Reaction by Oligoribonucleotides of the Type UAAN.

Reactions were carried out as described in Experimental (Section 2.2.5). Incubations contained 300 pmol of AUG, 100 µg of four times washed E. coli Q13 ribosomes, and 2.0 pmol of formyl-[^{35}S]met-tRNA$_f^{met}$. Termination reaction mixtures (30 minutes at 24°C) contained 14 µg of DEAE RF-1 (purified through steps 1, 2 and 4, Ganoza and Tompkins, 1970), and varying levels of UAAN. Unspecific release observed with AUG in the presence of RF-1 and no UAAN was subtracted.

size, as tetramers are larger and should be sterically hindered somewhat more than a triplet. Therefore, at limiting oligoribonucleotide concentrations, UAA should stimulate the termination reaction better than tetramers, and this effect is shown in Figure 8. Hence, one nucleotide 3′ to UAA does not affect the *in vitro* release reaction. These results suggest that the *E. coli* recognition system differs from that of eukaryotes because the latter responds to tetranucleotides better than to triplets (Beaudet *et al.*, 1971).

Using the trinucleotide assay (Caskey *et al.*, 1968), it was found that in contrast to the tetranucleotide oligomers, the hexaribonucleotides UAAUAG, UAAUGA and UAAUAA stimulated RF-1-mediated release to markedly different extents as shown in Figure 9. The aliquot of RF-1 used in this assay (which did not contain RF-2), was purified by DEAE-cellulose chromatography (see Figures 6 and 7). As illustrated in Figure 9, hexamer UAAUAG stimulated the reaction slightly better than UAAUGA but surprisingly, UAAUAA was at least five-fold less effective than the two other hexanucleotides or triplet UAA. It is of interest that UAAUAA has not yet been observed in prokaryotic termination regions (Steege *et al.*, 1979).

At limiting concentrations of oligoribonucleotide, hexamer UAAUAG causes more RF-1-mediated release of N-acetyl-[^{3}H]methionine than does triplet UAA. Since

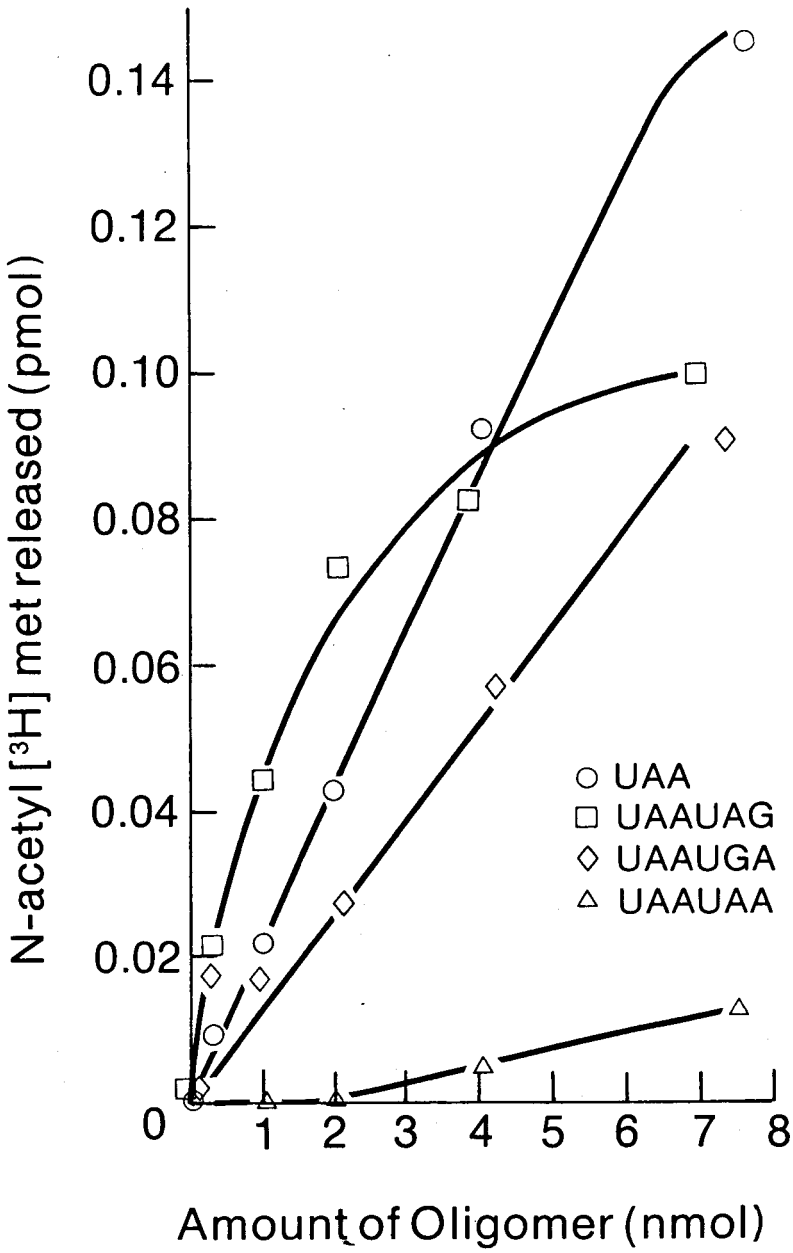


Figure 9. Stimulation of the Termination Reaction by Tandem Stop Polymers.

Conditions for initiation complex formation were as described in Experimental (Section 2.2.3) with 300 pmol of AUG and 2.5 pmol of N-acetyl-[^{3}H]met-tRNA$_f^{met}$. Termination reaction mixtures contained 14 µg of DEAE RF-1 (purified through steps 1, 2 and 4, Ganoza and Tompkins, 1970), and varying levels of UAA or of UAAUAA, UAAUAG, or UAAUGA. Incubation was for 5 minutes at 24°C. Unspecific release obtained with AUG in the presence of RF-1 and no UAA or UAA-containing polymer was subtracted.

UAAUAG contains two termination codons specific for RF-1 whereas triplet UAA only contains one, it seems plausible that at limiting oligomer concentrations UAAUAG would stimulate the termination reaction better than UAA. However, even at limiting concentrations of oligomer, UAAUGA has a decreased stimulatory capacity when compared to UAA. In this case, as the preparation of release factor did not contain RF-2 activity, UAAUGA only operates as a nonsense triplet with a 3′ nucleotide sequence composed of three base residues. On the basis of size, a hexamer would be more sterically hindered than a termination triplet and therefore, at limiting oligoribonucleotide concentrations, exhibit a decreased ability to stimulate the termination reaction. This effect is illustrated in Figure 9 with hexamer UAAUGA and UAA. This same size effect is found with UAAUAG and UAA, because although UAAUAG contains two nonsense triplets, at limiting oligomer concentration, this hexamer does not stimulate the termination reaction twice as well as UAA.

3.2 Investigation Of The Sequence 5′ To UAA.

The effect of sequences 5′ to UAA, on the termination reaction, could not be examined with the trinucleotide assay (Caskey *et al.*, 1968), as the tetraribonucleotide set, NUAA (where N is an adenosine, guanosine, cytidine or uridine

residue),was unreactive (Ganoza et al., unpublished). Hence, a set of longer oligomers was assembled (by Neilson and co-workers), each starting with AUG; AUGUAA, AUGUA, AUGUUA, AUGCUA, AUGAUG, AUGUUU, AUGAGC, AUGGCU, AUGUCU, AUGCUAA, AUGCUUA, AUGUUAA and AUGUUAU. Table 1 illustrates that each of these oligomers bound f-[^{35}S]met-tRNA$_f^{met}$ to approximately the same extent to ribosomes, supporting the previous conclusion that the nucleotide immediately following AUG does not affect ribosome•oligonucleotide interactions as much as the nucleotide 5′ to the initiator codon (Ganoza et al., 1982).

The ability of these oligomers to stimulate termination was tested: The oligomer was first bound, by its AUG sequence, to ribosomes with f-[^{35}S]met-tRNA$_f^{met}$, and subsequently incubated with RF-1 (purified by DEAE-cellulose, as illustrated in Figures 6 and 7). Since triplet UAA was not added, any release of formyl-methionine stimulated by the oligoribonucleotide must be caused by the portion of the oligomer which is 3′ to the AUG codon.

As shown in Table 1, hexamers AUGAUG, AUGUUU, AUGGUC, AUGUCU and AUGAGC were inactive in the termination assay, indicating that this assay system is free of unspecific hydrolases (Ganoza et al., 1976). In contrast AUGUUA, AUGUUAU, and AUGUUAA stimulated the release reaction to approximately the same extent, which suggests that the in

Table 1. Binding and Release with Various AUG-Containing Oligomers.

Oligomer bound	N-formyl-[^{35}S] met-tRNA$_f^{met}$ bound x 10^2 (pmol)		N-formyl-[^{35}S] met released x 10^2 (pmol)		%Release $\frac{\text{Amount of N-formyl-[}^{35}\text{S] met released}}{\text{Amount of N-formyl-[}^{35}\text{S] met-tRNA}_f^{met}\text{ bound}}$	
	Experiment Number					
	1	2	1	2	1	2
AUG	111.1	86.1	0.0	0.0	0.0	0.0
+UAA	111.1	86.1	1.2	0.0	1.1	-
AUGUAA	76.2	59.0	3.8	2.0	5.0	3.4
AUGUA	86.6	66.8	1.3	0.0	1.5	0.0
AUGUUA	61.2	47.7	4.3	5.2	7.1	10.9
AUGUUAA	58.5	46.6	5.5	4.2	9.5	9.0
AUGUUAU	60.9	48.2	5.1	5.3	8.4	11.0
AUGCUA	58.1	45.8	0.1	0.0	0.2	0.0
AUGCUUA	54.1	42.8	0.7	0.0	1.2	0.0
AUGCUAA	50.8	40.3	0.0	0.0	0.0	0.0
AUGAUG	86.8	68.8	0.7	0.0	0.8	0.0
AUGUUU	63.8	49.5	0.8	0.0	1.3	0.0
AUGGCU	45.0	34.8	0.5	0.0	1.1	0.0
AUGUCU	68.5	54.0	0.0	0.0	0.0	0.0
AUGAGC	55.3	44.1	0.0	0.0	0.0	0.0

300 pmol of oligoribonucleotide were bound to E. coli Q13 ribosomes (as described in Section 2.2.3 of Methods). After incubation for 15 min at 24°C, release factor and 300 pmol UAA were added (where indicated). Reaction proceeded 30 min at 24°C and was analyzed as described in Section 2.2.5 of Methods. In Experiment 1, reactions contained 5.2 µg of E. coli K12 RF-1 purified by DEAE-cellulose chromatography (see Figure 6). In Experiment 2, reactions contained 4.3 µg of E. coli B RF-1 purified by DEAE-cellulose chromatography followed by alumina Cγ chromatography (see Figure 6). Each experiment was done in duplicate and the results shown here are the average of these duplicates.

vitro assay for termination responds to oligomers which contain triplet UUA or UAA (Table 1). No release of formyl-methionine was observed with AUGUA, suggesting that either dinucleotide UA is not sufficient to stimulate termination or that UA may only enhance termination when positioned one nucleotide residue away from AUG.

Thus, it would appear that triplet UUA is able to increase the amount of termination observed. However, as shown in Figure 10 and as previously observed, neither dinucleotide UA nor trinucleotide UUA stimulates release of N-acetyl-methionine when added with RF-1 (purified by DEAE-cellulose chromatography) to AUG • N-acetyl-[^{3}H]met-tRNA$_{f}^{met}$ • ribosome complexes (Figure 10 and Caskey et al., 1968). Thus sequence UUA only causes release of N-acetyl-methionine when covalently linked to the initiation codon. Sequences UA and UAA appear to be more effective, at stimulating release of formyl-methionine, when either is positioned one nucleotide residue away from AUG (Table 1, oligomers AUGUA and AUGUUA; AUGUAA and AUGUUAA). If sequence UA is sufficient to stimulate termination (only when positioned one nucleotide residue away from AUG) then the "Termination site" is not the A site but rather, the T site overlaps the A site. However, if sequence UUA is required to enhance termination, then the T site is coincident with the A site. This UA-mediated hydrolysis is significantly depressed if a

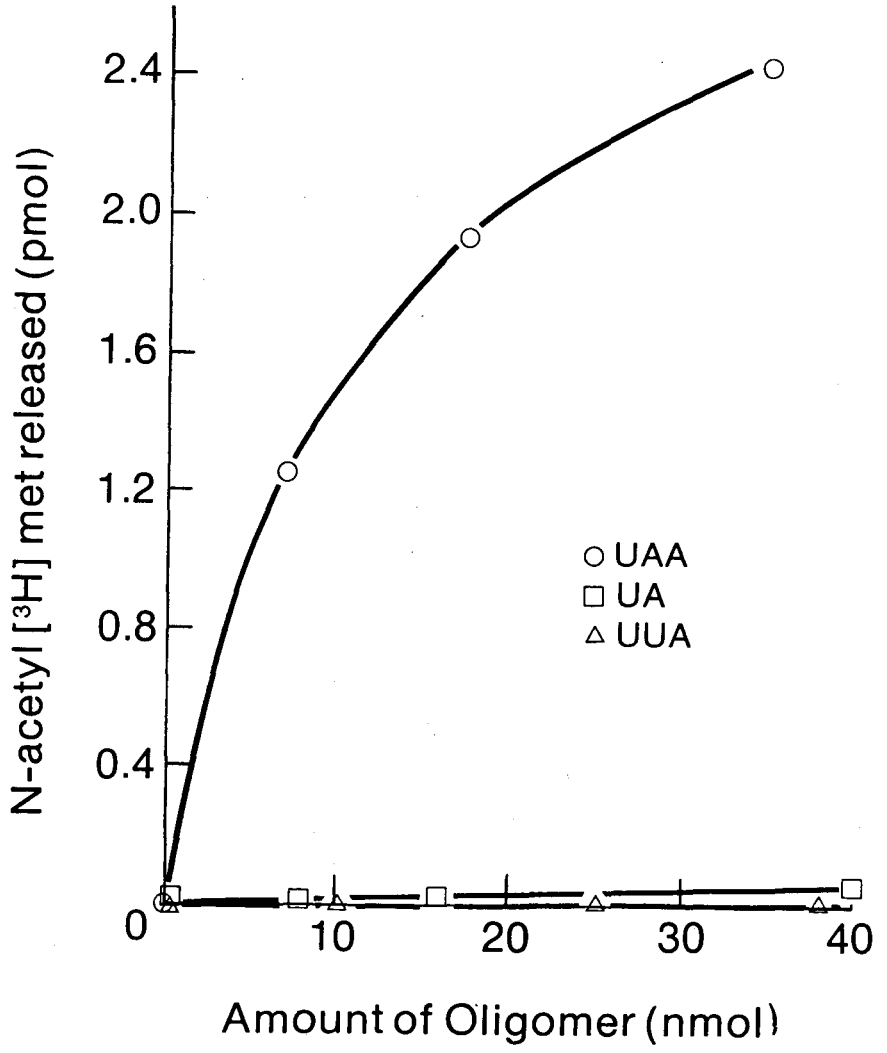


Figure 10. Lack of Stimulation of Release by UA and UUA.

Conditions for initiation complex formation were as described in Experimental (Section 2.2.3) with 300 pmol of AUG and 2.5 pmol of N-acetyl-[^{3}H]met-tRNA$_f^{met}$. Termination reaction mixtures contained 14 µg of DEAE RF-1 (purified through steps 1, 2 and 4, Ganoza and Tompkins, 1970), and varying levels of UAA, UA or UUA, and were incubated for 30 minutes at 24°C. Unspecific release obtained with AUG in the presence of RF-1 was subtracted.

cytidine residue preceeded it (see Table 1, oligomer AUGCUUA).

The amount and extent of termination stimulated by each of the oligoribonucleotides does not change when the strain of E. coli cells, used to prepare release factor, is switched. As illustrated in Table 1, in Experiment 1 the source of RF-1 was E. coli K12 cells, whereas in Experiment 2, RF-1 was prepared from E. coli B cells. Therefore, the behaviour exhibited by these RF-1 preparations did not depend on the strain, since both cause release of formyl-methionine with oligoribonucleotides which contain sequence UUA.

The time course of the termination reaction programmed by AUGUAA, AUGUUA and a mixture of free triplets AUG and UAA in the presence of RF-1 (purified by DEAE-cellulose chromatography) is shown in Figure 11. In each case 300 pmol of oligomer was bound and for AUG-bound complexes, 300 pmol of free UAA were added. The assay using the mixture of free triplets AUG and UAA was included as a control (Caskey and co-workers had already reported the results from the experiment using AUG and free UAA. They believe this reaction is due to release factor and it was therefore included in this experiment as a control). If the free triplet UAA (when added to AUG• N-acetyl-[^{3}H]met-tRNA$_{f}^{met}$• ribosomal complexes) exhibits the same behaviour previously reported when added

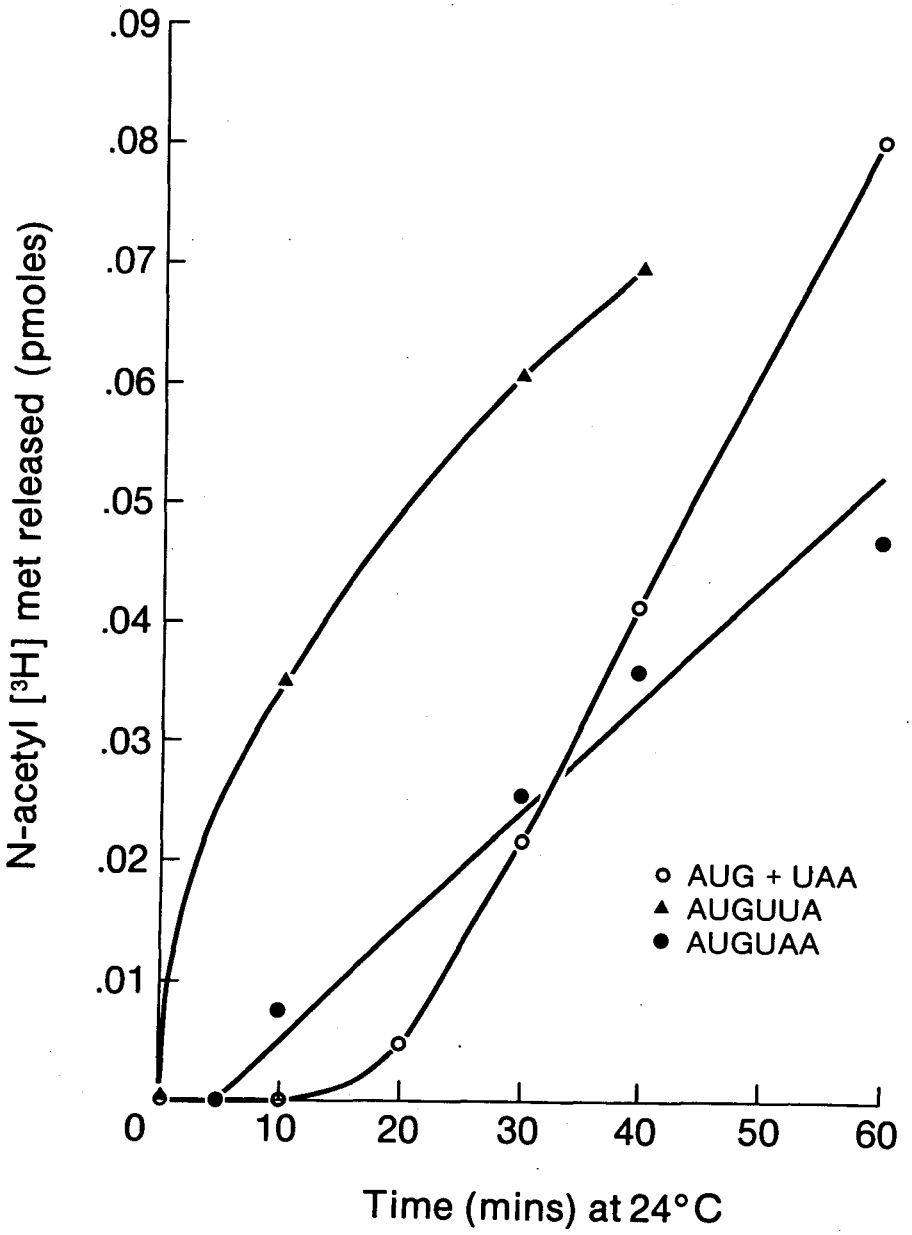


Figure 11. Stimulation of the Release Reaction with AUGUAA, AUGUUA or with Free AUG and UAA as a Function of Time.

Initiation complex formation was carried out as described in Experimental (Section 2.2.3) with 300 pmol of oligomer and 2.5 pmol of N-acetyl-[^{3}H]met-tRNA$_f^{met}$. Release was initiated by adding 14 µg DEAE RF-1 (purified through steps 1, 2 and 4, Ganoza and Tompkins, 1970) and 300 pmol UAA where indicated. Unspecific release observed in the absence of RF-1 was subtracted.

with RF-1, it would be plausible that the termination activities observed with AUGUUA and AUGUAA are also caused by RF-1 (Caskey et al., 1969). After addition of triplet UAA, the expected lag of approximately twenty minutes, before N-acetyl-methionine is released from AUG• N-acetyl-[^{3}H]met-tRNA$_f^{met}$• ribosome complexes, was observed, as previously reported. Therefore, the factor responsible for these activities appears to be the protein known as release factor which was isolated and characterized by Caskey and co-workers (1969).

When UAA is added in the trinucleotide assay, the lag (twenty minutes) which is observed before release of N-acetyl-methionine is not seen when the free triplets are covalently linked (as in oligomer AUGUAA). This result suggests that the release factor•ribosome•nonsense signal complex may react more efficiently when the termination codon is covalently linked to the mRNA. Addition of RF-1 (purified by DEAE-cellulose chromatography), to N-acetyl-[^{3}H]met-tRNA$_f^{met}$• AUGUUA•ribosome complexes, stimulates release of N-acetyl-methionine at a faster rate (see Figure 11). This result suggests a lack of specificity by this RF-1 preparation as triplet UUA (in oligomer AUGUUA) triggers the termination reaction far better than UAA (regardless of whether UAA is added as free triplet or covalently attached to AUG, as in AUGUAA).

3.3 Purification Of RF-1.

The possibility that a protein contaminant of RF-1 was responsible for the unspecific hydrolysis of formyl-methionine, in response to sequence UA, was examined with negative results. The release factors have never been purified to homogeneity however, these hydrolytic activities (both AUG + UAA- and AUGUUA-mediated release activities) co-purified through four purification steps (see Figure 6 and Table 2). Both activities were similarly inhibited by the antibiotic sparsomycin (Table 3), which interferes with peptidyl transferase activity. This antibiotic is reported to inhibit the termination reaction (Caskey et al., 1969 and Scolnick et al., 1968). These results suggest that the same protein is responsible for both the AUG + UAA- and the AUGUUA-mediated termination activities and that the factor, responsible for stimulating these reactions, is release factor.

Several other pieces of information also support the conclusion that the protein responsible is RF-1. The final product of the purification scheme (shown in Figure 6), when electrophoresed with denaturing conditions, revealed two bands (see Figure 7). The major band had a molecular weight of 45,500 whereas that of the minor band was 39,500 (see

Table 2. Effect of Purification of RF-1 on Release of N-Formyl-[^{35}S]Methionine.

Oligomer Bound	% Release $\frac{\text{Amount of f-[}^{35}\text{S]met released}}{\text{Amount of f-[}^{35}\text{S]met-tRNA}_f^{met}\text{ bound}} \times 100\%$		
	Experiment Number		
	1	2	3
AUG	0.0	0.0	0.0
AUGUUA	7.1	38.5	21.9
AUGUUAU	8.4	31.1	28.5
AUGCUA	0.2	1.7	3.0
AUGUCU	0.0	1.0	2.2

In experiments 1, 2 and 3, 300 pmol of oligoribonucleotide was incorporated into f-met-tRNA$_f^{met}$·oligomer·*E. coli* Q13 ribosome complexes during a 15 min incubation at 24°C (see Section 2.2.3 of Methods). Subsequently, approximately 5 µg of *E. coli* K12 release factor was added and reaction mixtures were incubated 30 min at 24°C. The extent of release of formyl-methionine was measured as described in Section 2.2.5 of Methods. Each experiment was completed at least twice (Experiment 1 was executed three times) and the results are those typically found during one experiment and are the average of duplicate reaction mixtures.

In experiment 1, the fraction of RF-1 used was purified by DEAE-cellulose chromatography (Figure 6) and 5.2 µg of this RF-1 preparation were added.

In experiment 2, the aliquot of RF-1 added (5.6 µg) to f-met-tRNA$_f^{met}$·oligomer·ribosome complexes was first purified by DEAE-Sephadex chromatography followed by purification using P-11 phospho-cellulose chromatography, (see Figure 6).

In experiment 3, 6.0 µg (purified by both DEAE-Sephadex and P-11 phosphocellulose chromatography, followed by purification of RF-1 by discontinuous gel electrophoresis, (see Figure 6), were added to oligomer·f-met-tRNA$_f^{met}$·ribosome complexes.

Table 3. Effect of Purification of RF-1 on Specific Activity Mediated by Free Triplets (AUG + UAA) and Oligomer AUGUUA.

Purified Fraction of RF-1 added (approx. 10 µg)	Total Units* of RF-1 activity obtained by the purification procedure	Specific Activity pmol of f[^{35}S]met released / min mg (of RF-1 fraction)			
		AUG + UAA Experiment 1		AUGUUA Experiment 2	
		Sparsomycin (-)	Sparsomycin (+)	Sparsomycin (-)	Sparsomycin (+)
Ammonium Sulphate Precipitation	1160 (only loaded 804 units onto DEAE-Sephadex A-50 column)	0.10	0.00	0.85	0.12
DEAE-Sephadex A-50	639 (loaded 568 units onto P-11 phosphocellulose)	0.51	0.00	1.46	0.18
P-11 cellulose phosphate chromatography	392 (in .1 M KCl fraction; 146 units obtained in .2 M KCl fraction, 112 units loaded onto discontinuous electrophoresis gel)	0.62	0.00	1.12	0.00
Discontinuous gel electrophoresis (followed by electro-elution)	112+	0.25	0.03	1.65	0.00

Termination assay and conditions were as described in Section 2.2.5 of Methods. Specific activities were performed in two different ways; the specific activity reported in columns 3 and 4 was obtained by adding the aliquot of E. coli K12 RF-1 indicated, and 7.6 nmol of triplet UAA to AUG·f-met-tRNA$_f^{met}$·ribosome complexes whereas the specific activity reported in columns 5 and 6 was obtained by adding only the fraction of RF-1 specified to AUGUUA·f-met-tRNA$_f^{met}$·ribosome complexes. Sparsomycin (5×10^{-4} M) was added where indicated. The termination portion of the assay proceeded for 15 min at 24°C (see Section 2.2.5 of Methods).

*Total units of RF-1 activity obtained by a purification procedure was calculated, using the trinucleotide assay with free triplets (AUG and UAA), as described in Section 2.2.5 of Methods. First, the amount of f-[^{35}S]met released by an aliquot of RF-1 was determined, under conditions where the only limiting factor was the amount of RF-1, and secondly, multiplying this amount of f-[^{35}S]met released by the total amount of RF-1-containing fraction.

+Note that total units of RF-1 activity obtained by the electrophoresis procedure could not be calculated by using the trinucleotide assay (with free triplets AUG and UAA) as this RF-1-containing fraction did not respond well to free UAA. Therefore, as RF-1 activity was only found in one portion of the gel (which is the fraction used in this experiment) it was assumed that all units of activity loaded on the gel were contained in this fraction. Each experiment was completed in duplicate and the values shown are the average.

Figure 12). The molecular weight of RF-1 is reported as 44,000 (Capecchi et al., 1969) therefore, the heavy band (from the denaturing gel) is much more likely to be RF-1. Figure 13 is a calculation of the number of copies of RF-1 per cell. By this method, it is estimated that there are 310 molecules of RF-1 per cell which compares favourably with values reported by Capecchi et al., (1969) of 600 copies per cell and Caskey et al., (1969) of 100 copies of RF-1 per cell. Thus, by the molecular weight of the protein (obtained by discontinuous gel electrophoresis) and the calculation of the number of molecules of this protein per cell, it seems reasonable to assume that this purification scheme (Figure 6) results in a very highly purified fraction of RF-1 (at least 90% pure, according to Capecchi et al., 1969).

This purification scheme results in a fraction of RF-1 which contains only one minor contaminant (see Figure 7), which compares very favourably with the scheme of Capecchi et al., (1969), whose purified RF-1 contains at least three contaminants. Other published purification schemes do not show denaturing gels of the purified fraction (Caskey et al., 1969 and Ganoza et al., 1970). Also, the purification schemes previously published contain many more steps than does that presented in Figure 6. For instance, the schemes of Capecchi et al., (1969) and Caskey et al., (1969) each contain seven steps and that of Ganoza et al.,

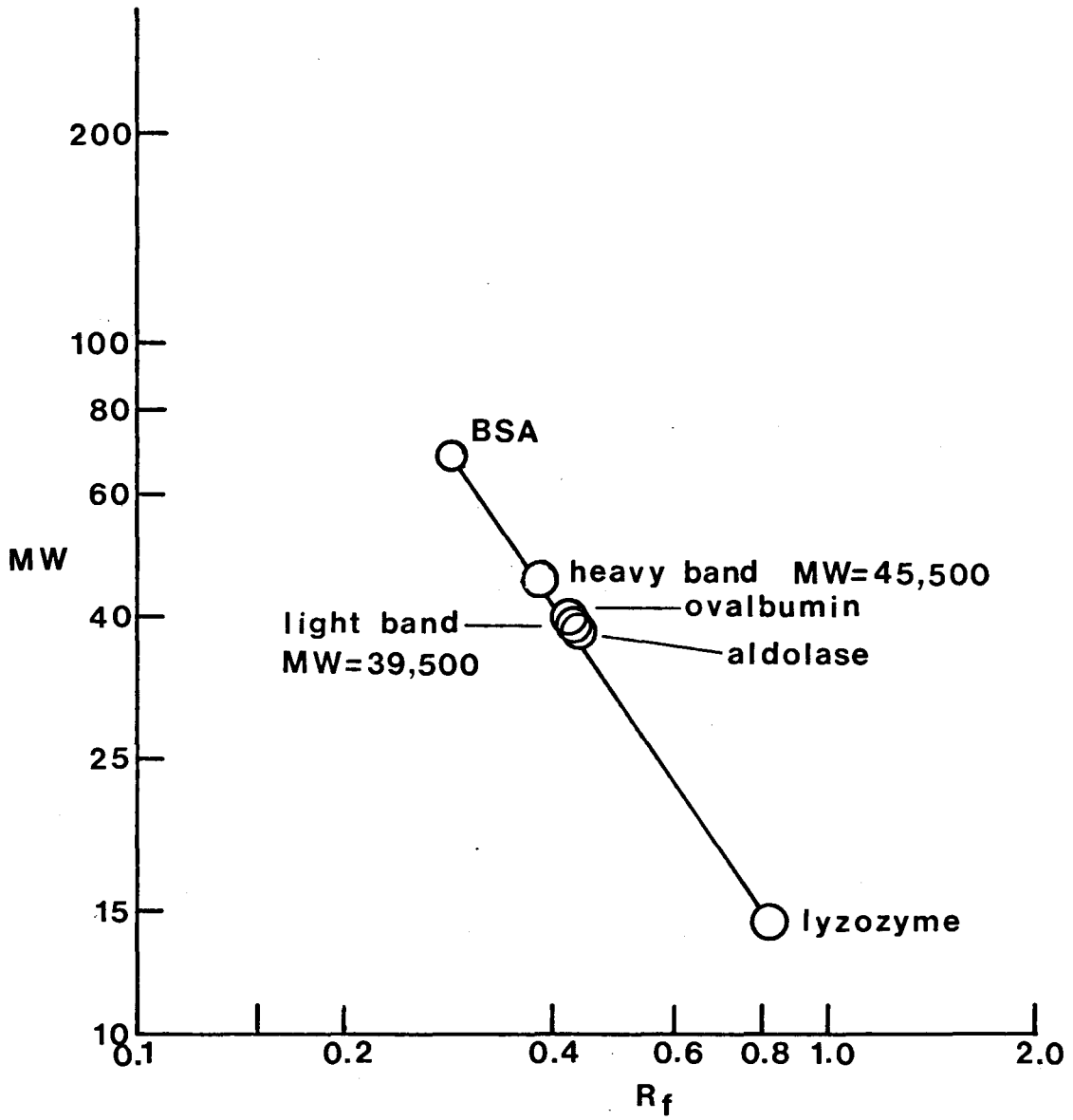


FIGURE 12.

Molecular Weight Determination
Using SDS Gel Electrophoresis.

Figure 13. Calculation of Number of Molecules of RF-1 per Cell.

259.5 g [2.595×10^{14} cells (Caskey et al., 1969)] E. coli K12 cells were broke in French press.

↓

S-100

↓

Ammonium Sulphate (0-80% precipitation)

(1547 mg)

(1) Assume this fraction contains 100% RF-1 activity (Caskey et al., 1969) (therefore this fraction contains 100% of RF-1 from 2.595×10^{14} cells).

(2) 580 mg of this fraction loaded onto DEAE-Sephadex A-50 column (and 580 mg of ammonium sulphate precipitate contains RF-1 from

$$\frac{1547 \text{ mg protein}}{2.595 \times 10^{14} \text{ cells}} = \frac{580 \text{ mg}}{x} = 9.70 \times 10^{13} \text{ cells}$$

DEAE-Sephadex A-50 purified-RF-1 (70.6 mg)

(1) Assume 100% yield of RF-1 from DEAE-Sephadex A-50 column (all fractions which contained RF-1 activity were pooled); therefore 70.6 mg of protein contains RF-1 from 9.70×10^{13} cells.

(2) Load 62.7 mg of this fraction onto P-11 phosphocellulose column and 62.7 mg of protein contains RF-1 from

$$\frac{70.6 \text{ mg protein}}{9.70 \times 10^{13} \text{ cells}} = \frac{62.7 \text{ mg}}{x} = 8.60 \times 10^{13} \text{ cells}$$

P-11 phosphocellulose column

1) 2.8 mg RF-1 in 0.2 M KCl fraction (26% of total RF-1 activity); 74% of RF-1 activity in .1 M KCl fraction. Therefore 2.8 mg of phosphocellulose purified protein contains RF-1 from

$$\frac{8.60 \times 10^{13} \text{ cells}}{100\% \text{ recovery}} = \frac{x}{26\% \text{ recovery}} = 2.2 \times 10^{13} \text{ cells}$$

2) Load 2.2 mg of this fraction onto discontinuous gel electrophoresis system and 2.2 mg of protein contains RF-1 from

$$\frac{2.2 \times 10^{13} \text{ cells}}{2.8 \text{ mg protein}} = \frac{x}{2.2 \text{ mg protein}} = 1.8 \times 10^{13} \text{ cells}$$

Discontinuous Gel Electrophoresis-purified RF-1

(1) Assume this fraction contains 100% of RF-1 activity (as only this portion of the gel contained RF-1 activity); therefore this fraction of protein (0.475 mg) contained RF-1 from 1.8×10^{13} cells.
(2) SDS gel of this discontinuous gel electrophoresis purified RF-1 revealed two bands. Major band has molecular weight of 45,500 whereas minor band has molecular weight of 39,500. Since molecular weight of RF-1 is reported as 44,000, assume heavy band (which is approximately 90% pure (according to Capecchi et al., 1969)) is RF-1.
(3) 90% of .475 mg of protein is RF-1 or

$$\frac{.475 \text{ mg protein}}{100\% \text{ RF-1 activity}} = \frac{x}{90\% \text{ of RF-1 activity}}$$

0.428 mg of this protein fraction is RF-1.
(4) Number of molecules of RF-1/cell may be calculated as follows: A) RF-1 obtained by this purification scheme has a molecular weight of 45,500, therefore each copy of RF-1 weighs

$$\frac{45{,}500 \text{ g}}{\text{mol of RF-1}} \div \frac{6.02 \times 10^{23} \text{ molecules}}{\text{mol}} = 7.60 \times 10^{-20} \text{ g}$$

(C) Number of molecules of RF-1 in the electrophoresis-purified fraction is

$$4.28 \times 10^{-4} \text{ g} \div \frac{7.60 \times 10^{-20} \text{ g}}{\text{copy RF-1}} = 5.63 \times 10^{15} \text{ molecules}$$

(C) Number of molecules of RF-1/cell is $\frac{5.63 \times 10^{15} \text{ molecules}}{1.8 \times 10^{13} \text{ cells}}$

310 molecules/cell

(1970) has five steps whereas, the scheme of Figure 6 has only four steps. On this basis, the purification scheme in Figure 6 results in the most purified fraction of RF-1 in the fewest steps.

3.4 A Possible Mechanism For UA-Mediated Termination.

Sequences UUA and UAA enhance termination which implies that RF-1 probably recognizes the dinucleotide UA, which is common to both of these triplets. This recognition is more successful when UA is positioned one nucleotide residue away from AUG however, this UA-mediated release is inhibited when a cytidine residue precedes it (see Table 1, AUGUUA and AUGCUA as well as AUGUUAA and AUGCUAA). Therefore, the context of UA is important in determining whether this sequence is capable of stimulating release of formyl-methionine.

Neilson and co-workers assisted me in searching for an explanation to these results by examining the conformation adopted by oligoribonucleotides in aqueous solution. They note that different conformations reflect the degree of oligomer base stacking, which in turn is dependent upon the percentage of 3′-endo ribofuranoside (which can be measured by proton NMR spectroscopy) (Alkema et al., 1982 and Everett et al., 1980). Base stacking

results because the bases stack on top of each other in order to maximize overlap of their p orbitals. On complete base stacking, in the A-helix of RNA, 100% 3´-endo form occurs. Inspection of various NMR signals in short oligomers, in the temperature range (70°C to 10°C), clearly demonstrates that the "rate" of base stacking differs for each base residue, consequently the overall stacking behaviour of oligomers varies greatly as a function of temperature. It has been determined that base residues stack in the order: Adenine > Guanine > Cytosine >> Uracil (Everett et al., 1980). Local conformations adopted by short sequences within a native RNA have been shown to be part of the recognition signal within protein-RNA interactions (Steckert et al., 1982), and it is likely that conformation can affect the termination response as well (Engelberg-Kulka et al., 1981).

Oligoribonucleotides are presumed able to exist in a variety of different conformations including bulge-loop conformations (Everett et al., 1980). For instance, in oligomer AUGUUAA, because the guanosine and adenosine residues tend to stack, it is quite plausible that one (or even both) of the intervening uridine residues will bulge-out to accommodate this stacking interaction. However, whether the intervening uridine residues loop-out or are just forced into a closer proximity with their neighbours, in stacked conformations, remains unknown,

although energetically, the bulging of the uridine residues appears to be more favourable. Tinoco and his group studied oligomers, which contained a uridine residue sandwiched between two residues with strong stacking tendencies, and found that the intervening uridine residue did, in fact, bulge-out (Lee et al., 1980). With the idea in mind (that intervening uridine residues will probably bulge-out when sandwiched between two residues with high base stacking abilities), the behaviour of UA-containing polymers may be explained.

The observations that AUGUUA, AUGUUAA and AUGUUAU stimulate the release reaction to about the same extent (see Tables 1 and 2), can now be explained. Each oligomer is bound to the ribosome through its AUG portion. If the 3′ unbound remainder of each oligomer is assumed to adopt the same conformations as that in solution, AUGUUA, AUGUUAA and AUGUUAU exhibit less extended conformations because the guanosine and adenosine residues will stack, forcing the intervening uridine residue, which is the fourth nucleotide of the oligomer, namely U-(4), to loop-out (see Figure 14). This is just one interpretation, as both intervening uridine residues could bulge, or just be compressed tightly between neighbouring guanosine and adenosine residues.

Since hexamer AUGUAA, causes only less than one-half of the release obtained with AUGUUAA, stacking between the guanosine and adenylyladenosine residues could force the

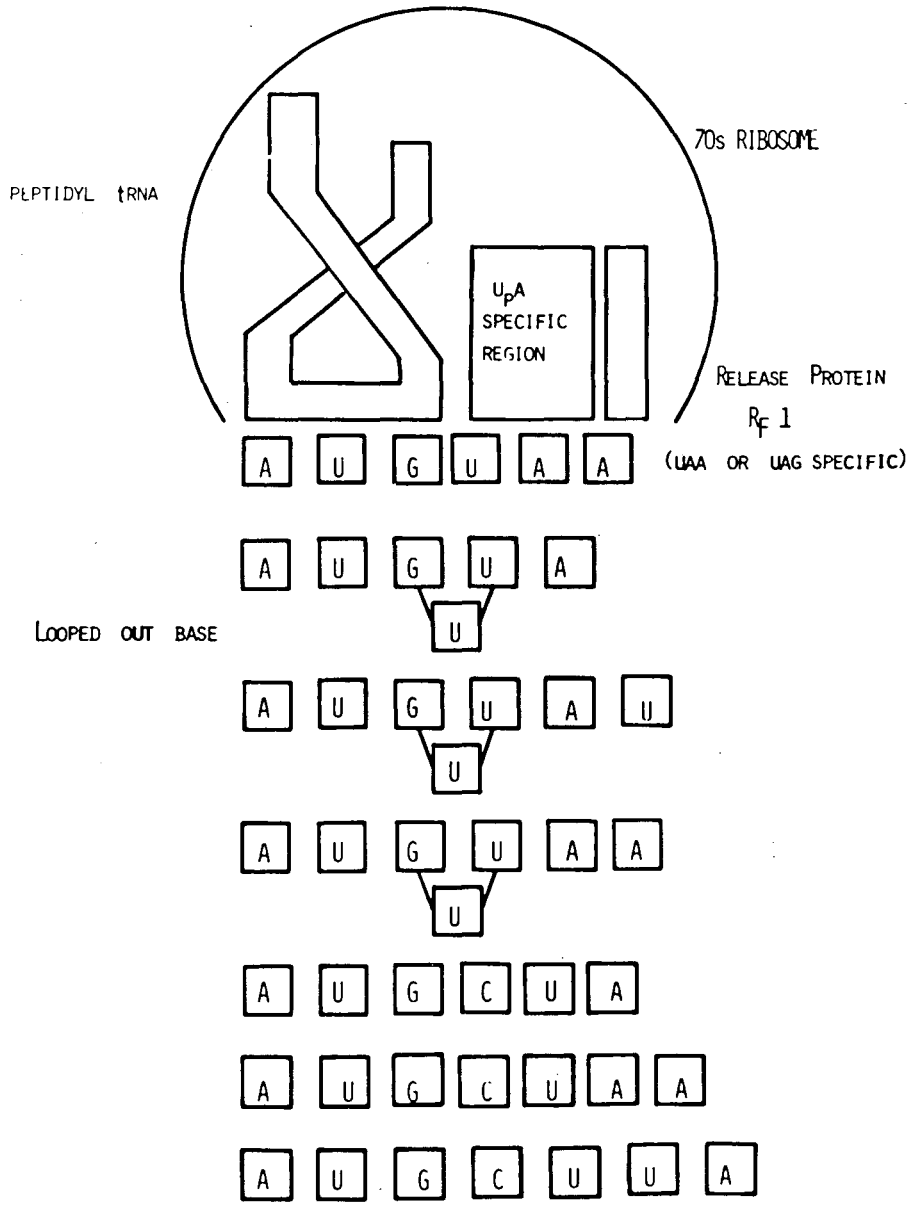


Figure 14. Schematic Representation of How Base Stacking Within Various RNA Oligomers May Affect the Position of the UA "Termination Signal" in the In Vitro Initiation Complex.

uridine residue to loop-out or alternatively, the adjacent adenylyladenosine residue increases the amount of stacking throughout the molecule, causing it in either case to be less extended, thus displacing the UA portion from its optimum position on the ribosome (see Figure 14). If this is correct, AUGUA should exhibit a more extended conformation than AUGUAA, and this is supported by variable temperature NMR analysis on AUGUA. However, although the sequence UA is aligned properly, AUGUA does not cause termination. This result suggests that UA must be part of a triplet in order to cause termination. Oligomers, AUGUUA and AUGUUAA, promote termination to an equivalent degree. In this case, an adenosine residue 3′ to UA does not enhance the termination reaction, which suggests that the third base interaction is not required by the ribosome• f-met-$tRNA_f^{met}$•mRNA•RF-1 complex.

The model messengers AUGCUA and AUGCUAA are expected to exhibit more highly stacked conformations in comparison to the other oligomers studied, because of the greater ability of a cytidine residue relative to a uridine residue, to base stack (Everett et al., 1980). The strength of the stacking interaction within these oligomers does not allow the cytidine residue to loop-out, thereby positioning the sequence UA incorrectly and so preventing termination (see Tables 1 and 2 and Figure 14). In contrast, in oligomers AUGUAA, AUGUUA, AUGUUAA and AUGUUAU, the recognition site,

UA on the messenger may be positioned in a favourable "configuration" on the ribosome for RF-1 to stimulate a termination response (Figure 14). Since UA stimulates the termination reaction when positioned one residue away from AUG it appears that the T site is different from the A site. However, the T site overlaps the A site.

An explanation for the results obtained with the various tandem stop codons shown is also possible (see Figure 9). In triplet UAA, base stacking between the adenosine residues weakly extends to the uridine residue. Apparently, this UA conformation is best recognized by RF-1 (Figure 9). Reinforced base stacking in hexamer UAAUAA, through four adenosine residues extends to uridine-(1) and probably loops-out uridine-(4). The more compressed U(1)AA is less suitable for RF-1 recognition. This explains why UAAUAA does not release N-acetyl-methionine well. It appears that RF-1 does not recognize sequences which are highly base stacked.

The results predicted by the model are not only in good agreement with those obtained in the present *in vitro* system, but also explain the *in vivo* result that adenosine residues (or purine residues in general) 3′ to nonsense codons, favour suppression of nonsense codons (Engelberg-Kulka *et al.*, 1981).

As previously discussed in Section 1.3.1 of the Introduction, release factors compete with suppressor-tRNAs

for nonsense codons (Bossi, 1983 and Ganoza et al., 1970). Release factors appear to favour nonsense codons whose context is such that the termination sequence is not highly base stacked whereas, suppressor-tRNAs favour nonsense codons situated in a highly base stacked sequence. This observation appears to be the basis for the competition exhibited by release factors and suppressor-tRNAs for nonsense codons.

In the case of UAAUGA, only the UA in UAA is recognized by RF-1. In UAAUAG, the guanosine residue does not stack as well as an adenosine, consequently the UAG function approximates that of UA and the complete hexamer operates as two UA units, so that UAAUAG can release N-acetyl-[^{3}H]methionine better than UAAUGA or UAA, at limiting concentrations of nonsense codon-containing oligomers. However, somewhat lower release is expected by a UAA-containing hexamer than by UAA on steric grounds, and hexamer UAAUAG is not expected to cause release of N-acetyl-[^{3}H]methionine significantly better than triplet UAA.

These results suggest that the RF-1 termination reaction responds to context, as suggested by Salser and co-workers (1969). A cytidine residue 5′ to sequence UA decreases release of formyl-methionine whereas, a uridine residue increases termination (See Table 1). The context 3′ to UA is important as well. A nucleotide sequence

containing several base residues, with a high ability to base stack, decreases release of formyl-methionine when present 3′ to sequence UAA (see Figure 9, oligomer UAAUAA). However, these studies do not provide evidence that the termination signal is longer than a triplet.

A serious consequence of this lack of specificity exhibited by RF-1 is the potential to elicit release of peptidyl-tRNAs in the neighbourhood of UA sequences other than UAA or UAG. This dinucleotide occurs at too high a frequency in mRNAs, to tolerate termination in response to UA only. Therefore the possibility that aminoacyl-tRNAs could effectively compete with the release factors during chain propagation was examined. It was found (see Table 4) that f-Met-leu-tRNA is synthesized in the presence of proteins, IF-1, IF-2 (initiation factors) and EF-T (elongation factor), in response to AUGUUA. Addition of DEAE cellulose-purified RF-1 decreased the level of f-Met-leu-tRNA synthesized and this effect was more pronounced when UAA and RF-1 had bound to ribosomes. This result is very significant; although release factor was known to compete against suppressor-tRNAs for nonsense codons (Bossi, 1983 and Ganoza et al., 1970), we have shown a direct competition between release factor and aminoacyl-tRNA for a sense codon. This result, along with the fact that release factor responds differently depending on whether a uridine or cytidine residue separates UA from

Table 4. Effect of Release Factor on the Synthesis and Release of F-Met-Leu Programmed by AUGUUA.

Additions	Total f[^{35}S]met Recovered From Paper (dpm)	Total f[^{35}S]met-leu Synthesized (dpm)	Hydrolysis f[^{35}S]met (dpm)	Hydrolysis f[^{35}S]met-leu
1. IF-1,IF-2,EF-Tu,EF-Ts	56,000	10,140	7,060	800
2. IF-1,IF-2,EF-Tu,EF-Ts + RF-1	51,000	5,000	7,800	1,300
3. IF-1,IF-2,EF-Tu,EF-Ts + RF-1+UAA	39,000	2,100	7,100	1,200
4. RF-1 + UAA	30,600	-	12,493	190+
5. None	30,600	-	944	190+

Dipeptide synthesis was analyzed electrophoretically as described in Section 2.2.4 of Methods. Recovery of total radioactivity was approximately 85% of added counts. Release of f[^{35}S]met with added factors was due to a trace of f-met-tRNA$_f^{met}$ hydrolase II (Ganoza et al., 1975) in the IF-1 preparations. Experiments 4 and 5 used AUG instead of AUGUUA and [^{35}S]met-tRNA$_f^{met}$ bound to ribosomes as substrates without IF-1 or IF-2. Where indicated 7 nmol UAA were added to the reactions. Non-specific hydrolysis may account for about 10% of the f-[^{35}S]met-leu released. Traces of EF-G in RF-1 could account for the remaining hydrolysis of f-met-leu in experiments 2 and 3. 14 µg of RF-1 (purified by DEAE-cellulose chromatography, see Figure 6) were added as indicated.

+Represents background radioactivity.

AUG (see Tables 1 and 2) suggest that it is the termination reaction triggered by release factor which responds to context, rather than the suppression reaction as suggested by Bossi (1983).

3.5 Discovery Of A Factor Which Confers Specificity To RF-1-Mediated Termination.

The purification scheme outlined in Figure 6 results in a fraction which exhibits the known properties of RF-1. However, Table 3 shows that the purification step of discontinuous gel electrophoresis results in a fraction with a decreased specific activity with free triplets AUG and UAA whereas the specific activity of AUGUUA is enhanced by this step. As the results above suggest that this electrophoresis-purified fraction contains highly purified RF-1, it seems plausible that RF-1 requires some other factor for specificity which is present in the P-11-phosphocellulose fraction but is subsequently removed by discontinuous gel electrophoresis. Such a factor, when added to the termination assay, would be expected to 1) enhance the amount of RF-1-mediated release observed with free triplets AUG and UAA and either 2) decrease, or have no effect on, the RF-1-mediated release observed with AUGUUA.

The possibility that one of the factors already implicated in protein synthesis (namely IF-1, IF-2, IF-3,

EF-T, EF-G, EF-P, W, rescue and the hydrolases) (Ganoza, 1977) could correct the lack of specificity exhibited by RF-1 was investigated. The effect of initiation factors on free triplets AUG and UAA as well as on UA-containing oligoribonucleotides AUGUA, AUGUUA and AUGUAA was tested using the assay system described in Section 2.2.5 of Methods. Table 5 illustrates that initiation factors do affect termination by inhibiting the amount of release of N-acetyl-[^{3}H]methionine caused by each oligomer. This inhibition does not seem to be significant in terms of contributing to the specificity of the RF-1-mediated reaction because the UA-mediated and UAA-mediated release reactions are inhibited to the same extent (compare triplets AUG (and free UAA) with AUGUA and oligomer AUGUUA with AUGUAA). As these initiation factors were not purified to homogeneity, they could contain some unspecific inhibitor of the termination reaction, or the initiation factors could compete with RF-1 for the "site" on the ribosome.

Elongation factors P, T and G and factors W and rescue were added separately during the termination assay in an effort to determine if any of these proteins contribute to the specificity of the RF-1-mediated reaction. Table 6 illustrates that EF-P, EF-T, EF-G and factors W and rescue inhibit the RF-1-mediated release triggered by AUGUUA and free triplets AUG and UAA to the same extent (within experimental error). Thus, none of these proteins is a

Table 5. Effect of Initiation Factors on the Release of N-Acetyl-[^{3}H]Methionine.

Oligomer	Amount of Inhibition* (percent)
AUG (+ UAA)	90.9
AUGUA	86.8
AUGUUA	37.8
AUGUAA	40.3

300 pmol of oligoribonucleotide were bound to E. coli Q13 ribosomes with N-acetyl-[^{3}H]-methionine as described in Section 2.2.3 of Methods. If initiation factors were added then ribosome complexes were formed as in Section 2.2.3 of Methods: With the following modifications; 1) ribosomal complexes were formed in the presence of a pH 7.4 buffer containing 22 mM Tris-HCl, 56 mM NH_4Cl and 5 mM $MgCl_2$ and 2) 2.5 µg IF-1 and 2.5 µg IF-2 were added (purified as described in Section 2.2.4 of Methods). The termination portion of the assay was carried out as described in Section 2.2.5 of Methods with 7.6 nmol of UAA (added only to AUG•ribosome•f-[^{35}S]met-tRNA$_f^{met}$ complexes) and 15 µg of E. coli K12 RF-1 (purified by DEAE-cellulose chromatography, see Figure 6). These results are the average of two experiments each of which was completed in duplicate.

*Percent inhibition is calculated according to

$$\left[1 - \frac{\text{Amount of N-acetyl-[}^3\text{H]met released in the presence of IFs}}{\text{Amount of N-acetyl-[}^3\text{H]met released in the absence of IFs}}\right] \times 100\%$$

Table 6. Effect of Elongation Factors T, G and P and Proteins Rescue and W on the Release of F-[^{35}S]Methionine from AUG•F-[^{35}S]Met-tRNA$_f^{met}$•Ribosomal Complexes.

Additional Factor Added (10 μg)	Amount of Inhibition of Termination (percent)+	
	Polymer Bound	
	AUG + UAA	AUGUUA
EF-P	39.5	27.6
EF-T	13.7	17.2
EF-G	2.6	5.0
W	21.1	16.4
Rescue	27.4	23.4

300 pmol of oligomer (either AUG or AUGUUA) were bound to E. coli Q13 ribosomes with f-[^{35}S]met-tRNA$_f^{met}$ as described in Section 2.2.3 of Methods. The termination portion of the assay was carried out as described in Section 2.2.5 of Methods, with the modification that 1 mM GTP was added. RF-1 (16 μg), purified by DEAE-cellulose chromatography from E. coli K12 cells, were added, and 7.6 nmol of UAA were added to AUG•f-[^{35}S]met-tRNA$_f^{met}$•ribosome complexes. Additional factors added (EF-P, EF-T, EF-G, W, and Rescue) were purified as discussed in Section 2.2.4 of Methods. These results are the average of duplicates.

+Amount of inhibition of termination (percent) was calculated according to

$$\left[1 - \frac{\text{Amount of f-[}^{35}\text{S]met released in the presence of additional factor}}{\text{Amount of f-[}^{35}\text{S]met released in the absence of additional factor}}\right] \times 100\%$$

likely candidate for the factor needed to confer specificity to RF-1-mediated termination.

Hydrolases are not expected to contribute to the specificity of the termination reaction. Peptidyl-tRNA hydrolase does not use N-acetyl-[^{3}H]met-tRNA$_{f}^{met}$ nor f-[^{35}S]met-tRNA$_{f}^{met}$ but rather peptidyl-tRNAs as substrate (Menninger et al., 1970). Therefore, the release of formyl-methionine observed, when RF-1 is added to ribosomal-bound AUGUUA, can not be due to peptidyl-tRNA hydrolase.

The other type of hydrolase, namely hydrolase II, does use f-[^{35}S]met-tRNA$_{f}^{met}$ or N-acetyl-[^{3}H]met-tRNA$_{f}^{met}$ as its substrate. It is possible that this hydrolase could be responsible for the specificity of RF-1-mediated release however, purification of ammonium sulphate fractions on DEAE-cellulose (or DEAE-Sephadex A-50) columns results in a fraction of RF-1 which is pure of hydrolase II. After DEAE-cellulose chromatography, hydrolase II is found in the void volume (Ganoza et al., 1975) whereas RF-1 is found in the 0.4 M KCl fraction. Therefore, hydrolase is present in ammonium sulphate fractions of RF-1, but not in DEAE-cellulose fractions of RF-1, yet the ammonium sulphate fractions had a high specific activity with oligoribonucleotide AUGUUA when compared to that specific activity obtained with DEAE-Sephadex A-50 RF-1 (see Table 3). The loss of

hydrolase II did not greatly decrease the specific activity with AUG + UAA nor greatly increase the specific activity with AUGUUA and therefore hydrolase II does not seem to be the factor responsible for conferring specificity to the RF-1-mediated reaction.

The third release factor, RF-3 could possibly be the factor which confers specificity. Since the discontinous gel electrophoresis step greatly decreased the specificity of the RF-1 reaction (as the specific activity of this RF-1 was greatly decreased with free triplets AUG and UAA but enhanced with AUGUUA) (see Table 3), the factor conferring specificity to the RF-1 reaction was eliminated by this purification step. Therefore, the partially purified fractions obtained before this purification step were tested for RF-3 activity.

RF-3, in the presence of GTP, inhibits termination at low nonsense codon concentration (300 pmol). At a high level of nonsense triplet (7.6 nmol), the addition of GTP is believed to enhance termination (Goldstein *et al.*, 1970a). The activity of RF-3 in RF-1 fractions (partially purified by DEAE-cellulose chromatography) was tested by the response of this protein to GTP in the trinucleotide termination assay (see Section 2.2.5 of Methods and see Table 7). The addition of GTP to the AUG-bound ribosomal complex caused inhibition of release of formyl-methionine regardless of whether the concentration of free UAA was low (300 pmol) or

Table 7. Effect of GTP on the Trinucleotide Termination Assay.

Amount of UAA (nmol)	Amount of Inhibition of Termination (percent)*
0.3	32.9
7.6	43.0

Triplet AUG was bound to E. coli Q13 ribosomes with f-[^{35}S]met-tRNA$_f^{met}$ as described in Section 2.2.3 of Methods. 15 μg of E. coli K12 RF-1 (purified by DEAE-cellulose chromatography) and triplet UAA, were added to AUG·f-[^{35}S]met-tRNA$_f^{met}$·ribosome complexes and the reaction mixtures were made 1 mM in GTP. The buffer, salt concentrations and conditions of the assay were as described in Section 2.2.5 of Methods. These results are the average of three experiments, each of which was completed in duplicate.

*Percent inhibition is calculated by

$$\left[1 - \frac{\text{Amount of f-[}^{35}\text{S]met released in the presence of GTP}}{\text{Amount of f-[}^{35}\text{S]met released in the absence of GTP}}\right] \times 100\%$$

high (7.6 nmol), which was not the expected result if RF-3 was present in the partially purified preparation of RF-1. Hence, RF-3 does not appear to be the factor which confers specificity to the RF-1-mediated reaction.

It would seem that some factor other than initiation or elongation factors or proteins W, rescue, hydrolases I and II or RF-3 is responsible for ensuring the specificity of the termination reaction. For this reason, the S-100 supernatant of E. coli K12 cells was prepared and added to the termination assay (as illustrated in Table 8).

Surprisingly, the S-100 contains a factor which seems to partially restore the specificity of the termination reaction, as this factor enhances the activity of electrophoretically-purified RF-1 (as shown with free triplets AUG and UAA and oligomer AUGUAA) however, UA-mediated release is inhibited substantially (oligomer AUGUUA). Oligomers which did not have the ability to enhance RF-1-mediated release do not seem to be affected by this factor (see oligomer AUGCUA in Tables 1 and 8). The enhancing effect, exhibited by this factor, is obliterated by sparsomycin (see Table 8). This result suggests that the stimulation of formyl-methionine release (observed when an aliquot of S-100 is added during UAA-mediated release) involves peptidyl transferase. The involvement of peptidyl transferase implies that the enhanced release of formyl-methionine is part of (or associated with) in vitro

Table 8. Effect of E. coli K12 S-100 Supernatant on the Termination Reaction.

Oligomer Bound to Ribosomes	Amount of RF-1 (μg)	Effect of S-100 on Release of f-[^{35}S]-methionine (percent)+	
		Experiment 1 (- Sparsomycin)	Experiment 2 (+ Sparsomycin)
AUG + UAA	3.4	47.2 (stimulation)	87.7 (inhibition)
AUG + UAA	6.8	69.1 (stimulation)	71.3 (inhibition)
AUGUUA	1.7	30.4 (inhibition)	91.6 (inhibition)
AUGUUA	3.4	62.1 (inhibition)	83.2 (inhibition)
AUGUUA	6.8	29.2 (inhibition)	92.6 (inhibition)
AUGCUA	4.4	2.7 (stimulation)	-
AUGUAA	8.8	44.5 (stimulation)	-

Oligoribonucleotide·ribosome·f-[^{35}S]met-tRNA$_f^{met}$ complexes were formed as described in Section 2.2.3 of Methods. Subsequently, discontinuous gel electrophoresis-purified RF-1 was added (in the amount indicated) to ribosomal-bound complexes, as well as 7.6 nmol of UAA (added only to AUG·ribosome·f-[^{35}S]met-tRNA$_f^{met}$ complexes) and 27 μg of E. coli K12 S-100. Reaction conditions were as described in Section 2.2.5 of Methods. Experiment 2 was performed with the same reaction conditions of Experiment 1, except for the addition of sparsomycin (5×10^{-4} M).

+Percent effect of E. coli K12 S-100 on the termination reaction was determined as follows;

$$\left[1 - \frac{\text{Amount of f-[}^{35}\text{S]met released in the presence of S-100}}{\text{Amount of f-[}^{35}\text{S]met released in the absence of S-100}}\right] \times 100\%$$

termination and not merely a non-specific hydrolysis independent of termination.

The nature of this factor was determined by temperature studies. Table 9 illustrates that boiling the S-100 obliterates its ability to affect the termination reaction. The activity of the factor is decreased gradually with temperature, with all activity obliterated by boiling at 60°C for five minutes. Since boiled samples were cooled slowly (allowing any denatured RNA to renature) and yet all enhancing activity is lost by boiling at 60°C, it seems that the factor responsible for conferring specificity to the RF-1-mediated termination reaction is either a protein, or a protein-containing complex (this factor has been tentatively designated Specificity Factor). The discontinuous gel electrophoresis purification step, used during the preparation of RF-1 (see Figure 6), appears to separate this Specificity Factor from RF-1 because this step greatly decreases the apparent functions of this factor (see Table 3). The Specificity Factor appears to enhance the ability of RF-1-mediated release caused by UAA as well as inhibits the ability of RF-1-mediated release by UA.

It is possible that the Specificity Factor affects the termination reaction by altering the oligomer• f-[^{35}S]met-tRNA$_f^{met}$•ribosome•RF-1 complex. Figure 14 shows a possible model for the mechanism of UA-mediated release of formyl-methionine. As the

Table 9. Effect of Temperature on S-100 Activity in the Termination Assay.

Experiment Number	Pretreatment of S-100	Amount of Stimulation of Termination (percent)+
1	none	101.7
2	50°C	81.0
3	55°C	33.9
4	60°C	0.6

Triplet AUG was bound to ribosomes with f-[^{35}S]met-tRNA$_f^{met}$ as described in Section 2.2.3 of Methods. An aliquot (8.8 µg) of DEAE-Sephadex purified RF-1 was added along with 7.6 nmol of UAA and 27 µg of E. coli K12 S-100, and release of formyl-[^{35}S]methionine was measured as described in Section 2.2.5 of Methods.

In Experiment 1, the aliquot of S-100 was not boiled, however, in Experiment 2, 3 and 4 the aliquot of S-100 was pre-treated by boiling for 10 min at 50°C, 55°C and 60°C respectively. Boiled samples were cooled slowly (allowing denatured RNA to renature). Any precipitated protein was eliminated by centrifugation and only the supernatant was added to reaction mixtures. These results are the average of duplicates.

+Percent effect on termination was determined as follows;

$$\left[1 - \frac{\text{Amount of f-[}^{35}\text{S]met released in the presence of S-100}}{\text{Amount of f-[}^{35}\text{S]met released in the absence of S-100}}\right] \times 100\%$$

Specificity Factor appears to confer specificity to the release reaction by both partially eliminating UA-mediated termination and stimulating UAA-mediated release (see Table 8), it is tempting to believe that Specificity Factor alters the UA-specific region of the ribosome in such a manner that the third base residue of the nonsense codon is not only required, but that it must either be an adenosine or guanosine residue.

From Table 3, it appears that the bulk of the Specificity Factor is eliminated by the discontinuous gel electrophoresis step. Therefore, the fractions of RF-1 obtained before this step must contain some of this factor, and yet these partially purified fractions exhibit UA-mediated hydrolysis (see Tables 1, 2 and 3). Thus, it would seem that one of the following situations exists; either 1) another factor (not yet discovered) exists which assists the Specificity Factor in eliminating UA-mediated release or 2) fractions of RF-1, obtained before purification by gel electrophoresis, do not contain one hundred percent of Specificity Factor activity or 3) the release is not fail-safe even <u>in vivo</u>.

It would be surprising if yet another factor exists, which assists in conferring specificity, as the aliquot, containing the Specificity Factor, was an S-100 fraction, which usually contains some fraction of all of the soluble cellular proteins. Using an aliquot of S-100 should be the

best method of scoring for an unknown protein, yet the addition of S-100 did not totally eliminate UA-mediated termination (see Table 8). Therefore, the possibility of yet another specificity factor does not seem substantial.

Similarly, the possibility that fractions of RF-1, obtained before gel electrophoresis purification, did not contain enough Specificity Factor to obliterate UA-mediated release, is improbable. Even the addition of excess S-100 could not totally diminish this unusual hydrolysis (see Table 8).

Perhaps the RF-1-mediated termination reaction is not fail-safe. The observed lack of specificity of RF-1 may be of advantage, in that, this protein could promote cleavage of peptidyl-tRNA during chain growth at proper termination sites or at out-of-phase UAs, when the supply of charged aminoacyl-tRNAs is limiting, or if the translation rate decreases for other reasons. The selective utilization within mRNAs of multiple termination signals which depend on base context, may allow protein synthesis to have many controllable stop signals. Nature could thus achieve a measure of fine tuning of gene expression at the termination level.

However, the unusual hydrolytic response of highly purified-RF-1 to the sequence UUA (when covalently attached to AUG) has not yet been found *in vivo*. As pointed out by Capecchi and Klein (1969), this lack of specificity by RF-1

may result because the assay does not involve a normal termination sequence. However, before this theory can be substantiated, another release assay must be developed which would not only incorporate a natural sequence but be simple as well.

CONCLUSIONS

The context of a nonsense codon has been proposed to affect the prokaryotic termination reaction (Fluck et al., 1977 and Salser et al., 1969). Using oligoribonucleotides of the form UAAX (where X is an adenosine, guanosine, cytidine or uridine residue), in the trinucleotide assay (Caskey et al., 1969), did not reveal a preference for a nucleotide residue 3′ to UAA.

Studies with UAAUAG, UAAUGA and UAAUAA were surprising, because UAAUAA was five-fold less effective than either the other two hexamers or UAA. It is interesting to note that UAAUAA has not been observed in prokaryotic termination regions (Steege et al., 1979). The nature of the sequence 3′ to UAA affects termination. Sequences 3′ to UAA which increase base stacking, decrease the extent of termination. Such an effect has been previously documented (Engelberg-Kulka et al., 1981).

The effect of sequences 5′ to UAA could not be examined using the trinucleotide assay, as the oligoribonucleotide set NUAA (where N is an adenosine, guanosine, cytidine or uridine residue) was unreactive (Ganoza et al., unpublished). For this reason, a set of longer oligoribonucleotides was built (by Neilson and co-workers), each of which began with AUG; AUGUAA, AUGUA,

AUGUUA, AUGCUA, AUGAUG, AUGUUU, AUGAGC, AUGGCU, AUGUCU, AUGCUAA, AUGCUUA, AUGUUAA and AUGUUAU. These oligomers were bound to E. coli Q13 ribosomes by their AUG portion, and subsequently, a crude fraction of RF-1 was added to these reaction mixtures. Oligomers, which contained either sequence UUA or UAA downstream from AUG, were able to cause release of f-[^{35}S]methionine. Since the portion common to both of these triplets is UA, it is probable that RF-1 responds to this dinucleotide. This stimulation of termination occurred only when sequence UA was not preceded by a cytidine residue. Oligomers which did not contain UA, or where UA was preceded by a cytidine residue, were not able to stimulate the termination reaction.

Dinucleotide UA or triplet UUA did not stimulate the release reaction when added with RF-1, to AUG• f-[^{35}S]met-tRNA$_f^{met}$• ribosomal complexes. These sequences (UA or UUA) must be covalently bound to AUG in order to enhance termination. UA appears to stimulate release better when positioned one nucleotide residue away from AUG (compare oligomers AUGUAA and AUGUUAA; AUGUA and AUGUUA).

Since these studies were begun with crude preparations of RF-1, the UA-mediated release reaction need not have been stimulated by RF-1, but rather a contaminating factor. For this reason RF-1 was purified. Denaturing gel electrophoresis of the purified protein revealed two bands.

The major band had a molecular weight of 45,500 whereas that of the minor band was 39,500. The molecular weight of RF-1 is reported as 44,000 (Capecchi et al., 1969). From these data, it would appear that the heavy band was RF-1. It was calculated that there are 310 molecules of RF-1 per cell, which compares well with those previously reported (100 copies of RF-1 per cell (Caskey et al., 1969) and 600 molecules of RF-1 per cell (Capecchi et al., 1969)). The results from the calculated molecular weight, as well as the number of molecules per cell, suggest that the majority of purified protein is RF-1. This fraction also enhanced UA-mediated release and this activity was inhibited by sparsomycin, which implies that the RF-1-mediated reaction is not absolutely specific.

A model was presented which accounts for the UA-mediated release. It is possible that UA only causes termination when it is part of a triplet. The fact that a cytidine residue 5′ to UA decreases termination may be explained in terms of base-stacking. Cytidine residues (which base stack better than uridine residues) do not "loop-out" to accommodate the base stacking of their 3′ and 5′ neighbours whereas uridine residues do. Therefore, oligomers with a cytidine residue 5′ to UA, do not stimulate termination because UA is not positioned properly on the ribosome.

All factors known to participate in protein synthesis

were screened unsuccessfully for their abilities to confer specificity to the termination reaction. Further investigation of an S-100 revealed a factor, protein in nature, which inhibits UA-mediated release but increases UAA-mediated release. This factor appears to confer specificity to RF-1-mediated release.

Context affects termination. Release factors favour nonsense codons whose context is such that the termination sequence is not highly base stacked. However, evidence that the termination signal is longer than a triplet was not found.

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