Multi-author Reviews

Genetic Code 1990

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Genetic code 1990. Introduction

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I still vividly remember the morning when our microbiology teacher, Urs Leupold, entered the class waving a newspaper with the headline 'Genetic Code cracked'. A furious race between competing laboratories had come to its end, the 'Code Sun' (C. Bresch) had been established. This sun shone, brilliant and immaculate; each amino acid had been assigned its codon(s), and that was that. However, as in real life, slowly, and in exponential order, sunspots began to appear. The classical wobble rules were questioned, and more and more exceptions found, and, with some delay, also accepted. However, these deviations from the wobble rules did not excite the general public, it was rather something for specialists. This situation has now drastically changed. Nowadays we cannot always simply deduce the protein sequence from the sequence of a mature message. Ribosome and tRNA hopping, frameshifting, readthrough, and RNA editing have to be considered. Furthermore, it was discovered that the code was not as universal as originally thought. Some organisms and organelles (e.g. ciliates, mycoplasma, mitochondria, chloroplasts etc.) have some codewords of their own. The field has become more complicated, but at the same time has also regained the original excitement.

At the 1989 tRNA workshop in Vancouver, I asked some friends and colleagues whether they would find it useful to compile an issue on the 'Genetic Code 1990'; they were enthusiastic, and the result is now presented in this multi-author review. I have asked the authors to put more emphasis on a personal view than on complete coverage. Thus, the blame for any omissions should fall on the coordinator of this review and not on the authors.

The accuracy of aminoacylation – ensuring the fidelity of the genetic code

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Summary. The fidelity of protein biosynthesis rests not only on the proper interaction of the messenger RNA codon with the anticodon of the tRNA, but also on the correct attachment of amino acids to their corresponding (cognate) transfer RNA (tRNA) species. This process is catalyzed by the aminoacyl-tRNA synthetases which discriminate with remarkable selectivity amongst many structurally similar tRNAs. The basis for this highly specific recognition of tRNA by these enzymes (also referred to as 'tRNA identity') is currently being elucidated by genetic, biochemical and biophysical techniques. At least two factors are important in determining the accuracy of aminoacylation: a) 'identity elements' in tRNA denote nucleotides in certain positions crucial for protein interactions determining specificity, and b) the occurrence in vivo of competition between synthetases for a particular tRNA which may have ambiguous identity.

Key words. trNA; recognition; identity; aminoacylation; mischarging.

Research is a cyclical process. Fields develop and mature and create unified concepts. Fashion and research then move to other areas. Meanwhile, with the development of new methods the original concepts may be reexamined by novel and more incisive techniques which often give rise to findings which challenge accepted notions. In just such a way, the methods of molecular biology, gene synthesis, and in vitro genetics have rejuvenated the field of protein biosynthesis, where new views on the structure and action of ribosomes[3,37], tRNA function[3,46,51], tRNA identity (see below), and coding for 'new' amino acids (reviewed in this volume) have emerged. The arti-

cles in this issue represent an interesting cross-section of our evolving and expanding view of the genetic code.

The faithful transmission of genetic information relies on the accuracy of protein biosynthesis in matching the codon of the messenger RNA with the correct amino acid. This process is mediated by complex formation of the messenger RNA codon with the anticodon of the aminoacyl-tRNA. Crucial to this scheme is the correct attachment of amino acids to their cognate tRNA species, a process whose underlying principles are sometimes unfortunately referred to as the 'second genetic code'[7, 59]. This aminoacylation reaction is catalyzed by a family of enzymes, aminoacyl-tRNA synthetases, which recognize specifically the amino acid and the cognate tRNA species[44, 53]. The basis for this highly specific recognition of tRNA by these enzymes (also referred to as 'tRNA identity') is currently being elucidated by genetic, biochemical and biophysical techniques. I would like to summarize briefly developments which have given a first insight into the processes by which tRNA molecules are specifically recognized by aminoacyl-tRNA synthetases.

Transfer RNA

Most cells contain 50–70 different tRNAs, the sizes of which range from 75 to 93 nucleotides except for some mitochondrial tRNAs which are slightly smaller. In *E. coli*, for example, there are 45 different tRNA species[23]. Generally there is more than one tRNA species for each amino acid used in protein synthesis. Leucine, for example, has 5 isoacceptor species in *E. coli*[56]. The sequences of several hundred tRNAs from many sources have now been established[54]. Although the sizes of tRNAs vary, they can all be folded into a common cloverleaf secondary structure (fig. 1 A). All tRNAs contain a common sequence (... CCA) at their 3'-end, the acceptor end. In addition, there are certain nucleotides which are highly conserved between different tRNAs (fig. 1 A). Most of these residues are involved in tertiary base-pairing interactions which stabilize the 3-dimensional structure of the tRNA. Transfer RNA is so far the only class of RNA molecules whose whole structure has been characterized by X-ray crystallography. All tRNA molecules form the characteristic L-shaped structure (fig. 1 B) in which the anticodon loop and the acceptor end are the furthest apart.

Aminoacyl-tRNA synthetases

Aminoacyl-tRNA synthetases are a structurally diverse family of enzymes[44, 53]. Each enzyme recognizes three substrates, the cognate amino acid, ATP and one or more isoacceptor RNAs. The enzymatic reaction is a two-step process; amino acid activation (measured by ATP-PP_i exchange) can be experimentally separated from aminoacyl transfer to tRNA[44, 53]. The amino acid is esterified to either the 2'- or 3'-hydroxyl group of the terminal adenosine depending on the nature of the synthetase[14]. Although all aminoacyl-tRNA synthetases perform the same reaction, i.e., the synthesis of aminoacyl-tRNA, they vary in molecular mass (from 59 to 380 kDa) and

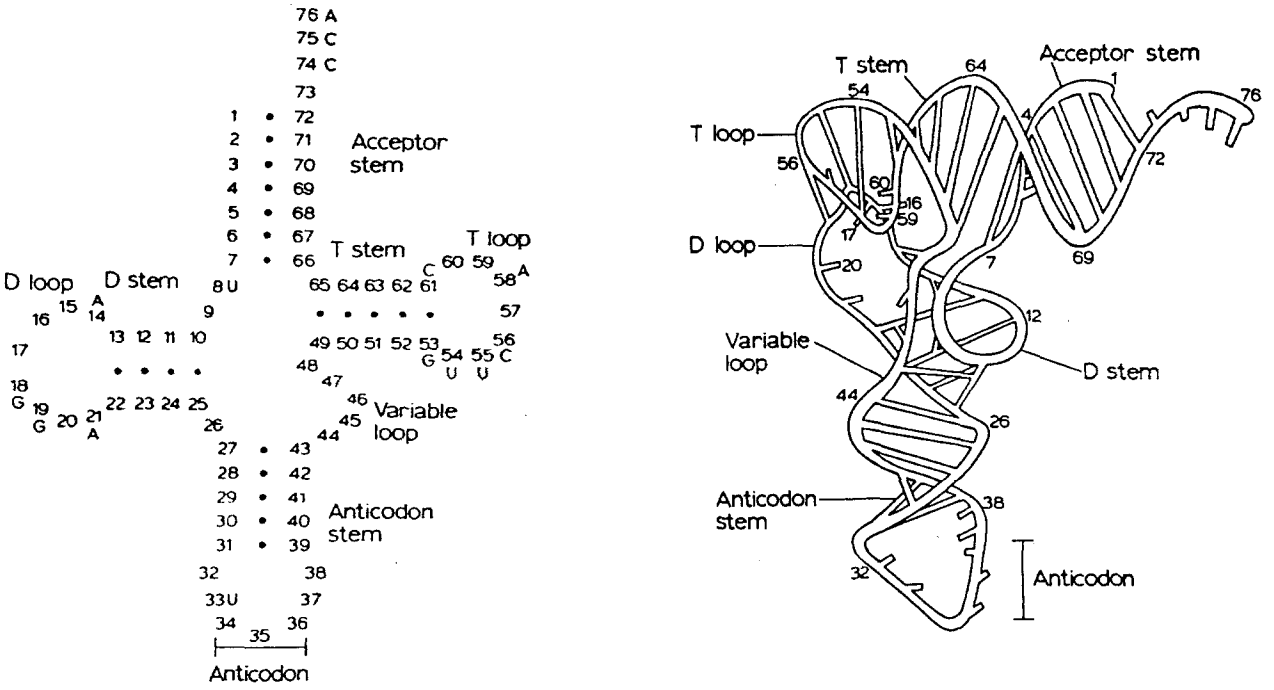

Figure 1. Secondary and tertiary structure of tRNA. (Left) Invariant nucleotides in the cloverleaf structure and base pairing (dots) are indicated. (Right) The L-shaped model of tertiary structure. Adapted from Normanly and Abelson[31].

quaternary structure features (α, α_2, α_4, α_2 β_2) which may be important to other roles these enzymes play [e.g. 'splicing'[1], amino acid transport[60]]. Recent X-ray crystallographic analysis of a number of aminoacyl-tRNA synthetases and computer comparison of amino acid sequences have revealed structural similarities between aminoacyl-tRNA synthetases which have led to the classification of this family of enzymes into two groups[9]. A major difference between the groups involves the protein motif which is responsible for ATP binding; a Rossmann fold in one case, while the other group possesses an ATP binding domain which has not been encountered previously. This suggests that the active site domains of aminoacyl-tRNA synthetases may have evolved from different ancestors. In addition, the division correlates with the position of aminoacylation at the 2'- or the 3'-hydroxyl of the tRNA's terminal adenosine[14].

The accuracy of aminoacylation depends on the precise discrimination by the aminoacyl-tRNA synthetases against similar amino acids and especially against structurally very similar tRNA molecules. For instance, *E. coli* leucyl-tRNA synthetase is able to discriminate between 45 different tRNA species and recognizes and charges specifically 5 leucine acceptor RNAs with leucine. Thus, these enzymes may have intricate proofreading mechanisms (reviewed in Freist[11]). The high specificity of these enzymes towards their substrates has attracted much experimental attention. The most detailed studies regarding the binding of ATP and amino acid were based on the known crystal structure[28] of *Bacillus stearothermophilus* tyrosyl-tRNA synthetase. Protein engineering experiments involving residues indicated by the structure to be critical gave some information on the binding of ATP and tyrosine (summarized in ref. 24), although there is some doubt about the interpretation of these results based on a refined crystal structure[5]. However, as the tRNA binding site of this enzyme was disordered in the crystal structure[4], the only detailed information on the binding of tRNA has come from the study of the co-crystal of *E. coli* glutaminyl-tRNA synthetase with $tRNA^{Gln}$[40].

E. coli glutaminyl-tRNA synthetase (GlnRS)

For the past decade our interest has focused on the *E. coli* GlnRS and its interaction with tRNA. The enzyme, a small monomer of 553 amino acids[57], belongs to a small subgroup of synthetases (including Arg, Glu, and Gln) which do not display the two-step mechanism of most other synthetases, the clear separation of amino acid activation (measured by ATP-PP_i exchange) and aminoacyl transfer to tRNA[44, 53]. Based on our current knowledge this feature seems to be conserved in evolution, as in all organisms studied so far these three small, monomeric enzymes require the presence of tRNA for ATP-PP_i exchange. Because of this property, one may expect to see a different arrangement of binding sites along the linear amino acid sequence of GlnRS compared to enzymes such as leucyl-tRNA synthetase[39] or alanyl-tRNA synthetase[21] with their linear, modular arrangement of tRNA and ATP/amino acid binding domains. There are a number of reasons why the GlnRS system is appealing to experimental work aimed at studying the process of tRNA selection. GlnRS is the only enzyme thus far for which mutants (mischarging mutants) exist with altered tRNA discrimination[19, 35]. Since GlnRS is linked to selectable phenotypes by virtue of being required to aminoacylate suppressor tRNAs[8, 34], there is a great deal of flexibility in the selection of mutants and revertants to fine-tune the analysis of the molecular nature of tRNA : synthetase interactions. The small size of the enzyme and the ability to produce a large amount of enzyme and tRNA from cloned genes[36] has allowed biophysical studies of the GlnRS : $tRNA^{Gln}$ complex by X-ray crystallographic analysis to near atomic resolution[35, 40]. As such, the *E. coli* GlnRS-$tRNA^{Gln}$ system is one of the best studied tRNA/synthetase systems to date and has yielded significant information on the question of tRNA recognition.

The GlnRS : $tRNA^{Gln}$ complex

The establishment of the high resolution crystal structure of *E. coli* GlnRS complexed with $tRNA^{Gln}$ and ATP[40] was a major step towards our understanding of the process of tRNA discrimination. The enzyme is made up of several domains (fig. 2). The active site which binds ATP and glutamine is made up of a nucleotide binding fold motif which includes the amino acid sequence 'HIGH' common to some aminoacyl-tRNA synthetases. This domain is structurally similar to the amino acid binding domains of *E. coli* methionyl-tRNA synthetase[63] and *B. stearothermophilus* tyrosyl-tRNA synthetase[5]. The tRNA in the complex is bound in the well-known L-shape (fig. 2) with the inside of the tRNA in contact with the protein. The 3'-terminal CCA sequence is hairpinned back (by G_{73} binding to the phosphate of A_{72}) and thereby breaking the U_1–A_{72} base pair. A similar opening of the terminal base pair was observed upon binding $tRNA^{Met}$ to its cognate *E. coli* synthetase as judged by fluorescence energy transfer studies[10]. Interactions that may play a role in specific recognition are found at the anticodon and acceptor stem region of the tRNA[40]. Refinement of the structure from X-ray crystallography will provide a basis for further experiments to distinguish nucleotides in $tRNA^{Gln}$ that provide specific contacts with GlnRS and those common to other tRNA/protein or tRNA/synthetase interactions.

Transfer RNA mischarging in E. coli

Although tRNA recognition by synthetases is remarkably specific, mischarging, the acylation of the wrong amino acid onto tRNA, was shown to occur both in vivo

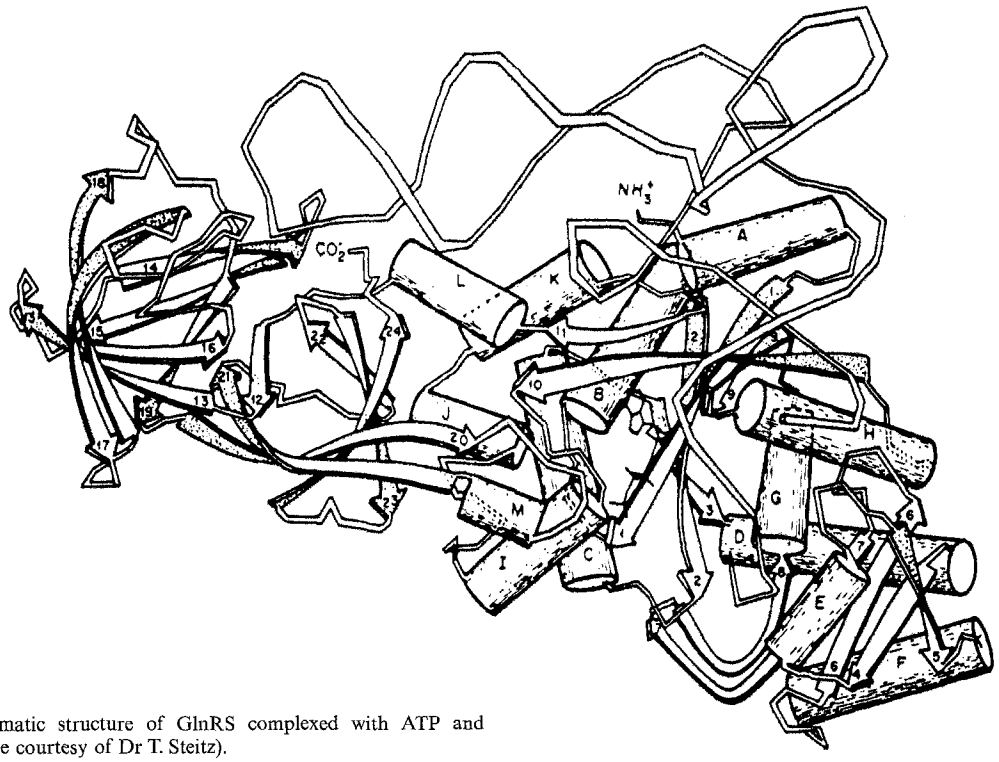

Figure 2. Schematic structure of GlnRS complexed with ATP and $tRNA^{Gln}$ (figure courtesy of Dr T. Steitz).

and in vitro in studies involving mutant tRNAs and wild-type or mutant GlnRS enzymes[19, 35, 55, 58]. Recently it came to light that the misacylation phenomenon is not restricted to GlnRS as mischarging by lysyl-tRNA synthetase has also been observed (see below).

How is mischarging detected? The early experiments[12, 15, 18, 50, 52] have relied on a genetic selection which utilizes amber suppressor tRNAs and amber mutations specific for a given amino acid. Thus, mischarging with glutamine of a mutated tyrosine amber suppressor tRNA *(supF)* was detected by suppression of certain amber mutations. For instance, suppression of the amber mutation $lacZ_{1000}$ (in the gene for β-galactosidase) yields active β-galactosidase only by glutamine insertion (mediated by the *E. coli supE* suppressor). The insertion of tyrosine *(supF)*, leucine *(supP)*, or serine *(supD)* into the polypeptide chain will not produce an active enzyme[34]. Therefore, this spectrum of suppression can be used as an assay for mischarging by GlnRS. A modern in vivo assay relies on the amino-terminal protein sequencing of mutated *E. coli* dihydrofolate reductase (DHFR). The gene for the mutant protein was engineered to have an amber codon at position 10[32]. In vivo suppression of the amber codon in *E. coli* strains carrying a suppressor tRNA gene will then give an enzyme with the amino acid at position 10 corresponding to the amino acid(s) charged on the suppressor tRNA. This approach is currently used for many of the in vivo identity experiments discussed below.

Mischarging caused by mutant tRNA genes. In classical experiments with the $lacZ_{1000}$ and amber mutations in other genes, mutations in suppressor tRNA genes were selected which could restore the function of the gene containing the amber mutation. In this way, it was determined that a $tRNA^{Tyr}$ and a $tRNA^{Trp}$ species could be mutated to glutamine acceptance. Sequence analysis showed that single base changes in the acceptor stem of the *supF* $tRNA^{Tyr}$ and in the anticodon of the *supU* $tRNA^{Trp}$ were sufficient to alter the 'identity' of these tRNAs to glutamine (fig. 3). In several cases the tRNAs still retained recognition by tyrosyl-tRNA synthetase and thus were dual identity molecules. Additional in vivo genetic experiments suggested that changes in the acceptor stem are important for recognition by other aminoacyl-tRNA synthetases (see Murgola[30]). The advent of gene synthesis techniques made possible the challenging approach of synthesizing amber suppressor tRNA genes with specificity for all 20 amino acids. This was done by artifical gene construction of normal tRNA genes with mutations in the anticodon to make the CUA amber suppressor tRNA anticodon. To date, 16 suppressors have been generated and shown to be viable. The others may not be good suppressors because of the altered anticodon context[62]. The fidelity of in vivo aminoacylation of these tRNAs was assessed in the DHFR test which showed that a number of these molecules were mischarged with glutamine or lysine (table 1). Thus, the anticodon is important for identity of $tRNA^{Lys}$ and $tRNA^{Gln}$.

Mischarging caused by mutant aminoacyl-tRNA synthetases. So far the only aminoacyl-tRNA synthetase mutants with altered tRNA discrimination derive from

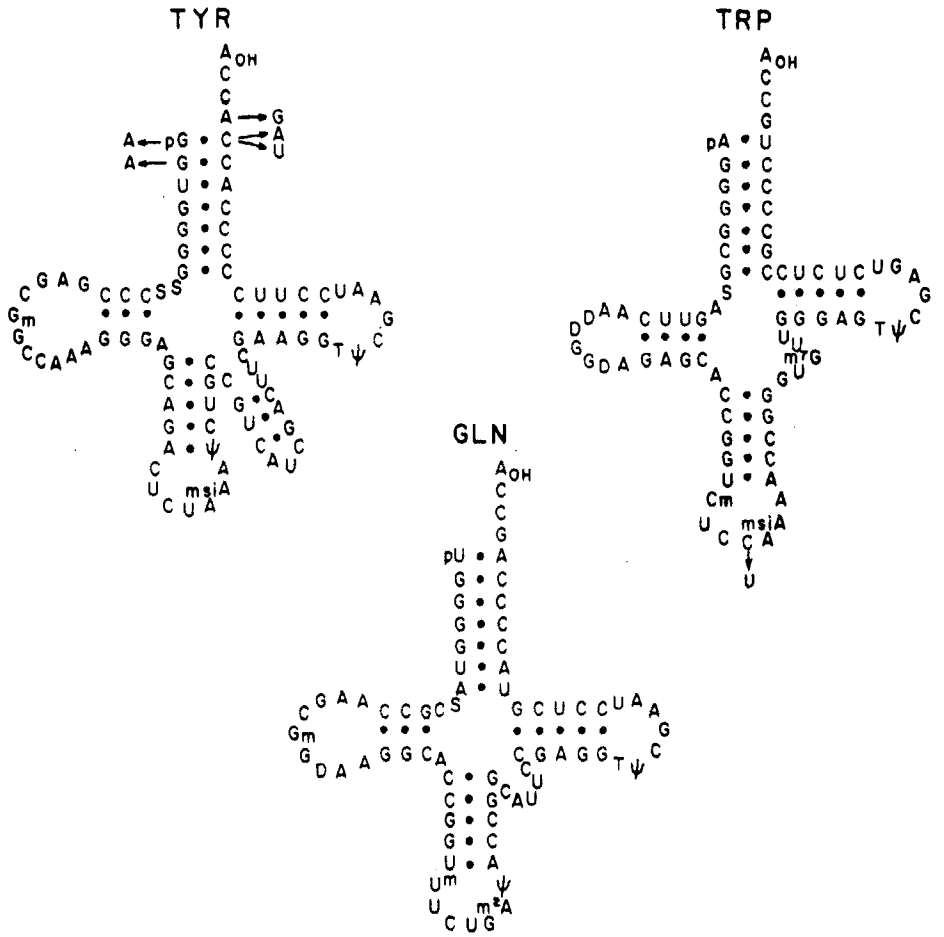

Figure 3. Mutations leading to Gln mischarging. The arrows indicate the nucleotide substitutions in certain positions of $tRNA^{Tyr}$ and $tRNA^{Trp}$ that allow these tRNAs to be charged with glutamine.

Mischarging of synthetic amber suppressor tRNAs

With glutamine	With lysine
Ile_1	Ile_2
Gly (*glyT* gene)	Arg
Met_1	Met_m
Glu	Asp
Trp	Thr
	Val

Correct aminoacylation was seen with tRNAs specific for: Ala, Cys, Gln, Gly (*glyU* gene), His, Leu, Lys, Phe, Pro, Ser, Tyr. Adapted from Normanly and Abelson[31].

E. coli GlnRS. The availability of the $lacZ_{1000}$ selection for mutants of *supF* mischarged with glutamine prompted the search for mutants of GlnRS that can misacylate *supF*. Using this approach three mutant alleles of *glnS* were isolated in vivo[19,35]. These mutants *(glnS7, glnS10, glnS15)* were cloned and sequenced and shown to result from single base changes. The corresponding amino acid changes were Asp235→Asn235 *(glnS7)*, Asp235→Gly235 *(glnS10)*, and Ile129→Thr129 *(glnS15)*. Since these experiments showed that position 235 was very important, additional mutants were made in vitro by substituting a variety of different amino acids (e.g., Glu, Lys, Ala, Val)[58]. Most of these mutant enzymes could misacylate tRNAs in vivo as shown by β-galactosidase assays. In addition, all these mischarging enzymes can still charge $tRNA^{Gln}$ in vivo and in vitro. The enzymes encoded by the mutant *glnS* genes were purified and shown to aminoacylate with glutamine a select number of *E. coli* tRNA species albeit with very much lower efficiency. The crystal structure of the GlnRS:$tRNA^{Gln}$ complex makes the importance of Asp235 clear. The carboxyl side chain of this amino is vital in binding to G_3 and via a bound water molecule also to G_2[35]. This interaction is different when the carboxyl group is 'lost' in the amino acid replacements Gly or Asn. Based on the knowledge of the structure of the GlnRS:$tRNA^{Gln}$ complex[40], directed in vitro mutagenesis studies can now be performed to generate more mischarging mutants.

Mischarging of normal tRNAs caused by wild-type aminoacyl-tRNA synthetases. In order for the cell to survive, the error rate in vivo in protein biosynthesis obviously must be very low. However, there is a finite rate; it has been estimated that valine is incorporated instead of isoleucine in vivo in a ratio of 1:3000[25]. Thus, it was not

surprising to discover during the characterization of the mutant *glnS* alleles that the mischarging in vivo phenotype could also be caused by overproducing the wild-type *glnS* gene product. This indicates that when *E. coli* overproduces wild-type GlnRS it can apparently misacylate *supF* tRNA (we consider naturally-occurring suppressor tRNA as normal tRNAs). This phenomenon has not been observed to date in other aminoacyl-tRNA synthetase systems. Competition between synthetases in vivo was also shown to help the discrimination of Gln/Ser[38] and Ala/Tyr[17]. There is a likely explanation for this phenomenon. It is assumed that in vivo aminoacyl-tRNA synthetases and tRNAs are complexed. In *E. coli* (and presumably in other cells too) the cognate pairs of macromolecules exist in a well-defined ratio[20]. Thus, in the normal situation in vivo most of the GlnRS and $tRNA^{Gln}$ molecules are complexed with each other. However, elevation of the GlnRS levels in the cell results in un-complexed GlnRS molecules that are free to interact with non-cognate tRNAs and thus cause mischarging. If this notion is correct, then concomitant overproduction of the cognate $tRNA^{Gln}$ would restore the ratio of complexed GlnRS with cognate tRNA and abolish mischarging. Similarly, overproduction of TyrRS at the same time as GlnRS would complex more completely *supF* tRNA which would then no longer be available for mischarging by GlnRS. Experiments confirmed these predictions[55]. This means that an important factor in maintaining accuracy of aminoacylation in vivo is the ratio of synthetase to its cognate tRNA.

To maintain this accuracy, the level of each aminoacyl-tRNA synthetase must be regulated within certain limits, as the presence of excess enzyme can act on uncomplexed non-cognate tRNA. The regulation may be more important for GlnRS than for other synthetases, as GlnRS is unusual in that the ratio of GlnRS:$tRNA^{Gln}$ is only about 1:1, which is much lower than the tRNA:synthetase ratios of other parallel tRNA:synthetase systems studied to date in *E. coli*[20]. Therefore, the unusually low ratio of synthetase:tRNA molecules for the glutamine system may account for the observed mischarging by overproduction of GlnRS, and explain why so many mutant tRNAs are aminoacylated by GlnRS in vivo (summarized in Normanly and Abelson[31]).

Identity of tRNA

A particularly active current topic of investigation concerns 'tRNA identity', the question of which positions/bases in tRNA determine the specific interaction with the cognate aminoacyl-tRNA synthetase[31]. These elements may determine positive or negative interactions. The results of the genetic line of experimentation were presented above. The application of chemical/biochemical techniques in resectioning tRNAs has shown that the anticodon is important for both methionine and glutamine identity in *E. coli*[47]. In a different approach computer analysis of tRNA sequences was used to determine which positions in tRNA might correspond to those of the identity elements in various acceptor RNAs[27]. Upon mutagenesis of the appropriate genes some of the predictions were shown to be significant[26]. More recently, the facile production of in vitro transcribed unmodified tRNA molecules[16,26,32,42,48] and the fact that unmodified tRNA can actually be aminoacylated very well[43] has really spurred the field. By making a large set of mutants, it was shown for $tRNA^{Phe}$ that a small number of identity elements are required for specific recognition by yeast phenylalanyl-tRNA synthetase[42]. These studies are being extended currently to other cognate systems. However, a different and unexpected result emerged from studies with a minor *E. coli* $tRNA^{Ile}$ species[29]. In this tRNA the first anticodon base (C) can be modified with lysine to create the modified nucleotide lysidine. The modified tRNA can specifically recognize the isoleucine codon AUA and can be aminoacylated with isoleucine. However, if the tRNA contains the unmodified C it can be aminoacylated with methionine and must also recognize the AUG codon. Thus, a post-transcriptional nucleotide modification determines both codon specificity and tRNA identity. The fact that nucleotide modification is important to tRNA identity (in one case so far) may limit the use of unmodified T7 RNA polymerase transcripts of tRNA genes in such studies.

A number of conclusions have emerged so far: a) it is clear that the anticodon is an important recognition element for many synthetases, although some of them (e.g., $tRNA^{Ala}$) do not use this feature, b) the 'discriminator base' (the fourth nucleotide from the 3'-end of the tRNA[6], is also important for recognition of a number of tRNAs, and c) the position of other identity elements varies between different acceptor RNAs.

Based on the available genetic[15,38,50,61], biochemical[49], and biophysical[40] studies the probable view of the nucleotides of $tRNA^{Gln}$ important for recognition by GlnRS is given in figure 4. As discussed above the acceptor stem and the anticodon are the two major regions shown thus far to be involved in specific interaction with the enzyme. The nucleotide modification at position 34 in $tRNA_1^{Gln}$ was shown by chemical modification[49] to be essential for aminoacylation with GlnRS. The importance of the discriminator nucleotide G_{73}[6] was first recognized in genetic experiments[38]; in the structure of the tRNA:synthetase complex this nucleotide participates in a sequence-specific interaction with the acceptor stem of tRNA[40]. Biochemical studies of aminoacylation with both mutant tRNAs and mutant enzymes are needed to determine the contribution of the individual changes to the observed specificity.

Outlook

How common is mischarging in nature in vivo and how can it be corrected? Low levels of misacylated tRNAs are

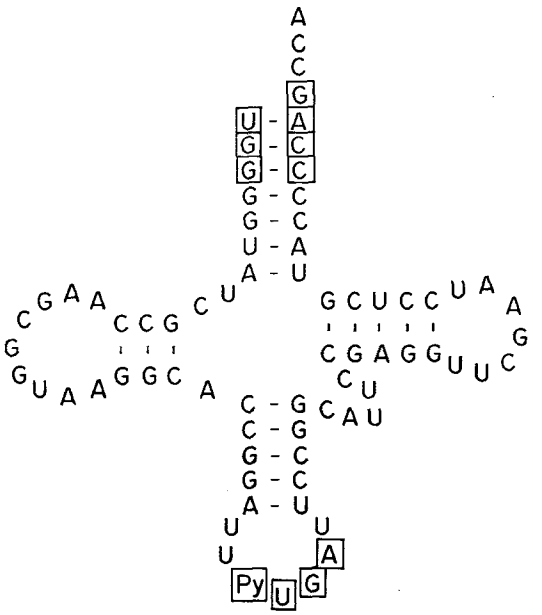

Figure 4. Identity elements in $tRNA^{Gln}$. The sequence shown is of $tRNA_2^{Gln}$ in cloverleaf form; the nucleotides boxed are implicated in identity.

tolerated in *E. coli* as the occurrence of missense suppression demonstrates [13]. However, mischarging of tRNA to a greater extent is lethal to the cell as it interferes with the correct production of proteins. But there may be vestiges of older, less precise synthetase systems present today. One way of ensuring correct charging is to build into aminoacyl-tRNA synthetases specific mechanisms for error correction. Thus, in an ATP-dependent step, non-cognate aminoacyl-tRNAs are hydrolyzed before release of the incorrectly aminoacylated product from the enzyme [11] or in an editing mechanism in which the non-cognate amino acyl-adenylate is hydrolyzed prior to acylation onto tRNA [2]. Conformational changes upon amino acid binding and aminoacyl-adenylate formation may also contribute to the accuracy of aminoacylation [41].

There is a well-established case in nature for mischarging in the glutamine system. In gram-positive eubacteria, archaebacteria as well as in organelles there is no detectable GlnRS activity [45]. Gln-$tRNA^{Gln}$ is formed by amidation of Glu-$tRNA^{Gln}$ which is synthesized by glutamyl-tRNA synthetase in a misacylation reaction of $tRNA^{Gln}$ with glutamate. Therefore, in these organisms glutamyl-tRNA synthetase is a naturally-occurring mischarging enzyme. It then seems likely that the mischarging and amidation pathway preceded the pathway of direct formation of Gln-$tRNA^{Gln}$, as the presence of GlnRS activity is limited to gram-negative eubacteria and the cytoplasm of eukaryotic cells [45]. It will be interesting to compare the recognition of the tRNA-dependent amidotransferase and of the mischarging glutamyl-tRNA synthetase with what we have learnt about the recognition process of *E. coli* GlnRS.

Research in the field of aminoacyl-tRNA synthetase: tRNA recognition is again vigorous and active. By now all *E. coli* aminoacyl-tRNA synthetases have been cloned and all *E. coli* tRNA genes have been characterized. The determination by biochemical and genetic techniques of the number and nature of these identity elements and the individual contribution of each position/base to the overall process of discrimination will be a fascinating study, especially with more crystal structures of aminoacyl-tRNA synthetases ready to be solved in the near future. Given the multifaceted experimental approaches currently underway the identity of all acceptor RNAs in *E. coli* very likely will be determined soon. The interesting questions are: Is there a general mechanism for identity/recognition or are the various tRNA/synthetase systems different? Is there a common ancestor to tRNA binding domains in synthetases? Is tRNA identity conserved among organisms? Was aminoacylation always so specific? How did it evolve and will it tell us about the evolution of this class of enzymes and of tRNA? What are the principles of RNA-protein recognition which emerge from this work? The answer to these questions may be relevant to the understanding of other RNA-protein recognition processes.

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