NONSENSE MUTATIONS AND tRNA SUPPRESSORS

EDITED BY

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PREFACE

This book is a record of the proceedings of the EMBO Laboratory Course and Aarhus University 50 Years Anniversary Symposium on "Nonsense mutations and tRNA suppressors" held in Aarhus in July 1978.

Nonsense mutations and their suppressors have played a key role in the genetics and study of gene expression in bacteria and yeast. Also our knowledge of transfer RNA genetics, biosynthesis and structure-function relationships has depended to a large extent on the tRNA suppressors. The possibility of developing similar suppression systems in higher eukaryotes has stimulated extensive research on the characterisation of nonsense mutants and attempts to isolate nonsense suppressors in animal cells.

The main aim of the book is to introduce the reader to the field of translational suppression, specifically to nonsense mutations and tRNA suppressors. The book covers classic work on nonsense suppressors in prokaryotes and yeast as well as the latest developments in the search for nonsense mutations and tRNA suppressors in higher eukaryotes. To help the reader the book contains a few general chapters dedicated to tRNA and its role in protein synthesis as well as a compilation of wild type and mutant tRNA sequences.

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The Editors.

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STRUCTURE AND FUNCTION OF tRNA

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Introduction

In the late 1950s no one could envisage how nucleic acids could programme the synthesis of protein by direct structural interactions with amino acids. However, Crick (1957) proposed his 'Adaptor Hypothesis' whereby an adaptor molecule (rather vaguely defined in terms of structural properties) would mediate between the amino acid and the nucleic acid which carried genetic information for specifying the amino acid sequence during polymerisation into protein. Shortly afterwards the Adaptor Hypothesis was confirmed by the discovery in rabbit liver extracts of a class of small RNA molecules capable of specifically binding amino acids (Hoagland *et al.*, 1959). Each example of this class of RNA molecule consists of a polynucleotide chain about 80 units long starting with a 5'-phosphate at one end and ending with a common sequence CpCpA at the other (3') end. Later this class of small RNA molecule became known as transfer RNA describing its role in protein biosynthesis.

Transfer RNA functions in protein biosynthesis by carrying esterfied, activated amino acids to the ribosomal site in the correct order for peptide bond formation. During this central biological function the tRNA plays its role as an adaptor by decoding the genetic information carried by mRNA. Indeed, in modern terminology one could consider the tRNA as a molecular interface between the protein and nucleic acid languages.

The total tRNA in a cell makes up about 1% of dry weight in the case of a bacterial cell so that there are about 4×10^5 tRNA molecules of perhaps 55 different types in such a cell. Thus tRNA content corresponds to a concentration of about 0.5 mM, a figure to be borne in mind when trying to relate *in vitro* results to reality. Estimates of

how much of the cellular tRNA is charged with an amino acid are notoriously difficult to obtain because of the lability of the aminoacyl bond to the tRNA. Probably estimates such as about 80% of the tRNA being charged (Lewis and Ames, 1972) are on the low side since it is likely that the catalytic activity of the adequate amount of aminoacyl-tRNA synthetase (activating enzymes) molecules in the cell keeps the tRNA fully charged provided that sufficient free amino acids are available.

Now that a three-dimensional structure is known for one tRNA species and more than 100 primary structures all with predictable secondary structures in clover leaf forms (Holley *et al.*, 1965) there is considerable motivation for attempting to explain tRNA function in terms of structure. At present, naturally, most of this work concentrates on explaining tRNA function during protein biosynthesis because so much more has been discovered about the biochemical processes involved, thanks to the research stimulus during the elucidation of the genetic code in the 1960s. Although the availability of the crystal structure of a tRNA provides a new meaningful basis for discussion of function it must be remembered that this structure gives only one conformation or view of the tRNA in its most stable or resting conformation (Ladner, 1978). How this conformation changes during biological function especially during protein biosynthesis is an intriguing and tantalizing problem currently engaging many different types of research workers using X-ray crystallography, chemical modification studies, enzyme kinetic measurements and physical chemical techniques such as proton magnetic resonance, electron spin resonance, laser light scattering and fluorescence.

In this chapter I shall concentrate on the role of tRNA in protein biosynthesis, but in the last section it will be pointed out that tRNA is becoming more and more fascinating as it is being implicated in more and more biological activities other than its traditional adaptor role. Possibly due to evolutionary pressure, biological economy and tRNA probably being as old as life itself, tRNA has become imbued with its multifunctional role.

This chapter does not aim to be all embracing of the extensive tRNA literature so I apologize for being arbitrarily selective in reference citations. A much more extensive literature survey will be found in a recent review by Rich and RajBhandary (1976).

Role of tRNA in Protein Biosynthesis

A summary of the current knowledge about the biological function or, more precisely, biological activities of tRNA in protein biosyn-

thesis is given in Table I. Although much is known about the role of tRNA in protein biosynthesis even in this area some of the activities listed have not been characterized adequately enough to elevate them to functions.

TABLE I

Biological Activities of tRNA in Protein Biosynthesis

1.	Activation of amino acids	
Elongation		*Initiation*
2. Recognition by EF-Tu		6. Recognition of initiator tRNA by IF
3. Location in A-site		7. Location in I-site (part of P-site)
4.	Decoding mRNA	
5. Signal for 'magic spot'		8. Recognition by transformylase
9.	Regulation	
	a) Repressor b) Feedback Inhibitor c) Suppression	

In summary for normal protein biosynthesis each tRNA species is charged with an amino acid (activity 1 of Table I) by an aminoacyl-tRNA synthetase ('activating enzyme'), and then the charged species is carried to the ribosome in the form of a ternary complex made with the elongation factor Tu (EF - Tu) and GTP (activity 2). In similar fashion the unique tRNA species, the initiator tRNA, a special class of methionine tRNA, is thought to be carried to the ribosome by an initiation factor and probably GTP as well (activity 6). The aminoacyl-tRNA is located by an uncharacterized mechanism in the A-site of the ribosome (activity 3) where it decodes mRNA via its anticodon triplet (activity 4). In contrast the initiator tRNA, formylmethionyl-$tRNA_f^{Met}$ in prokaryotes, and methionyl-$tRNA_f^{Met}$ sometimes called Met-$tRNA_i$ in eukaryotes, is located in the initiation (I)-site on the small ribosomal subunit (activity 7) for decoding the initiator triplet codon. This site becomes part of the ribosomal P-site. Since the prokaryotic Met-$tRNA_f$ has to be formylated for its initiator activity, it is also recognized by a special enzyme for this, the transformylase (activity 8).

When prokaryotic cells are starved for amino acids an unusual role has been detected for uncharged tRNA (Pedersen *et al.*, 1973). The uncharged tRNA is bound to the ribosomal A-site as if in mRNA decoding, but sets off a signal for the formation, by the so-called

stringent factor, of unusual guanosine nucleotide derivatives, ppGpp and pppGpp, called 'magic spots' (activity 5).

A group of assorted roles for tRNA in the regulation of protein biosynthesis has been collected as activity 9. These include bacterial roles as a repressor of the histidine operon (Lewis and Ames, 1972) a less defined regulator of amino acid biosynthesis (Allende, 1975) and the well characterized suppressor of nonsense mutations (Smith, 1972 and this volume) and a possible role as a feedback inhibitor in yeast (Bell *et al.*, 1976). In eukaryotes, there are additional non definitive roles relating to evidence on the binding to tryptophan pyrrolase in Drosophila (Rich and RajBhandary, 1976) and the inhibition of protein synthesis in virally infected animal cells by the degradation of one or more essential tRNA species, a process that seems to accompany interferon production (Revel, personal communication).

Another set of phenomena concerning amounts of various isoaccepting species existing in different types of cells at various stages in growth or transformation can be classified under a general regulatory role. Obviously restricting the amount of one specific decoding aminoacyl-tRNA will control protein biosynthesis at this point in translation (Smith, 1975; Sharma *et al.*, 1976). However, despite the large literature on the subject, especially in connection with control of protein synthesis in carcinogenic states the regulatory role of tRNA has not been clearly characterized (Rich and RajBhandary, 1976).

Because of our present structural knowledge of tRNA it is now appropriate to study the molecular mechanism of protein biosynthesis in structural terms. We wish to know what happens to the tRNA structure during charging of the tRNA with an amino acid, what happens to the tRNA on the ribosome and how the tRNA gets to the ribosome.

A tRNA is enzymically linked to its specific amino acid at the 2′ or 3′ hydroxyl group on its 3′ - terminal adenosine, by its specific (cognate) aminoacyl-tRNA synthetase (activity 1 of Table I). Another chapter in this volume (by Bruton) describes what is known about the structure of this class of enzymes. Figure 1 summarizes the 2 step reaction concerned in charging the tRNA.

Since there is only one activating enzyme for charging several different tRNAs (isoaccepting species) with the same amino acid it was hoped that a knowledge of the several tRNA primary structures would reveal how the activating enzyme recognized them. Unfortunately, however, these studies have not been very helpful. Similar

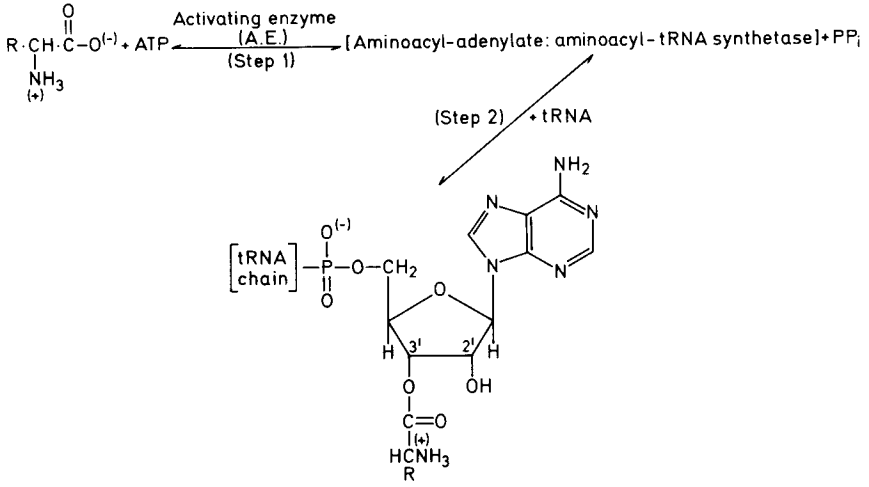

Fig. 1 A two-step scheme for charging a tRNA with an amino acid by an activating enzyme (an aminoacyl-tRNA synthetase).

feelings have been expressed for the reciprocal comparative studies of the enzymes' amino acid sequences. Clearly the folded tertiary structure provides the important points of contact for recognition and it is still an intriguing problem how such a synthetase recognises a particular tRNA when we believe that many of the tRNA tertiary structures are similar. This enzyme specificty for tRNA and amino acid clearly is an important feature in ensuring fidelity of translation of the genetic code. What we really do need to solve this problem is an X-ray crystallographic analysis of a complex of an amino-acyl-tRNA and its cognate synthetase. So far no crystals containing such a complex have been reported so other physical and chemical methods are being used to gather useful information.

When the recognition site of a tRNA for its activating enzyme is discussed an uncertainty arises. There is no guarantee that every tRNA is recognized in a similar fashion by some property of a similarly located set of nucleotides. Perhaps the recognition of particular tRNAs by their specific activating enzymes has evolved differently, so that by now there are different classes of tRNA recognition features. If this is correct no generalization about the recognition process will be possible when details of recognition of one tRNA by its activating enzyme are known.

The possible different recognition features have even been observed in the different points of attachment of different amino acids to their specific tRNAs (for a review, see Sprinzl and Cramer, 1978). In recent years the problem of the point of attachment has been dramatically solved for a series of synthetases. At first it was thought that the amino acid was attached to the 2′ -OH group of the 3′ terminal A, as for example is the case for methionine and phenylalanine. The story has been complicated by other studies where the attachment point is the 3′ -OH group (as shown in Figure 1) as in the case of amino acids such as lysine and serine. In addition there are a few cases where the attachment point occurs at both 2′ and 3′ -OH groups as in the case of tyrosine. A reasonable explanation is that the stereochemical details of the substrate binding site of a cognate tRNA:synthetase pair have evolved independently of other cognate pairs. Indeed, the actual point of attachment may not be very important because in solution aminoacyl migration can occur between the two 2′ and 3′ -hydroxyl groups of the terminal A about 1000 times faster than peptide bond formation on the ribosome; the latter rate is about 10 per sec. There is good biochemical evidence that the amino acid must be attached to the 3′ -OH group for peptide bond formation on the ribosome so the amino acid ends up in this position even if it was originally attached to the 2′ -OH.

Our knowledge of what happens to the tRNA on the ribosome (activities 3 and 4 of Table I) is rather meagre in spite of a great body of results in this field, but it is likely that with our increasing knowledge about the ribosome there will soon be some clarification.

After polypeptide chain initiation a series of peptide chain elongation steps occurs before the process of chain termination releases the completed protein.

The generally accepted scheme for peptide chain elongation which involves relative movement of the mRNA and ribosome is shown as a cyclic scheme for peptide bond formation in Figure 2. This scheme is simplified in that a static view of the ribosome involving special tRNA binding sites is assumed. At present there is no strong evidence to persuade us to drop this view and think in terms of a more dynamic situation involving activated binding states.

The cyclic scheme shown is quite self-explanatory, starting with a situation in state A with a growing peptide attached to $tRNA_n$ in the peptidyl-tRNA binding site (P-site) of a 70 S bacterial type ribosome decoding codon **n** of the mRNA and, an aminoacyl-$tRNA_{n+1}$ decoding codon n + 1 in the aminoacyl-tRNA binding site (A-site). The mRNA is bound to the 30 S subunit and the tRNA stretches across both 30 S and 50 S subunits. The peptide bond is made by the enzyme peptidyl

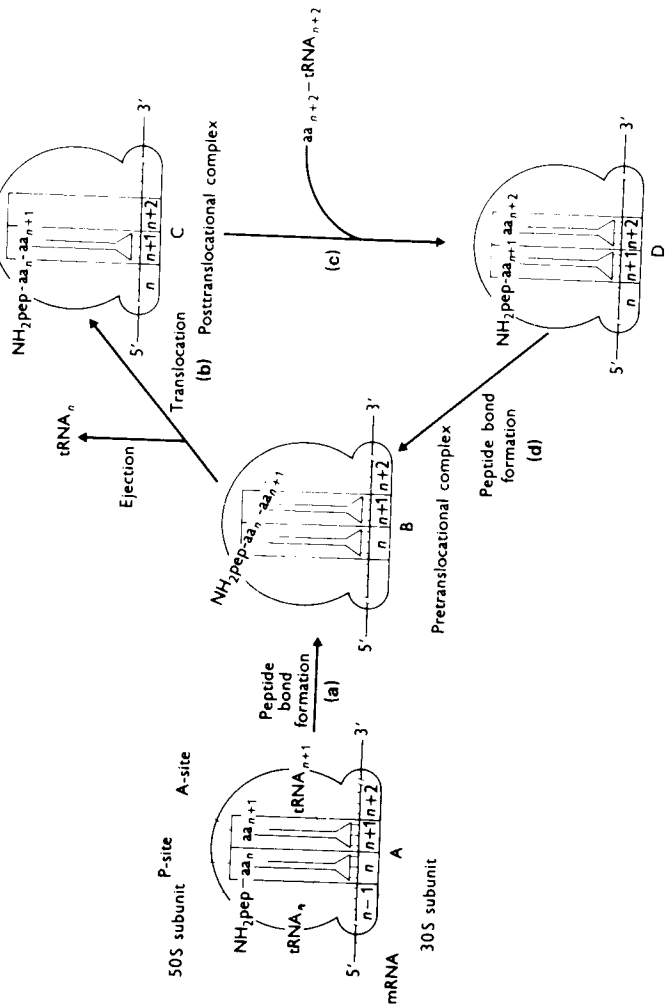

Fig. 2 Cyclic scheme for peptide bond formation on the ribosome in terms of two tRNA binding sites.

transferase on the 50 S subunit in step (a) leaving, in state B, an uncharged $tRNA_n$ in the P-site and a new peptide extended by one amino acid, aa_{n+1}, attached to $tRNA_{n+1}$ in the A-site. Movement of the $tRNA_{n+1}$ and mRNA occurs in step (b) to free the A-site state C for a new incoming aminoacyl-$tRNA_{n+2}$ in step (c). Step (b), involving movement of $tRNA_{n+1}$ with concomitant ejection of $tRNA_n$, is usually called translocation. What causes the ejection and relative motion is a stimulating area of research. When the new aminoacyl-$tRNA_{n+2}$ is bound in the A-site as in state D, the ribosome is back to a state equivalent to A ready for a new round of peptide bond formation and translocation giving the cyclic feature to the scheme.

Much work is in progress using physical chemical techniques, affinity labelling and chemical crosslinking to identify the protein and nucleic acid environments for the tRNAs bound in the different ribosomal sites.

Actually the situation is more complicated than in the simplified version of protein synthesis shown in Figure 2 because in this scheme the role of supernatant factors is ignored. Two protein elongation factors EF - T and EF - G are involved in the elongation step. It is believed that a subunit of EF -T called EF -Tu actually carries the aminoacyl-tRNA to the ribosomal A-site in the form of a ternary complex composed of aminoacyl-tRNA, GTP and EF - Tu. The cyclic scheme shown in Figure 3 is generally accepted (see a review by Miller and Weissbach, 1977) for the way in which EF - Tu plays a role in the elongation step. There is currently much research into the biochemistry and structure of EF - Tu.

EF - Tu provides a good contrast in the problem of specific recognition of aminoacyl-tRNA to an aminoacyl-tRNA synthetase because EF - Tu recognizes all aminoacyl-tRNAs whereas a synthetase recognizes at most a set of isoaccepting species. The details of how the ternary complex binds to the ribosome and how the EF - Tu:GDP is released after hydrolysis of the GTP are not known. However, the regeneration of EF - Tu:GTP is thought to be brought about catalytically by another protein factor, EF - Ts, which binds to EF - Tu to make the bimolecular EF - T. Interestingly there has been found a large amount of EF - Tu in bacterial cells - about 5% of total protein giving a concentration of about 0.3 mM which is of the same order as the aminoacyl-tRNA concentration. Perhaps the high amount of EF - Tu is needed to sequester the aminoacyl-tRNA during protein biosynthesis. Although no X-ray pictures of ternary complex crystals have yet been produced there is good progress towards solving the three-dimensional structure of EF - Tu:GDP.

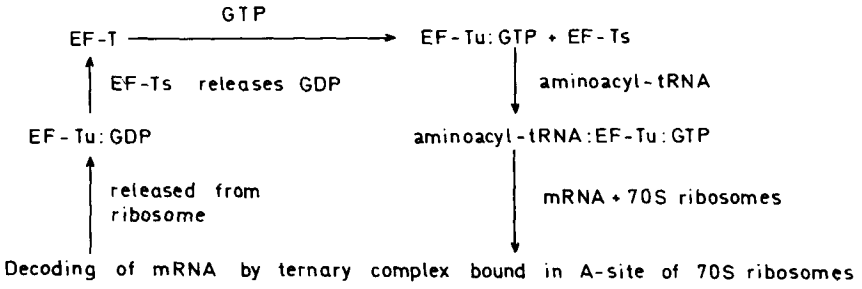

Fig. 3 Cyclic scheme for role of EF-Tu in protein biosynthesis.

A 6Å low resolution model has been described by Kabsch *et al.*, (1977) and a 2.7Å high resolution map showing the position of GDP and part of the polypeptide backbone has been reported by Morikawa and coworkers (1978).

Very little is known so far about how the tRNA could be complexed with EF - Tu (for review see Miller, 1978). However, an interesting speculation on the role of EF - Tu in providing a checking mechanism to increase fidelity of decoding has been proposed by Sprinzl and Cramer (1978). Based on some biochemical studies with analogues of normal aminoacyl-tRNAs to prevent aminoacyl migration, they have suggested that EF - Tu holds the aminoacyl-tRNA in the ternary complex with GTP so that the amino acid is linked to the 2′ -OH group of the 3′ -terminal A. Only after codon checking has taken place would aminoacyl migration occur prior to peptide bond formation.

Generalized Primary Structure of tRNA

The information from 101 different tRNA sequences (primary structures) known in July 1978 and listed in Table II (Barrell and Clark, 1974; Clark, 1977 and 23 new structures, Sprinzl *et al.*, 1978 and 10 structures, Sprinzl *et al.*, this volume) has been conveniently incorporated into standard 'cloverleaf' forms as shown in Figure 4. This remarkable feature of all the primary structures was first proposed by Holley and his colleagues (1965) and is based on Watson-Crick base pairing which forms double stranded helical stretches (the secondary structure). The simple classification shown in Figure 4 is based on size (see Table II for species).

Thus we have small and large tRNAs dependent upon the size of the extra arm (see also Figure 5 and Table II). The 10 new structures not listed by Sprinzl et al., (1978) are T4 Arg (Mazzara *et al.*, 1977)

TABLE II

Classes of tRNA according to Arm Sizes and Structure Correlations

Class 1 (86)	
A (50)	4 base pairs in b-stem (D stem) 5 bases in extra loop and containing m^7G
(and with A9)	Ec Ala_1, Sw Ala_1, Sw Ala_2, Ec Arg_1, Ec Asn, Ec Asp_1, Ec Gly_1, Ec Gly_3, Ec + Sal His_1, Ec Ile_1, Ec Lys, Bsu Lys_1, y Lys, Rbl $Lys_{2A(2B)}$, Rbl Lys_3, Svt Lys_4, An Met_f, Ec Met_m, y Met_3, Mye + Rbl Met_4, Ec Phe, Mp Phe, Bs Phe, Bsu Phe, y Phe, Wg + Ps Phe, Egc Phe, Bc Phe, Rbl + Hup Phe, T4 Pro, Ec Thr, Bsu Thr, T4 Thr, Ec + su^+ Trp, Ec Val_1, Ec Val_{2A}, Ec Val_{2B}, Bs Val_{2A}, Mye + Rbl + Hup Val_1.
(and with m^1G9 or G9)	R1 Asn, y Cys, Ec Met_{f1}, Ec Met_{f2}, Bsu Met_f, Tt Met_f, Nc Met_f, Mye + Rbl + St + X1 + Smg + Hup Met_f, yp Phe, y Trp, Chi + B1 + Rbl Trp_1.
B (7)	4 base pairs in b-stem (D stem) 5 bases in extra loop III without m^7G
	y Ala_1, Tu Ala_1, y Arg_3, Hay Lys, Mp Met_f, B1 + Rbl Trp_2.
C (10)	4 base pairs in b-stem (D stem) 4 bases in extra loop
	T4 Arg, y Asp_1, Ec Glu_1, Ec Glu_2, Ec + Sal Gly_1, Sta Gly, Wg Gly, y Gly, Sw Gly_1, Sw Gly_2.
D (19)	3 base pairs in b-stem (D stem) small extra arm with no. of bases (3-5)
	y Arg_2 (5), Ec Cys (4), Ec Gln_1 (5), Ec Gln_2 (5), T4 Gln (5), y Glu_3 (4) Ec Gly_2 (4), T4 Gly (4), Tu Ile (5), Ncm Met_f (4), Mul Pro (5) y Thr_{1A}(5), y Thr_{1B} (5), y + su^+ Tyr (5), Tu Tyr (5), y Val_1 (5), y Val_{2A} (5), y Val_{2B} (5), Tu Val (3)
Class 2 (15)	
	3 base pairs in b-stem (D stem) large extra arm with no. of bases (13-21)
	Ec + Sal Leu_1 (15), Ec Leu_2 (15), T4 Leu (14), y Leu_3 (13), y Leu_4 (13), Ec Ser_1 (16), Ec Ser_3 (21), T4 Ser (18), y Ser_1 (14), y Ser_2 (14), R1 Ser_2 (14), R1 Ser_3 (14), Ec + $su_3^+Tyr_1$ (13), Ec Tyr_2 (13), Bs Tyr (13).