Testing the viability of the Ambiguous Intermediate mechanism of codon reassignment with *in vitro* experiments: A proposal

Abstract

Different evolutionary mechanisms have been proposed to explain changes in the genetic code that have been observed in several mitochondrial and a few nuclear genomes. One of them is the Ambiguous Intermediate (AI) mechanism which argues that the codon reassignment process goes through a transient intermediate stage in which the codon is read ambiguously as two distinct amino acids. In cases where AI seems to be a plausible mechanism, the evidence for it can only be inferred indirectly. In this proposal we suggest some *in vitro* experiments to test the viability of the AI mechanism. These experiments rely on differential reaction rates for the various steps in the translation process to distinguish between the effectiveness of the two alternative modes of decoding in the AI stage. We further argue that some of the reassignments of a sense to a stop codon may have occurred as a consequence of low forward reaction rates and premature peptide release resulting from a mispairing between the codon and the anticodon. Finally, we discuss how these *in vitro* experiments can also be used to shed light on the Unassigned Codon (UC) mechanism, which is another possible mechanism of codon reassignment.

Introduction

Understanding the evolution of the genetic code has been a challenging area of research (Osawa and Jukes 1989, Shultz & Yarus 1996, Knight *et al.* 2001a, 2001b Santos *et al.* 2004) in molecular evolution over the past three decades. A codon reassignment would bring about changes in the amino acid sequence of every protein that uses that codon and is expected to be deleterious. It is therefore difficult to understand how a codon reassignment can become fixed in the population. Despite this perception, several examples of codon reassignments have been observed, primarily in mitochondrial genomes, but also in some nuclear and bacterial genomes. Several mechanisms have been proposed to explain codon reassignments. Earlier, we have shown (Sengupta & Higgs 2005) that the different mechanisms of codon reassignment can be integrated into a single gain-loss framework. This framework allows for four distinct mechanisms of codon reassignment depending on the sequence of gain (creation of a new tRNA or modification of an existing tRNA leading to the ability to recognize a new and previously non-cognate codon) or loss (deletion of a tRNA or modification of an existing tRNA leading to change in codon recognition capacity) events. In the Codon Disappearance (CD) (Osawa and Jukes 1989), the codon disappears from the genome *before* the gain/loss event occurs. The Ambiguous Intermediate (AI) mechanism (Schultz and Yarus 1994,1996) is characterized by the gain preceding the loss, while the Unassigned Codon (UC) mechanism (Yokobori *et al* 2001; Sengupta & Higgs 2005; Sengupta *et al* 2007) is characterized by the loss preceding the gain. All these mechanisms are characterized by the presence of an intermediate state where the gain (for AI) or loss (for CD) is fixed in the population. In contrast, for the Compensatory Change (CC) mechanism (Kimura 1985; Higgs 1998; Sengupta & Higgs 2005), the gain or loss never gets fixed in the population. Gain only (or loss only) states are selectively deleterious and remain at low to moderate frequencies in the population and when a subsequent compensatory loss (or gain) occurs, the gain in fitness can be large enough for the progeny of that individual to sweep through the population and go to fixation. In a recent paper (Sengupta *et al* 2007), we analyzed the different mechanisms of codon reassignment in mitochondrial genomes. By locating the exact position of the reassignment on a phylogenetic tree and correlating it with codon usage and tRNA content, we were able to infer the mechanism of reassignment in many of the cases.

In deciphering the mechanism of change leading to alternative genetic codes, the AI and UC mechanisms could only be inferred indirectly after ruling out the CD mechanism. In the case of the UC mechanism, the absence of a cognate-tRNA specific to a codon in some lineages, closely related to the ones which have reassigned that codon, can act as an indicator of the plausibility of the UC mechanism (Sengupta, Yang & Higgs 2007). However, no such indicator exists for the AI mechanism. (The only exceptions correspond to the cases where evidence for ambiguous translation exists as in *Bascillus subtilis* and some *Candida* species). In view of the lack of direct evidence for the AI mechanism, it would be interesting to test the viability of ambiguous translation using *in vitro* experiments. We propose that the in vitro experiments used to study fidelity of the translation process can easily be adapted to test the viability of the AI mechanism.

Processes ensuring translational fidelity

The accuracy of the translation process depends upon the proper recognition of a codon by its cognate tRNA and the rejection of a non-cognate or mischarged tRNA which would insert a "wrong" amino acid into the sequence. Every organism has developed sophisticated proof-reading mechanisms (Ibba & Soll 1999, Ling, Reynolds & Ibba 2009) to ensure translational fidelity. Since, both the AI and the UC mechanism involve the recognition of (and pairing with) a codon by a previously non-cognate tRNA, the experimental techniques (Rodnina & Wintermeyer 2001, Gromadski & Rodnina 2004, Cochella & Green 2005a, 2005b, Gromadski, Daviter & Rodnina 2006, Zaher & Green 2009) developed to understand miscoding and error correction during translation can be used to get insights into the viability of the AI and UC mechanisms.

Several conditions need to be fulfilled to ensure accurate translation. These involve the accurate recognition and acylation of isoacceptors tRNA's by the corresponding aaRS; competition between cognate and non-cognate tRNA's to arrive at the ribosomal A-site; accurate recognition and pairing between the codon of the mRNA and the anticodon of the cognate tRNA at the ribosomal A-site (with rate k_2); GTP activation/hydrolysis (rate k_3) or dissociation (rate k_{-2})of the non-cognate tRNA from the A-site, accommodation (rate k_5) and peptide transfer (rate k_{pep})

between the amino acid attached to the tRNA at the A-site and the partially formed peptide chain attached to the tRNA at the P-site and finally the accurate recognition of the stop codon by the appropriate RF protein leading to the hydrolysis of the fully-formed polypeptide from the tRNA at the P-site, leading to termination (Schmeing & Ramakrishnan, 2009) of translation.

Translation errors are usually rectified by a combination of mechanisms referred to as editing, kinetic proofreading and induced fit (Hopfield, 1974, Rodnina & Wintermeyer, 2001). Editing prevents misacylation of tRNA's. Kinetic proofreading (Gromadski & Rodnina, 2004, Gormadski, Daviter & Rodnina 2006) inhibits non-cognate tRNA's from binding to the codon in the mRNA and the induced-fit mechanism (Pape, Wintermeyer & Rodnina, 1999) ensures that cognate tRNA's undergo GTP hydrolysis and peptidyl transfer reactions with a greater rate than non-cognate tRNA's. Conformational changes at the ribosome and in the cognate tRNA are induced by the correct pairing between the codon and the anticodon and this favours GTPase activation and hydrolysis, accommodation and peptidyl transfer reactions. In contrast, conformational changes at the ribosome and the non-cognate tRNA brought about by mispairing between the codon and the anticodon decrease the forward reaction rates and increase the rejection rates (k_{-2}, k_7) of the non-cognate ternary complex from the ribosome. All these mechanisms of error correction prevent the insertion of a wrong amino acid into the peptide sequence. Recently, Zaher and Green (2009) discovered a mechanism which retrospectively corrects a wrong amino acid insertion (due to codon-anticodon mispairing) by inducing the premature release and degradation of the partially-formed erroneous peptide sequence. They found that the release factor RF2 catalyzed premature release rate at the A-site sense codon was significantly larger when mispairing resulted in the wrong amino acid insertion. Their experiments suggest that RF2 induced premature release of the partially formed erroneous peptide sequence from the ribosome acts as the final proof-reading step in ensuring translational fidelity.

Testing the AI mechanism

The AI mechanism is interesting because it appears to avoid the typical translation errors which can be corrected by editing, kinetic proofreading and induced fit but can nevertheless result in

the insertion of a "wrong" amino acid. This is because the new cognate tRNA which gains the ability to translate an originally non-cognate codon usually does so by a mutation or base-modification at the first anti-codon position. Such a change does not lead to mischarging of the tRNA. Secondly, wobble pairing between the first anticodon position and the third codon position is permissible and rules out mispairing error between the codon and the anticodon.

Sense to Sense Reassignments

As a specific example consider the reassignment of AAA: Lys to Asn observed in Echinoderms (Tomita *et al* 1999a) and Platyhelminthes mitochondrial genomes. After reassignment, the new cognate tRNA-Asn has the anticodon GUU. Since G.A wobble pairing is sometimes allowed (Yokobori *et al* 2001), the tRNA-Asn(GUU) can decode AAA as Asn. In the case of some Echinoderms, this pairing is facilitated by the additional modification of the second anticodon base from U to Ψ (pseudouridine). Moreover, tRNA-Lys has the anticodon CUU (Castrasena *et al.* 1998) which can correctly pair with the AAG codon only and precludes its pairing with the AAA codon. It is interesting to note that the base at the 37'th position of the tRNA-Lys(CUU) is still t^6A (N^6-threonylcarbamoyladenosine) instead of A (Tomita *et al* 1999a, Yokobori *et al* 2001) which can sometimes enable C.A mispairing at the wobble position even though in this case its presence appears to be insufficient to allow tRNA-Lys(CUU) to decode AAA as Lys.

Before reassignment, the only tRNA-Asn has the anticodon QUU can decode AAU and AAC only as Lys. Q is a Queuosine modification of G at the first anticodon position of tRNA-Asn which suppresses the affinity of the tRNA-Asn(QUU) for the AAA codon (Morris *et al.* 1999). There exists only one tRNA-Lys with anticodon UUU which can pair with both AAA and AAG codons and decode them as Lys. The structural ramifications of base modifications on codon-anticodon pairing and its effect on the translation process have recently been worked out in great detail (Phelps *et al* 2002a, 2002b; Murphy *et al* 2004). The presence of t^6A at the 37'th position of tRNA-Lys(UUU) is insufficient for it to decode the AAG codon and also hampers A- to P-site translocation (Phelps *et al* 2002b). An additional modification of the base at the 1'st anticodon position from U to 5-methylaminomethyluridine (mnm^5U) is necessary for the tRNA-Lys(mnm^5UUU) to successfully decode AAG as Lysine. These studies indicate that an increase

in the number of contacts between the decoding centres in the ribosome and the codon-anticodon duplex increases the stability of the latter. This increase is often achieved by suitable nucleoside modifications in the tRNA.

The intermediate AI stage corresponds to a situation in which the Queuosine base modification of G in tRNA-Asn has been lost and its anticodon is now GUU which has some affinity for pairing with the previously non-cognate AAA codon. Furthermore, the wobble position of the tRNA-Lys anticodon has not yet mutated from U to C. tRNA-Lys has the anticodon UUU which can pair with both AAA and AAG codons. Hence the codon AAA now has two distinct cognate tRNA's (tRNA-Lys(UUU) and tRNA-Asn(GUU)) both of which can pair with it albeit with differential affinities.

By producing two distinct ternary complexes for EF-Tu-GTP-Lys-tRNA-Lys(UUU)[1] with the t^6A37 modification and EF-Tu-GTP-Asn-tRNA-Asn(GUU) for the AAA codon, and using them alternatively on a ribosomal complex containing the AAA codon at the A-site (Fig.1), it would be possible to study the differential rates of the AAA codon recognition by the two tRNA's. Specifically, it should be possible to determine how the forward reaction rates (codon recognition, GTP hydrolysis and peptidyl transfer rate) as well as the rejection rate for the new cognate tRNA-Asn(GUU) compares with the original cognate tRNA-Lys(UUU) . Since G.U mispairing at the wobble position does not lead to RF2 stimulated premature peptide release (Zaher & Green, 2009), a comparison of the differential rates of translation by tRNA-Asn(GUU) and tRNA-Asn(UUU) would then be sufficient to reveal the extent of ambiguous decoding of the AAA codon. A complimentary method would involve incubating an RNC complex containing an AAA codon at the A-site with the ternary complexes EF-Tu-GTP-Lys-tRNA-Lys(UUU) and EF-Tu-GTP-Asn-tRNA-Asn(GUU) and analysing the peptide products to ascertain the relative efficiencies of AAA decoding by the above two competing ternary complexes.

[1] Since the decoding of the AAA codon does not depend on nucleoside modification from U to mnm^5U at the first anticodon position, it is ok to work with a tRNA-Lys having the UUU anticodon.

Reassignments involving gain of stop codons

Three reassignments which lead to the introduction of new stop codons are UUA: Leucine to stop in *T. Aureum* , UCA: Serine to Stop in *S. Obliquus* and AGR : Serine to stop in craniates. The precise mechanism of reassignments is unclear in those cases (Sengupta *et al* 2007). In the former case, one of the Leucine tRNA's has the anticodon CAA which can pair with the UUG codon only. If the mutation in the first anti-codon position of the tRNA-Leu, from U to C, occurred before the gain in specificity of the release factor for the UUA codon; the codon would have to be translated by the tRNA-Leu(CAA). Since this involves a mispairing between the codon and the anticodon, the chance of rejection of this tRNA before as well as after peptide bond formation is likely to be high. This can be tested *in vitro* by measuring the reaction rates associated with codon recognition, GTP hydrolysis, accommodation, peptidyl transfer and tRNA rejection. The codon-anticodon mispairing may also result in RF2 induced premature termination of the peptide sequence (Fig. 2(a)). The resulting effect would be the same as reassigning UUA to stop even without the RF acquiring specificity for the UUA codon. It would therefore be interesting to determine if translation of UUA by tRNA-Leu(CAA) is at all viable.

For the case of UCA: Serine to Stop reassignment in *S. Obliquus*, the situation is similar. The Serine tRNA anticodon has mutated from UGA to GGA thereby becoming incapable of pairing with the UCR codons. If this event initiated the codon reassignment process, the UCR codons would have to be inefficiently translated as Ser by the tRNA-Ser(GGA) at the cost of G.A and G.G mispairing at the wobble position. By forming di-peptidyl tRNA-ribosome complex with the amino acids Met-Ser attached to the tRNA-Ser(GGA) which pairs with the UCA (or UCG) codon at the P-site (Fig.2(b)), it would be possible to determine if the mispairing induces premature peptide release despite the presence of a sense codon at the A-site. If that is the case, the U to G mutation at the first anticodon position of the tRNA-Ser would effectively amount to a reassignment of UCA from Serine to stop.

Testing the UC mechanism

In the case of the UC mechanism, the deletion of a tRNA, cognate to a specific codon, forces a near-cognate tRNA to decode that codon albeit with less efficiency due to mispairing at the

wobble position. For example, the deletion of the tRNA-Ile with anticodon K_2CAU, cognate to the AUA codon results in the tRNA-Ile with anticodon GAU decoding the AUA codon despite the G.A mispairing at the wobble position. Such a situation does not result in the insertion of a wrong amino acid, but the mispairing is likely to affect the GTP hydrolysis rate (k_3), the peptidyl transfer rate (k_{pep}), the dissociation rate (k_{-2}) of the near-cognate tRNA from the ribosome without undergoing GTP hydrolysis and the rejection rate (k_7) of the near-cognate tRNA from the ribosome before peptidyl transfer. By using a ternary complex consisting of the near-cognate, charges Ile-tRNA-Ile(GAU), GTP and the elongation factor protein EF-Tu on a RNC complex containing the tRNA-fMet bound to the AUG codon at the P-site and an unpaired AUA codon at the A-site (Fig.3(a)), it should be possible to determine how these rates change in the case of G.A mispairing at the wobble position brought about by the pairing of the Ile-tRNA-Ile(GAU) with the AUA codon.

Even though there is strong evidence to suggest that the reassignment of AUA: Ile to Met was initiated by the deletion of the tRNA-Ile(K_2CAU) and therefore occurred via the UC and not the AI mechanism, a direct comparison of the effectiveness of UC and AI mechanisms in this case can also be easily carried out by replacing the above ternary complex by one which consists of Met-tRNA-Met(UAU), GTP and EF-Tu (Fig.3(b)). Decoding of AUA at the A-site by the Met-tRNA-Met(UAU) would avoid any mispairing and is therefore likely to occur with different rates compared to the decoding of AUA by Ile-tRNA-Ile(GAU).

Discussion and Conclusions

Codon reassignments which lead to alternative genetic code are rare events and the reason for their occurrence, if any, still remains mysterious. While much insight about the evolutionary mechanisms of the reassignment events can be gained by analysing sequence data, sometimes it is nevertheless difficult to determine the precise evolutionary pathway that eventually led to the codon reassignment event. This is because information about ancestral species in which the transient intermediate stages (AI or UC) of codon reassignment is observed is unavailable in almost all cases. One way to resolve this problem is by studying the effects of codon ambiguity and codon disappearance in model organisms by expressing a new cognate tRNA or deleting an

existing cognate tRNA. While such experiments can reveal important clues about the evolution of codon reassignments, they are nevertheless limited in their scope. The reason for this is genome size, codon usage patterns, tRNA types available, vary dramatically between organisms. Hence the evolutionary pressures of making a codon ambiguous or deleting a tRNA, in say *E.coli*, may be very different from the evolutionary pressures that the now extinct ancestral organism representing the transient intermediate stage of code evolution, was subject to.

The *in vitro* methods recently developed to study the accuracy of the translation process can also be easily adapted to test the viability of the AI and UC mechanisms. The answers are likely to depend on the codon being reassigned and the tRNA's involved in the decoding process during the transient intermediate phase. Previous work (Gromadski, Daviter & Rodnina, 2006) indicates that even though the rates of codon recognition (k_2) were similar for cognate as well as non-cognate codons; the dissociation rate (k_{-2}) due to mispairing was approximately a thousand times higher compared to the cognate codon. Similarly, the rejection rate (k_7) for near-cognate codons was about a hundred times larger compared to the cognate counterpart. However, the type or location of mismatch did not have a significant effect on the dissociation rates. Under LoFi conditions, the GTP hydrolysis (k_3) and accommodation (k_5) rates were largest for the non-cognate codon with G.A mispairing at the wobble (third) codon position, even though it was about ten times smaller than the cognate codon. However, these results may depend significantly on the type of tRNA and codon being decoded as well as on the tRNA bound to the neighbouring P-site. There is evidence (Yokobori *et al.* 2001, Sengupta *et al.* 2007) to suggest that G.A wobble pairing is tolerated for decoding of AUA by tRNA-Ile(GAU) , AAA by tRNA-Asn(GUU) and AGA by tRNA-Ser(GCU). Hence the forward reaction rates may not be very low and the rejection rates may not be very high in these cases. However, even if the experimental results indicate that the rates of forward reaction for some of these cases are indeed very low, it would have important implications. Such results would imply that G.A mispairing must be stabilized by nucleoside modifications either in or close to the anticodon. Structural studies of the type described in Murphy *et al* (2004) should also provide information on the structural constraints involved in decoding AAA by tRNA-Asn(GAA), UUA by tRNA-Leu(CAA) and UCA (or UCG) by tRNA-Ser(GGA). We believe that the experiments proposed here will provide substantial

new insights into the translation process and the evolutionary mechanisms which give rise to alternative genetic codes.

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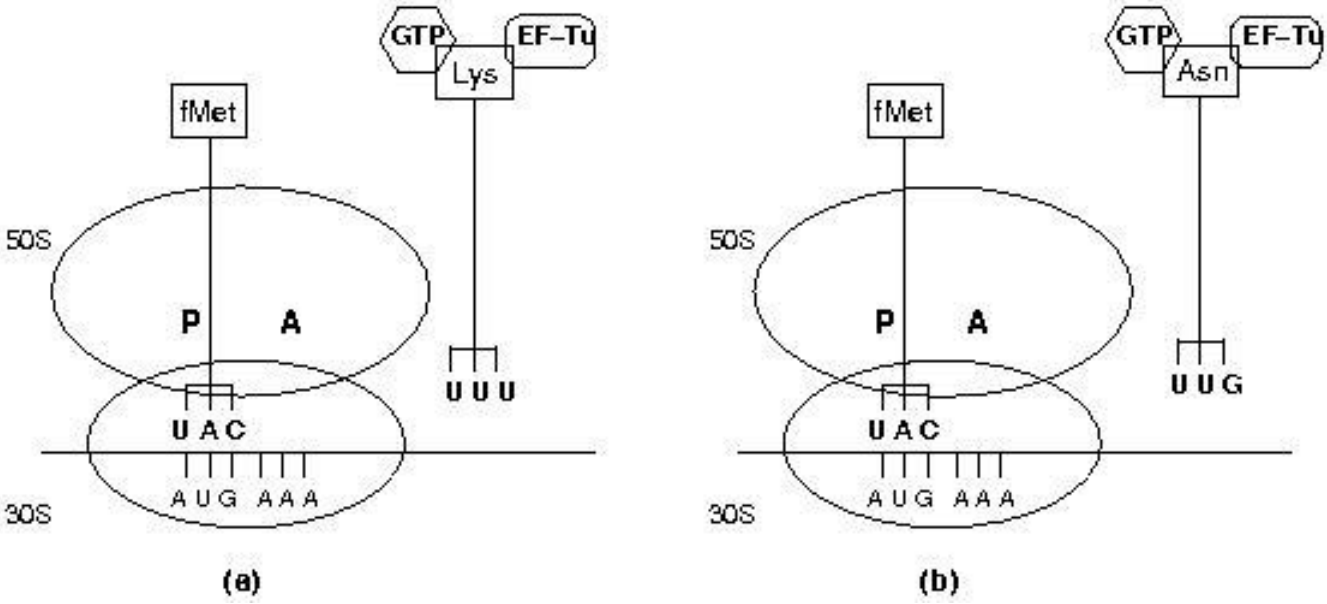

Figure 1

Setup to compare the forward reaction rates of AAA decoding by tRNA-Lys(UUU) and tRNA-Asn(GUU) to determine the extent of ambiguous translation in case both tRNA's are present. A ribosomal nascent chain complex (RNC) consisting of the ribosome, the mRNA with codons AUG and AAA at the P and A-site respectively and the amino-acylated initiator tRNA-fMet(CAU) attached to the start codon at the P-site is shown. Two distinct ternary complexes, (a) EF-Tu-GTP-Lys-tRNA-Lys and (b) EF-Tu-GTP-Asn-tRNA-Asn are capable of binding to the codon AAA at the A-site and producing a ribosomal complex with the di-peptidy tRNA carrying the peptide sequence fMet-Lys and fMet-Asn respectively.

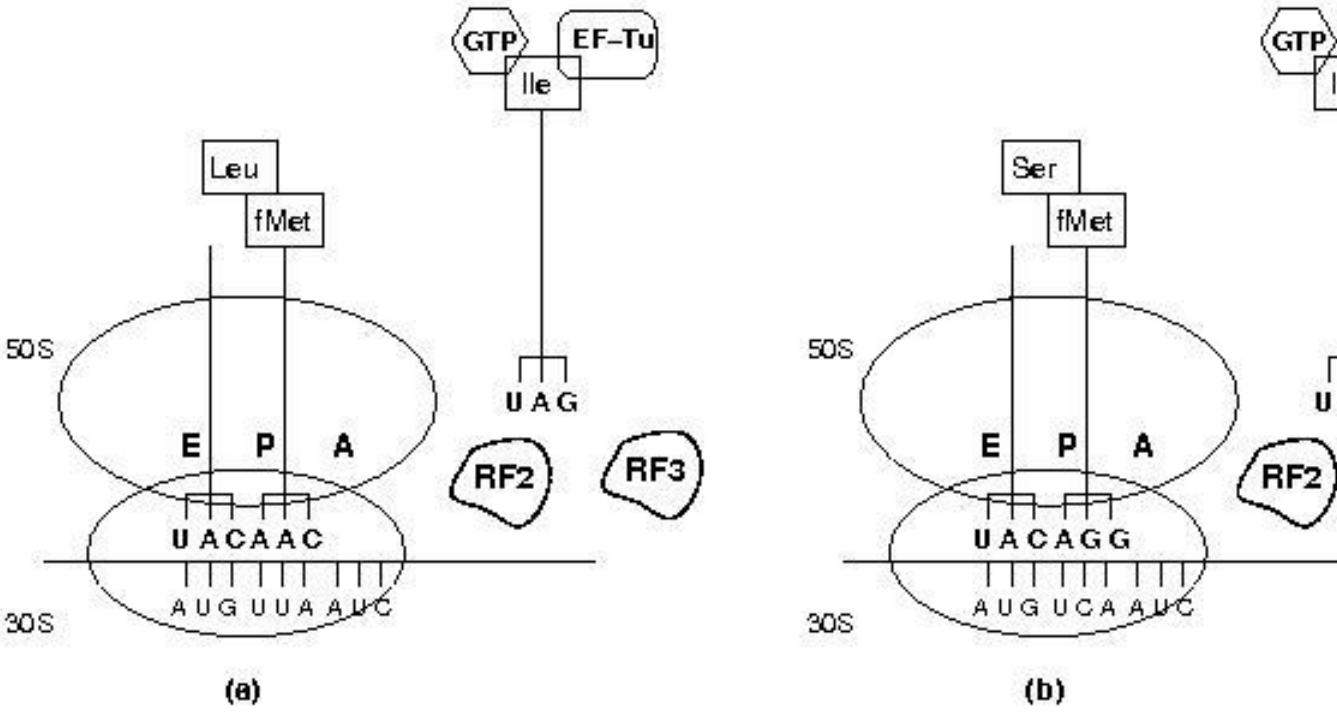

Figure 2

The figure shows a di-peptidyl tRNA-ribosome-mRNA complex with a codon-anticodon mismatch at the P-site and an Isoleucine codon at the A-site. In (a) the C.A mispairing is due to the decoding of UUA by tRNA-Leu(CAA) while in (b) the G.A mispairing is due to the decoding of UCA by tRNA-Ser(GGA). In both cases, the mispairing can lead to RF2 induced premature peptide release. The rate of premature peptide release can be determined by using the P-site mismatched complexes in a mixture with RF2, RF3 and the EF-Tu-GTP-Ile-tRNA-Ile ternary complex that would normally have a higher binding affinity for the AUA codon at the A-site.

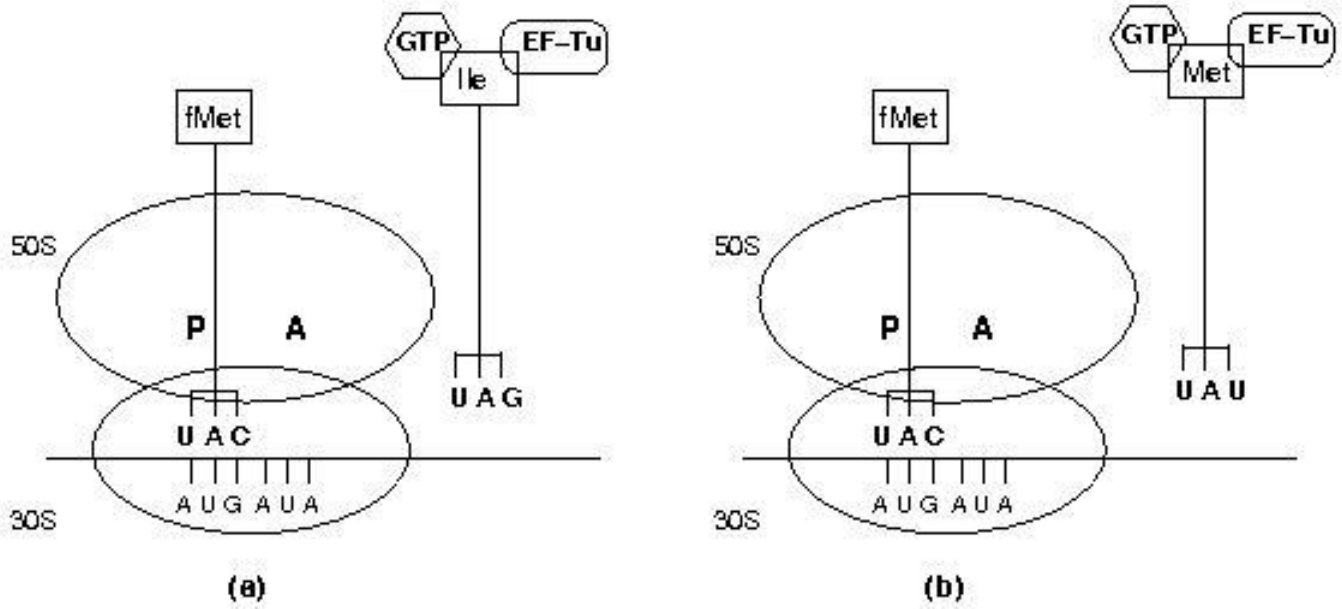

Figure 3

Setup to test the relative efficiencies of AUA decoding by tRNA-Ile(GAU) and tRNA-Met(UAU) when both tRNA's are present and can ambiguously decode AUA as Ile and Met respectively. A RNC complex consisting of the ribosome, the mRNA with codons AUG and AUA at the P and A-site respectively and the amino-acylated initiator tRNA-fMet(CAU) attached to the start codon at the P-site is shown. Two distinct ternary complexes, (a) EF-Tu-GTP-Ile-tRNA-Ile and (b) EF-Tu-GTP-Met-tRNA-Met are capable of binding to the codon AUA at the A-site and producing a ribosomal complex with the di-peptidyl tRNA having amino acids fMet-Ile and fMet-Met respectively. The efficiency of AUA decoding by either tRNA can be determined by estimating the forward reaction rates.