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Strategies for *in vitro* and *in vivo* translation with non-natural amino acids

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Summary

The use of site-directed mutagenesis (Smith, 1985) to replace amino acids at any chosen position in a protein, coupled with advances in analytical procedures, has greatly advanced our understanding of biological structure-function relationships in recent years. The only limitation of conventional site-directed mutagenesis is that substitutions are restricted to the 20 naturally occurring amino acids. However, the discovery of a 21st amino acid, selenocysteine, and the development of novel *in vitro* translation techniques have demonstrated that considerably more site-specific replacements are possible during protein engineering. These techniques have already found a wide range of applications and have shown that the translational machinery is able to accommodate an enormously divergent range of aminoacylated tRNAs.

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Although these techniques are mainly restricted to *in vitro* systems, recent progress in our understanding of aminoacyl-tRNA synthetase-catalyzed tRNA charging suggests that it may ultimately be possible to extend this technique to growing cells.

Introduction

Protein engineering has revolutionized the study of structure and function in proteins in recent years (Winter *et al.*, 1982; Creighton, 1993; Matthews, 1993). The insights which it has provided have already allowed the development of proteins displaying both modified and novel activities. During the same period it has also become clear that although most functional requirements can be fulfilled with the standard set of twenty amino acids, this is not always so. Apart from post-translational modifications, which will not be dealt with here, nature has found other means of achieving functional diversity by incorporating into proteins amino acids which lie outside the canonical 20. The best known of these is selenocysteine, which has become known as the 21st amino acid (Böck *et al.*, 1991a,b), although other examples of the use of modified amino acids in nature have also been observed. Selenocysteine is also one of many examples of context-dependent deciphering of the genetic code and it is this flexibility in the code which is essential during the incorporation of other non-canonical amino acids. The possibilities and limitations of exploiting the genetic code to include additional amino acids will be reviewed.

Both the ability of the translational machinery to accommodate amino acids outside the canonical 20 and the possible consequences of this have long been known. Over 30 years ago Richmond (1962) stated, 'Certain analogues . . . become incorporated into proteins in the place of the natural amino acids. The proteins formed in this way are sometimes altered in their specific enzyme activity'.

The use of amino acid analogues, which mimic their natural counterparts during translation, to modify proteins has found a number of applications. However, this approach has had less impact than protein engineering by site-directed mutagenesis as it did not allow site-specific replacements to be made. To overcome this problem, a number of techniques have been developed which allow the site-specific incorporation of novel, non-natural amino acids into proteins *in vitro* while overcoming restrictions of protein size associated with chemical peptide synthesis (Noren *et al.*, 1989). These methods are finding an increasing range of applications due to the vast range of novel structural and functional groups they offer the protein engineer. Currently, the only significant drawback of these approaches is their limitation to *in vitro* systems. The possibility of extending this approach to living cells, albeit for a far more limited range of non-natural amino acids, will be discussed in the light of recent progress in our understanding of enzymatic tRNA aminoacylation. The most successful existing techniques and the potential for future development of respective approaches will be reviewed below.

Naturally occurring non-canonical amino acid translation

N-FORMYLMETHIONINE

An invariant feature of all known protein-coding genes is that they start with an

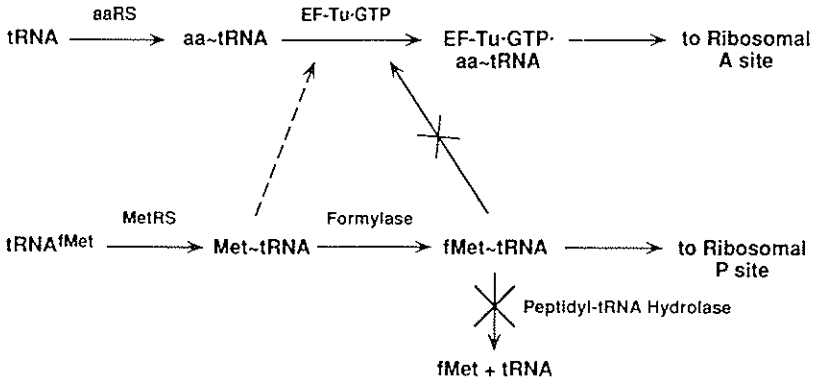

Figure 1. Utilization of N-formylmethionine during protein synthesis in eubacteria. $tRNA^{fMet}$ is aminoacylated by methionyl-tRNA synthetase (MetRS; aaRS = aminoacyl-tRNA synthetase) followed by formylation by methionyl-tRNA transformylase (formylase). Met-$tRNA^{fMet}$, in contrast to other aminoacylated tRNAs (aa-tRNA), is not a substrate for either elongation factor TU (EF-TU) or peptidyl hydrolase. (From RajBhandary and Chow, 1995, with permision).

initiator codon, usually AUG, or rarely GUG or UUG. This is decoded in eubacteria and eukaryotic organelles as N-formylmethionine (fMet), which provides perhaps the most common example of the incorporation into proteins of an amino acid outside the canonical 20. Initiator tRNA ($tRNA^{fMet}$) is first aminoacylated with methionine and then formylated by methionyl-tRNA transformylase (*Figure 1*). The resulting fMet-$tRNA^{fMet}$ is then incorporated into the nascent polypeptide chain and is later deformylated in most of the cases leaving methionine at the protein's N-terminus (Mazel, Pochet and Marlière, 1994; RajBhandary, 1994; RajBhandary and Chow, 1995). This decoding of AUG as fMet instead of methionine is only observed at the first codon and does not ultimately result in the presence of a non-canonical amino acid in the mature protein. Another example of an alternative reading of the genetic code which, however, does result in the incorporation of a non-canonical amino acid is the case of selenocysteine.

SELENOCYSTEINE, THE 21ST AMINO ACID

It has been known for almost 20 years that proteins may contain the highly reactive element selenium covalently bound to cysteine in place of sulfur in their active sites in the form of the amino acid selenocysteine (Cone *et al.*, 1976). The discovery that this is not a post-translational modification but the result of context-dependent decoding of the termination codon UGA (Zinoni *et al.*, 1987) represented an expansion of the genetic code (Böck *et al.*, 1991a). Selenocysteyl-tRNA synthesis occurs by the modification of a canonical amino acid after its attachment to a non-canonical tRNA species, analogous to the formation of fMet-$tRNA^{fMet}$. A selenocysteine-specific tRNA ($tRNA^{Sec}$) is initially charged with L-serine by seryl-tRNA synthetase and this species is then converted to selenocysteyl-$tRNA^{Sec}$. The incorporation of selenocysteine at UGA codons preceding specific stem-loop structures is then mediated by the translation factor SELB (Rinquist *et al.*, 1994) (*Figure 2*). Recent studies suggest that

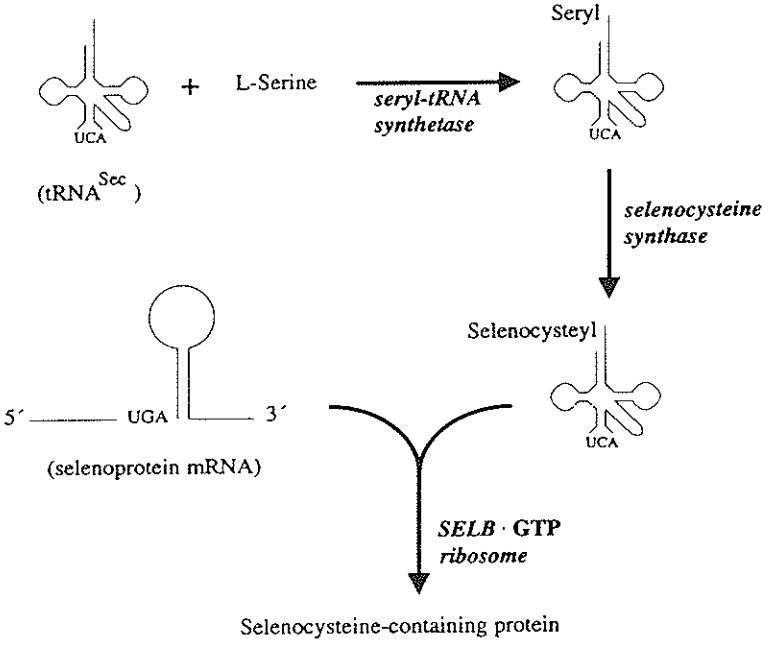

Figure 2. Pathway of selenocysteine incorporation into selenoproteins in *E. coli*. For details see text.

the use of selenocysteine during translation is ubiquitous, supporting the notion that there are in fact 21 canonical amino acids (Baron and Böck, 1995).

SYNTHESIS AND APPLICATIONS OF SYNTHETIC SELENOPROTEINS

Selenium, in the form of the amino acid analogue selenomethionine, is also a potentially important tool for the structural analysis of proteins by multiwavelength anomalous X-ray diffraction (MAD) (Hendrickson, Horton and LeMaster, 1990; Hendrickson, 1991; Skinner *et al.*, 1994). In this method, methionine auxotrophic *Escherichia coli* cells are grown on minimal medium containing selenomethionine but not methionine. This leads to the replacement of methionine residues by the selenium-containing analogue, a substitution tolerated by *Escherichia. coli*. Proteins isolated from such cells are then suitable for crystallization and MAD analysis. Highly efficient incorporation of selenocysteine at cysteine codons in a non-selenoprotein (*E.coli* thioredoxin) has also been accomplished using cysteine auxotrophic *E. coli* in a method analogous to that described above for selenomethionine (Müller *et al.*, 1994). Interestingly, the resultant protein contained a novel structural feature, a diselenide bridge, in place of the normal disulfide bridge. This suggests a possible use of selenocysteine incorporation in the assignment of unknown disulfide connections during structure determination. Selenoenzymes have also been generated by chemical modification of serine, as has been described for subtilisin (Bell and Hilvert, 1993). This is an example of semisynthesis (reviewed by Hilvert, 1991) a technique more comparable to post-translational modification which will not be discussed here. It

should be emphasized that none of these techniques yet offer the possibility of site-specific selenocysteine replacement. However, progress in the understanding of UGA decoding by selenocysteyl-tRNA suggests that this may eventually be possible (Berry *et al.*, 1994; Baron and Böck, 1995).

Besides opening up many possibilities for both studying the structure of proteins and modifying their activity, the naturally occurring selenocysteine insertion system also illustrates several important points in the design of non-natural amino acid incorporation systems. First, how to encode additional amino acids within the existing genetic code; second, how to achieve incorporation only at specific, selected codons; third, the synthesis of a novel aminoacyl-tRNA complex able to utilize such modifications to the genetic code. Our current knowledge of these areas and how this can be exploited to allow the incorporation of non-standard amino acids is discussed below.

Utilization of non-canonical codons and amino acids *in vivo*

THE NON-UNIVERSAL GENETIC CODE AND CODON CONTEXT EFFECTS DURING TRANSLATION

The non-universality of the genetic code was first observed in eukaryotic mitochondria (Barrell, Bankier and Drouin, 1979) and since then numerous examples have been described (e.g. Suzuki *et al.*, 1994). As discussed above, UGA can act both as a terminator and a selenocysteine codon depending on its context, but it can also serve as a tryptophan codon in mitochondria and *Mycoplasma capricolum* and a cysteine codon in *Euplotes octocarinatus* (Hatfield and Diamond, 1993). The other terminator codons, UAA and UAG, also have additional functions, for example in *Tetrahymena thermophila* where they are both able to encode glutamine (Hanyu *et al.*, 1986; Schüll and Beier, 1994). The extent and significance of these and other deviations from the genetic code have been extensively reviewed by Osawa *et al.* (1992) who conclude that such deviations are both a result of and contribute to biodiversity. The nascent peptide itself acts as an additional codon context determinant (Lovett, 1994; Mottagui-Tabar, Bjornsson and Isaksson, 1994) and the variable translation of some codons has also been observed during production of recombinant proteins in *E.coli* due to imbalances in amino acid pools (Jakubowski and Goldman, 1992). With the exceptions of selenocysteine and formyl-methionine, none of these genetic code modifications result in the incorporation of amino acids outside the canonical 20.

The diversity (Farabaugh, 1993), and indeed redundancy, of certain elements within the genetic code suggest it may be possible to accommodate additional amino acids. The most widespread example of this has been amber suppression of the UAG termination codon, which is discussed later. It is worth noting that the efficiency of such termination is itself also strictly context dependent (Poole, Brown and Tate, 1995). However, the incorporation of non-proteinogenic amino acids can most easily be achieved without any genetic manipulation simply by supplying cells with amino acid analogues which mimic their natural counterparts during protein synthesis.

NON-CANONICAL AMINO ACIDS AS SUBSTRATES FOR TRANSLATION

It has long been established that a number of both naturally occurring and synthetic

amino acid analogues can be taken up by cells and directly incorporated into proteins (Richmond, 1962) and that this may play a role in biological control, for example by providing the mode of action of some antibiotics (e.g. azaleucine, Stieglitz and Calvo, 1971) and plant anti-predatory compounds (e.g. canavanine, Pines, Rosenthal and Applebaum, 1981). These and other examples, such as synthetic selenoprotein synthesis (see above), illustrate the various criteria which must be met for successful incorporation of an amino acid analogue during *in vivo* translation.

(i) *Uptake of the amino acid analogue.* The analogue must be recognized and transported across the cytoplasmic membrane into the cell either by the machinery used for the uptake of its natural counterpart or by the general import machinery (Reizer *et al.*, 1993; Tam and Saier, 1993). In addition it should not be degraded within the cell.

(ii) *Stable formation of an aminoacyl analogue-tRNA complex.* The analogue must be a suitable substrate for aminoacylation by an aminoacyl-tRNA synthetase (for example, *p*-fluorophenylalanine, Hennecke and Böck, 1975). Once formed, the aminoacyl-tRNA complex must avoid the editing pathways which normally prevent misacylation of tRNA (Jakubowski and Goldman, 1992; Kim *et al.*, 1993; Gao, Goldman and Jakubowski, 1994; Schmidt and Schimmel, 1994). It has been shown for a mutant of *E. coli* phenylalanyl-tRNA synthetase (PheRS) that while both tyrosine and the analogue *p*-chlorophenylalanine are activated by the enzyme, only the naturally occurring non-cognate amino acid is edited (Ibba, Kast and Hennecke, 1994).

(iii) *Formation of a stable ternary complex with elongation factor Tu (EF-Tu)-GTP.* The final requirement is that the aminoacyl analogue-tRNA complex must be an efficient substrate for EF-Tu (Thompson, 1988; Sprinzl, 1994) which will then channel it to the ribosomal decoding site (Barciszewski, Sprinzl and Clark, 1994). Thus, the aminoacyl analogue-tRNA must avoid discrimination by EF-Tu, as has been observed for Glu-tRNA^{Gln} (Stanzel, Schön and Sprinzl, 1994), fMet-tRNA^{fMet} (Seong and RajBhandary, 1987) and Sec-tRNA^{Sec} (Baron and Böck, 1991). Discrimination must also be avoided at the ribosomal A site (Roesser *et al.*, 1989; Hohsaka *et al.*, 1993).

Surprisingly, given the extremely high fidelity of cellular protein synthesis, a reasonably large number of analogues have been identified which meet these criteria. This appears to result from the comparative insensitivity of the aminoacyl-tRNA synthetase editing mechanism to amino acids not within the canonical 20 and the apparent promiscuity of EF-Tu towards the amino acid moiety of aminoacyl-tRNA. The incorporation of such analogues has found a number of applications (extensively reviewed by Hortin and Boime, 1983, and Wilson and Hatfield, 1984) the more recent of which are outlined in *Table 1*. The usefulness of such applications is, however, limited to techniques requiring widespread protein modification since any given analogue may be incorporated at any codon for its naturally occurring counterpart. One consequence of this is that where an analogue is significantly structurally different from its natural counterpart a high level of incorporation may ultimately prevent growth, as for example in the case of the amino acid antibiotic azaleucine (Lemeignan, Soligo and Marlière, 1993). Thus, in many cases it would be far more desirable to incorporate analogues at specific positions only, as for conventional site-directed mutagenesis (Ross *et al.*, 1992), which would then give rise to more subtle

changes in structure and function.

Table 1. Examples of recent studies which have made use of the *in vivo* incorporation of amino acid analogues into proteins

Amino acid	Analogue	Application	Reference
Arginine	Canavanine	Stress induction in *Saccharomyces cerevisiae*	Heinemeyer *et al.*, 1991
Cysteine	Selenocysteine	Introduction of diselenide bridges into thioredoxin	Müller *et al.*, 1994
Isoleucine	Furanomycin	Incorporation studies	Kohno *et al.*, 1990
Leucine	Azaleucine	Phenotypic suppression of mutant thymidylate synthase	Lemeignan, Sonigo and Marlière, 1993
Methionine	Selenomethionine	Multiwavelength anomalous diffraction	Hendrickson, 1991
Methionine	2-Amino hexanoic acid	Modification of human epidermal growth factor	Koide *et al.*, 1988
Phenylalanine	3-Fluorophenylalanine	^{19}F n.m.r. studies of cyclic AMP receptor protein	Hinds, King and Feeney, 1992
Proline	Azetidine	Stress induction in *E. coli*	Kanemori, Mori and Yura, 1994
Tryptophan	5-Hydroxytryptophan	Spectral enhancement of proteins	Ross *et al.*, 1992
Tyrosine	3-Fluorotyrosine	Electronic and dynamic studies of high-potential iron protein	Lui and Cowan, 1994

CHEMICAL MODIFICATION OF INDIVIDUAL AMINO ACID RESIDUES

As outlined above, one of the key requirements for increasing the usefulness of amino acid analogue incorporation is site-specificity. One technique which can circumvent this problem for certain proteins is cysteine mutagenesis, which exploits the reactivity of this residue towards alkylating and oxidizing reagents. The residue of interest is first mutated to cysteine, and the protein then purified and subjected to chemical modification. This approach has been successfully employed in the biophysical characterization of a number of proteins including steroid isomerase (Holman and Benisek, 1994), aspartate aminotransferase (Kim *et al.*, 1994) and bacteriorhodopsin (Steinhoff *et al.*, 1994). The latter example illustrates one of the most successful applications of cysteine mutagenesis, the site-specific introduction of spin labels into membrane proteins and the subsequent determination of electron paramagnetic resonance spectra.

The degree of specificity that can be attained with this technique is strictly dependent on the number and location of cysteine residues in the protein of interest and consequently it does not provide the flexibility of a method such as site-directed mutagenesis. The incorporation of non-natural amino acids at specific sites has been most successfully accomplished *in vitro* by recruiting the amber terminator codon UAG.

Expansion of the genetic code *in vitro*

SUPPRESSION OF THE AMBER TERMINATOR CODON UAG

The translation of termination codons by suppressor tRNAs containing anticodons complementary to the appropriate terminator and the subsequent insertion of an amino acid at a stop site is a well characterized phenomenon (Murgola, 1995). Based on this, amber suppressor alleles for 17 tRNAs were constructed, all containing the anticodon CUA, which could mediate incorporation of their respective amino acids at a UAG codon (Normanly *et al.*, 1990). Site-directed mutagenesis of a codon of interest to TAG followed by transformation into various backgrounds harboring different suppressor tRNA genes then allows the rapid generation of a large number of natural amino acid substitutions. This approach has been used to characterize the functional and structural roles of numerous residues in a range of proteins including thymidylate synthase (Michaels *et al.*, 1990), β-galactosidase (Cupples and Miller, 1990) and the *lac* repressor (Markiewicz *et al.*, 1994). The translational machinery is able to utilize a broad range of aminoacyl-tRNAs as it is apparently insensitive to most changes in either the aminoacyl (described above) or tRNA (Nazarenko, Harrington and Uhlenbeck, 1994) moieties. Consequently, a wide variety of both chemically (Heckler *et al.*, 1984) and enzymatically aminoacylated amber suppressor tRNAs are suitable substrates for protein synthesis, and it is this which provides the basis for the method of site-directed non-native amino acid replacement (SNAAR) (Noren *et al.*, 1989; Ellman *et al.*, 1991). In this procedure a tRNA species containing the anticodon CUA is first purified, either by chemical modification of the commercially available yeast $tRNA^{Phe}$, or by runoff transcription of a suitably modified tRNA suppressor gene (Nowack *et al.*, 1995). This tRNA is then used as a substrate for chemical

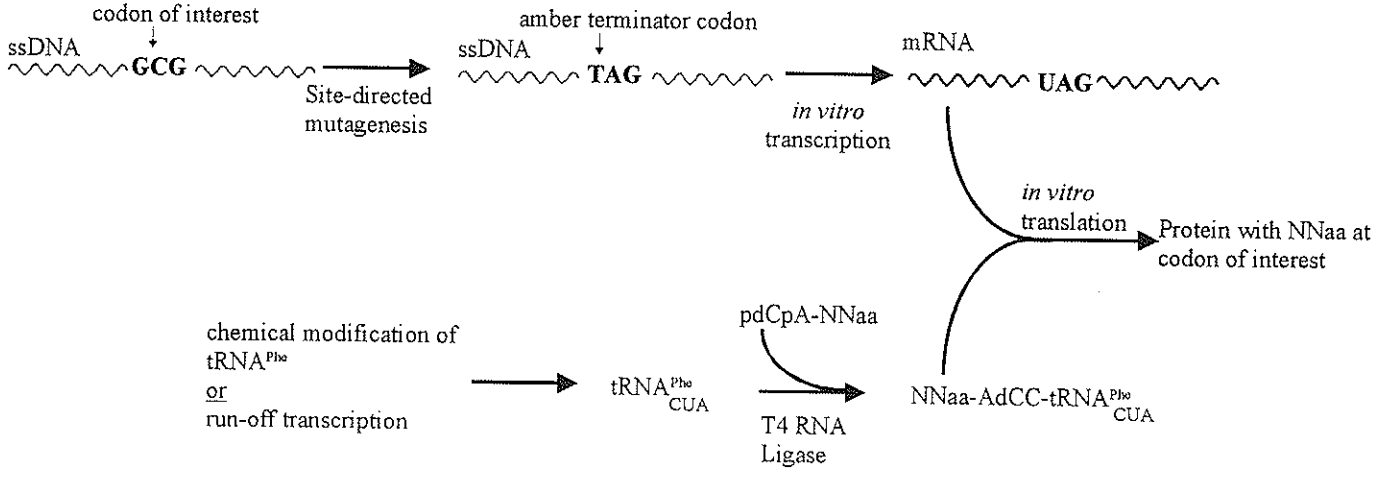

Figure 3. Experimental procedure for site-directed nonnatural amino acid replacement (SNAAR). NNaa = nonnatural amino acid.

Table 2. Applications of *in vitro* non-natural amino acid replacement. Where a range of amino acids were used, only characteristics are given

Characteristics of non-natural amino acids	Target Protein	Reference
Protein stability determinants	β-lactamase	Noren *et al.*, 1989
Novel backbone structures	T4 lysozyme	Ellman, Mendel and Schultz, 1992
Protein stability probes	T4 lysozyme	Mendel *et al.*, 1992
Photocross-linking amino acids	Various	Brunner, 1993a,b; High *et al.*, 1993; Martoglio *et al.*, 1995
Modified main chain, H-bonding and steric conformation	Ras protein	Chung, Benson and Schultz, 1993
Glutamate analogues	Staphylococcal nuclease	Judice *et al.*, 1993
Biophysical probes, including spin and fluorescent labels	T4 lysozyme	Cornish *et al.*, 1994a
β-branched amino acids	T4 lysozyme	Cornish *et al.*, 1994b
Deuterated tyrosine	Bacteriorhodopsin	Sonar *et al.*, 1994
Various	DHFR, HIV-1 protease	Karginov *et al.*, 1995

aminoacylation: the non-natural amino acid of interest is first chemically acylated to the dinucleotide pdCpA and this species is then attached to the suppressor tRNA by T4 RNA ligase. The resulting aminoacyl-tRNA is then added to an *in vitro* translation reaction containing an mRNA where the codon UAG has been inserted at the desired site of non-natural amino acid incorporation (*Figure 3*). A comparable procedure has also been described where the tRNA is instead enzymatically aminoacylated (Sonar *et al.*, 1994). The technique has been successfully used in a wide range of investigations in the fields of both cell biology and biochemistry (*Table 2*) and its more critical aspects have been extensively reviewed (Cornish and Schultz, 1994). The scope of this system has recently been extended to intact cells (*Xenopus* oocytes, Nowack *et al.*, 1995) which, together with the increasing range of non-natural amino acids available (see for example, Burgess, 1994), should now allow its application to an even broader range of biological problems. The major advantage of this system is that it allows the incorporation of a vast range of non-natural amino acids (Mendel, Ellman and Schultz, 1993) at any codon amenable to mutagenesis to TAG. The major disadvantage is that while it can be applied to intact cells, the requirement for significant quantities of charged tRNA imposes limitations on the scale and scope of target protein production. Nevertheless, improvements to existing *in vitro* translation methodologies may help to alleviate these problems (Spirin *et al.*, 1988; Resto *et al.*, 1992; Ying, Zhang and Kramer, 1992; Sonar *et al.*, 1993).

One further limitation of this system is that it only allows the site-specific incorporation of one particular non-natural amino acid per protein, albeit at any number of positions. It is conceivable that for some applications it may be desirable to incorporate a variety of non-natural amino acids at defined locations. A number of approaches may be applicable to this problem: the recruitment of rare codons, such as AGG, which has been successfully used to incorporate non-natural amino acids through *E. coli in vitro* translation (Hohsaka *et al.*, 1994); the future exploitation of terminator codons other than UAG; and the expansion of the genetic code beyond 64 codons. While the first option provides a means of incorporating two different non-natural amino acids into a protein, the incorporation of further novel residues depends on the availability of other sufficiently redundant codons. The second option may be limited to UAA, believed to be the least efficiently suppressible terminator (Bain *et al.*, 1991), since the other possibility, UGA, is already used for selenocysteine and thus its potential for exploitation is also limited. The third option, expansion of the genetic code, would instead offer an enormous number of new, and therefore by definition unused, codons.

(*iso*-C) AG. THE 65TH CODON

The demonstration that the complementary nucleotides *iso*-C and *iso*-G can form a novel Watson-Crick base pair and can be incorporated into duplex DNA and RNA by the respective polymerases represented a potential means of expanding the genetic code (Piccirilli *et al.*, 1990; Benner, 1994). This potential was later realized by the synthesis of an mRNA containing a 65th codon, (*iso*-C)AG, which was exclusively decoded by a synthetic tRNA containing the anticodon CU(*iso*-dG); chemical charging of the tRNA with iodotyrosine was used to incorporate this non-natural amino acid into a peptide at an (*iso*-C)AG codon (Bain *et al.*, 1992). Release factors were apparently unable to recognize the novel codon, resulting in a higher level of

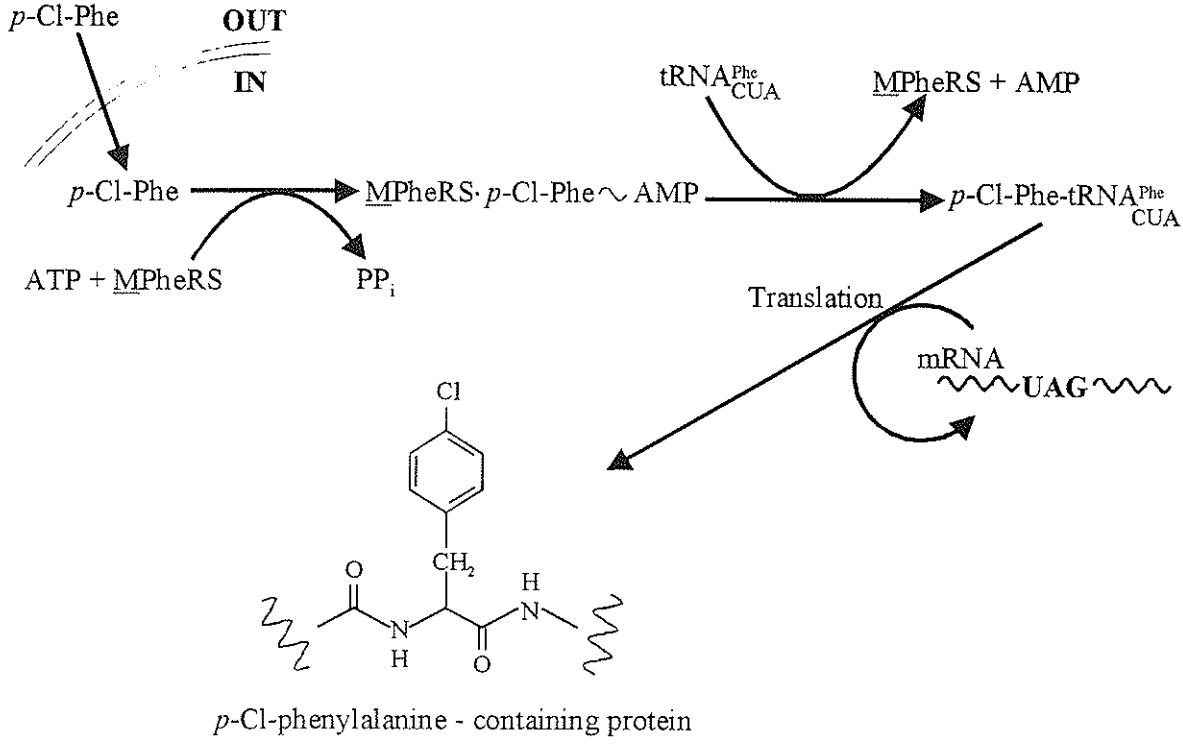

Figure 4. Possible scheme for the site-directed incorporation of *p*-chlorophenylalanine (*p*-Cl-Phe) *in vivo*. *M*PheRS refers to a postulated phenylalanyl-tRNA synthetase mutant which recognizes both *p*-Cl-Phe and amber suppressor $tRNA^{Phe}$ but no longer recognizes phenylalanine and wild-type $tRNA^{Phe}$.

termination suppression, and hence of amino acid incorporation, than at a comparable UAG codon. Thus the use of these nucleotides offers a means to increase both the scope and efficiency of SNAAR, particularly in the light of recent progress in the replication of DNA containing non-standard H-bonding patterns (Horlacher *et al.*, 1995). However, the (*iso*-C)AG approach suffers from the drawback that the majority of its components must be chemically synthesized *de novo*, which is currently beyond the capabilities of all but the most specialized laboratories. This is also true to a lesser extent for SNAAR which, as mentioned previously, is strictly an *in vitro* system. Ultimately it would be desirable to have an *in vivo* system available of comparable site-specificity to SNAAR for the incorporation of non-natural amino acids (Ibba and Hennecke, 1994). Analysis and modification of certain components in the cellular translation machinery is currently being directed towards this end and to date this has mainly concentrated on the aminoacylation of tRNAs with amino acids other than their cognate substrates.

Aminoacylation of tRNA with non-natural amino acids *in vivo*

MISCHARGING OF tRNA

As described above, many tRNAs aminoacylated *in vitro* with non-natural amino acids are stable. Similarly, the proofreading of tRNAs mischarged with non-cognate natural amino acids is not universal. For example, the mischarging of $tRNA^{Gln}$ with glutamate followed by the conversion of Glu-$tRNA^{Gln}$ to Gln-$tRNA^{Gln}$ by a specific amidotransferase is an essential process in chloroplasts, mitochondria and Gram-positive eubacteria (Schön *et al.*, 1988), all of which lack glutaminyl-tRNA synthetase. This necessity for mischarging *in vivo* suggests that it should also be possible to accommodate non-natural amino acids within the existing cellular translational machinery without detrimental effects on the cell. The only caveat is that the non-natural amino acid should meet the criteria outlined above for *in vivo* amino acid analogue incorporation (see 'Non-canonical amino acids as substrates for translation').

The expansion of the range of amino acids routinely used during *in vivo* translation has been demonstrated in *E.coli* using the amino acid antibiotic azaleucine (Lemeignan, Sorigo and Marlière, 1993). After an essential arginine codon (Arg 126) in thymidylate synthase was mutated to a leucine codon, cells were only able to grow in the presence of azaleucine. This was shown to work because only azaleucine, and not leucine, was able to substitute functionally for arginine at this particular position, increasing the number of amino acids required during protein synthesis in this particular strain. Thus, given that non-natural amino acid incorporation can be sustained *in vivo*, further modifications in the substrate specificity of aminoacyl-tRNA synthetases are required in order to achieve the site-specificity characteristic of SNAAR.

SUBSTRATE SPECIFICITY IN AMINOACYL-TRNA SYNTHETASES

The aminoacyl-tRNA synthetases are an extremely well characterized group of enzymes both in their structure and function and much is known about their mecha-

nisms of substrate recognition and catalysis (Carter, 1993; Delarue, 1995). They catalyze the two-step aminoacylation of tRNAs with their cognate amino acids:

$$E + AA + ATP \rightleftharpoons [E{\cdot}AA\text{-}AMP] + PP_i \quad \text{Eq. (1)}$$

$$[E{\cdot}AA\text{-}AMP] + tRNA \rightleftharpoons AA\text{-}tRNA + AMP + E \quad \text{Eq. (2)}$$

(AA = amino acid; E = aminoacyl-tRNA synthetase)

The first step required for redesigning an aminoacyl-tRNA synthetase to be used in an *in vivo* SNAAR is to modify its amino acid substrate specificity to include non-natural amino acids. Broadening of the amino acid substrate specificity has been achieved with phenylalanyl-tRNA synthetase where a mutation in the active site results in a number of phenylalanine analogues becoming suitable substrates for activation (Equation (1); Kast and Hennecke, 1991; Ibba, Kast and Hennecke, 1994) and subsequent attachment to $tRNA^{Phe}$ (Equation (2); Ibba *et al.*, 1995). Phenylalanine analogues, including *p*-chloro- and *p*-bromophenylalanine, can then be incorporated into target proteins both *in vitro* and *in vivo* (Ibba and Hennecke, 1995). However, further work is required to modify the enzyme further such that phenylalanine itself is no longer a substrate for aminoacylation. It is worth noting that if such an enzyme were to be used *in vivo* it would be essential to maintain the wild-type protein to ensure the continued incorporation of phenylalanine.

The second requirement of an *in vivo* SNAAR is that the activated amino acid should be attached to a tRNA for a unique codon such as one of the terminators UAG or UAA. In order to achieve site-specific incorporation it would be essential that these tRNAs were only recognized by the aminoacyl-tRNA synthetase which had activated the non-natural amino acid. Much is known about both the identity sites in tRNA (McClain, 1993; Saks, Sampson and Abelson, 1994) and how they interact with recognition domains in aminoacyl-tRNA synthetases (Rould, Perona and Steitz, 1991; Weygand-Durasevic, Schwob and Soll, 1993; Schimmel and Ribas de Pouplana, 1995). Therefore it is possible that by mutating the tRNA, or possibly a smaller RNA substrate (Hipps *et al.*, 1995), and the aminoacyl-tRNA synthetase in tandem a unique interaction could be obtained which, in combination with the modified amino acid substrate specificity, would provide the necessary requirements for an *in vivo* SNAAR. This scheme is summarized in *Figure 4*. Recent developments also suggest that other potential routes to the same goal may also be possible. One is the designing of antibodies able to catalyze aminoacylation (Jacobsen *et al.*, 1992), while another is the *in vitro* selection of RNAs able to catalyze RNA aminoacylation (Illangasekare *et al.*, 1995). Either of these may provide a pathway to attach a non-natural amino acid to RNA independently of aminoacyl-tRNA synthetases, thereby circumventing specificity considerations.

Future prospects

The systems described here offer a number of ways to incorporate non-natural amino acids into proteins. The use of such methods is becoming increasingly widespread with cloning systems (Kast, 1994) and commercial *in vitro* translation kits (Kurzchalia *et al.*, 1988) now available that exploit the incorporation of *p*-chlorophenylalanine and biotin-lysine respectively. The reported applications of SNAAR are also becoming

more widespread and it seems likely that in the near future the technique will become as routine as conventional site-directed mutagenesis. The only requirement is for the commercial availability of the necessary aminoacylated tRNAs. The scope of SNAAR may also be broadened by advances in chemical peptide synthesis (Dawson *et al.*, 1994) which will allow the combination of synthetic, SNAAR-modified and conventional peptides to produce novel proteins (Gaertner *et al.*, 1994). The prospects for transferring SNAAR to replicating *in vivo* systems are also good in view of the rapid recent increase in our understanding of aminoacylation mechanisms.

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