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MEETING REVIEW

Aminoacyl-tRNA synthetases: Versatile players in the changing theater of translation

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ABSTRACT

Aminoacyl-tRNA synthetases attach amino acids to the 3′ termini of cognate tRNAs to establish the specificity of protein synthesis. A recent Asilomar conference (California, January 13–18, 2002) discussed new research into the structure–function relationship of these crucial enzymes, as well as a multitude of novel functions, including participation in amino acid biosynthesis, cell cycle control, RNA splicing, and export of tRNAs from nucleus to cytoplasm in eukaryotic cells. Together with the discovery of their role in the cellular synthesis of proteins to incorporate selenocysteine and pyrrolysine, these diverse functions of aminoacyl-tRNA synthetases underscore the flexibility and adaptability of these ancient enzymes and stimulate the development of new concepts and methods for expanding the genetic code.

Keywords: aminoacyl-tRNA synthetases; genetic code

INTRODUCTION

Accurate translation of the genetic information into proteins is a complex ensemble performance by essential cellular players: the ribosome, messenger RNAs, aminoacylated tRNAs, and a host of additional protein and RNA factors. Among the latter are the aminoacyl-tRNA synthetases (aaRS[1]), which join amino acids with their cognate transfer RNAs in a high-fidelity reaction. Although the principal functions of the aaRS in translation were established decades ago, these enzymes have continued to surprise us with their idiosyncratic origins, mechanistic complexities, and unexpected connections to other critical aspects of cellular function. Like a venerable character actor playing against type in a new production, the aaRS and their close relatives are emerging with new functions in biology. These include direct participation in amino acid biosynthesis, DNA replication, RNA splicing, and aspects of eukaryotic cell biology related to cytokine function and cell cycle control. Many of these roles were discussed at the Fourth International Conference on Aminoacyl-tRNA Synthetases in Biology, Medicine, and Evolution, which was organized by the authors and held earlier this year at Asilomar. The remarkable functional diversity of tRNA synthetases hints at the underlying flexibility and adaptability of the translation apparatus, a feature also highlighted by the recent report of a new amino acid, pyrrolysine (Hao et al., 2002; Srinivasan et al., 2002). This "22nd amino acid" is likely incorporated into proteins by use of the same strategy employed for the "21st amino acid," selenocysteine. Here we summarize recent findings that strengthen our understanding of the catalytic mechanisms and substrate recognition properties of tRNA synthetases, particularly with regard to induced-fit conformational changes and amino acid editing. We also describe new and highly significant developments in the field. The emerging picture is of a family of enzymes distinguished by a multiplicity of biological roles, potential for impact in the evolution of biotechnology, and the ancient function in translation that sheds light on the molecular evolution of life (Fig. 1). Readers should be advised that space limitations pre-

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[1]Aminoacyl-tRNA synthetases are abbreviated as aaRS, and individual enzymes are abbreviated by the three-letter code of the appropriate amino acid followed by the "RS" suffix.

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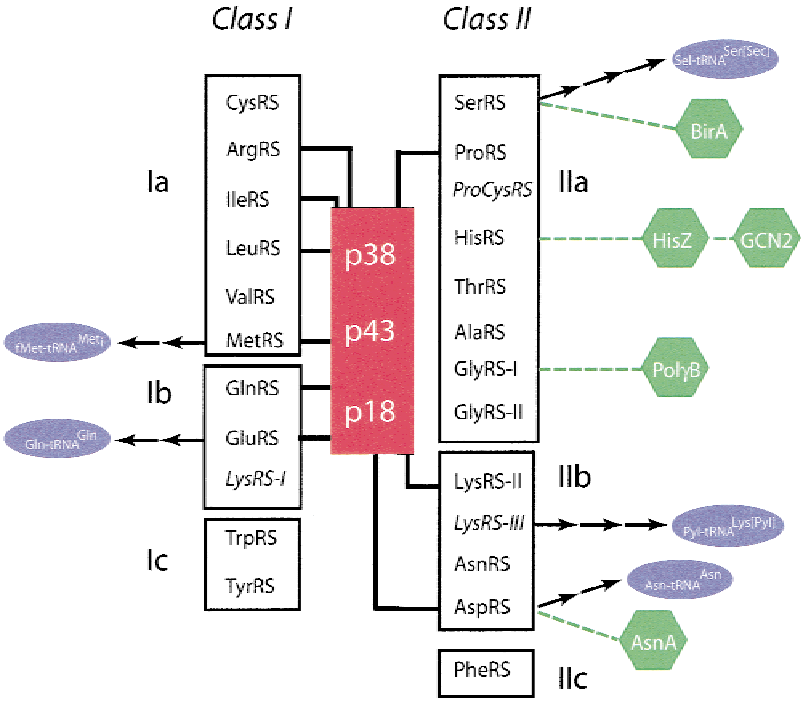

FIGURE 1. Schematic table of the two classes of tRNA synthetases, which are further divided into classes Ia, Ib, and Ic, and classes IIa, IIb, and IIc. The nine synthetases in higher eukaryotic cells that constitute the multisynthetase complex are indicated by the thick lines connected to the three auxiliary proteins p38, p43, and p18. Synthetases that participate in amino acid biosynthesis that involves transformation of a standard amino acid on a tRNA are indicated by arrows. These include GluRS and AspRS in the synthesis of Gln-tRNA^{Gln} and Asn-tRNA^{Asn}, respectively, and SerRS and LysRS-III in the synthesis of Sel-tRNA^{Sec} and Pyl-tRNA^{Lys}, respectively. Also, bacterial MetRS attaches methionine to the initiator tRNAi, which serves as the intermediate for converting methionine to formyl-methionine (fMet) to synthesize fMet-tRNA^{Meti}. Synthetases SerRS, HisRS, and AspRS that are structurally related to metabolic enzymes BirA, HisZ, and AsnA, respectively, are indicated by broken arrows. HisRS is also related to the regulatory protein GCN2. Finally, GlyRS-1, which is structurally related to the mitochondrial polymerase PolyγB, has the standard class II quarternary α_2 structure, whereas GlyRS-II has the unusual $\alpha_2\beta_2$ structure.

clude us from discussing here many of the interesting program areas covered at the meeting, including aaRS–tRNA interactions, aaRS as therapeutic targets, aaRS evolution and phylogenetics, and other aspects of tRNA synthetase structure and function.

All aaRS catalyze a two-step aminoacylation reaction. This entails condensation of the amino acid with ATP to form an activated aminoacyl adenylate intermediate, followed by transfer of the amino acid to the 3′-terminal ribose of tRNA to generate the aminoacylated product (Ibba & Soll, 2000). In this way, each amino acid becomes associated with one or more anticodon sequences in the cognate tRNA isoacceptor set, and thus a corresponding set of codons. In the original adaptor hypothesis proposed by Francis Crick, each amino acid is associated with its unique cognate aaRS, such that a typical cell would possess a full complement of 20 different aaRS to accommodate all of the standard amino acids used in translation (Crick, 1958).

A major insight gained in the last decade has been that these 20 canonical enzymes are divided evenly into two classes, each of which represents a distinct evolutionary solution to the requirement for the aminoacylation reaction. Enzymes of the same family share a characteristic catalytic fold, identifiable peptide sequence motifs, and distinctive mechanistic features (Ibba & Soll, 2000). Thus, the catalytic domains of class I enzymes are based on a Rossmann dinucleotide binding fold, whereas those of class II enzymes are organized around a six-stranded antiparallel β-sheet flanked by three α-helices. Recently, exceptions to the general paradigm have emerged, complicating efforts to construct simple evolutionary scenarios.

NATURAL EXPANSIONS OF THE GENETIC CODE: SELENOCYSTEINE AND PYRROLYSINE

Although alternative assignments of certain codons are characteristic of some cellular organelles (Fox, 1987), selenocysteine represents the first known authentic expansion of the genetic code. Selenocysteine is found in a select group of enzymes, including formate dehydrogenases, glutathione peroxidase, and the type I iodothyronine deiodinase of thyroid. The specialized translational machinery required for selenocysteine insertion in all three kingdoms is almost completely described (Commans & Bock, 1999; Hatfield & Gladyshev, 2002). Selenocysteine incorporation depends critically on tRNA^{Sec}, an unusually large UGA-decoding tRNA that possesses a UCA anticodon and an extended acceptor arm. Selenocysteine-tRNA^{Sec} is produced in a pathway that features enzymes encoded by the *sel* gene cluster. First, SerRS aminoacylates tRNA^{Sec} to synthesize seryl-tRNA^{Sec}, which is subsequently converted into aminoacryl-tRNA^{Sec} by the SelA selenocysteine synthase. The conversion of the acrylate product into the authentic selenocysteine-tRNA^{Sec} is subsequently catalyzed by the SelD gene product. The specific mechanisms that dictate insertion of selenocysteine at specific UGA codons in the appropriate target genes differ between prokaryotes and eukaryotes,

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and require special elongation factors that interact with mRNA. In prokaryotes, the SelB translation protein associates with GTP and selenocysteine-tRNA^{Sec} to target a stem-loop structure adjacent to the site of UGA insertion. In eukaryotes, several elongation factors are necessary to recognize an RNA hairpin structure (selenocysteine insertion sequence, SECIS) in the 3′ untranslated region of the mRNA carrying the UGA codon for selenocysteine.

In contrast to the widespread occurrence of selenocysteine, the distribution of pyrrolysine is so far known only in a handful of methanogenic archaea and bacteria (Galagan et al., 2002; Srinivasan et al., 2002). The central metabolism of these organisms is the catabolic conversion of methylamines, which requires a family of mono-, di-, and trimethylamine methyltransferases. Many of the methyltransferase genes of the methanogen *Methanosarcina barkeri* are interrupted by in-frame amber codons; tryptic peptide sequencing of one such protein (MtmB) showed that the amber codon is decoded as lysine (James et al., 2001). The determination of the X-ray structure of MtmB to a resolution of 1.55 Å showed additional electron density best described as a lysine in an amide linkage to a 4-substituted-pyrroline-5-carboxylate (Hao et al., 2002). This unusual amino acid suggests a plausible (though as yet unproven) mechanism for the methyltransferase reaction (Hao et al., 2002). The machinery necessary for pyrrolysine insertion in *M. barkeri* appears to reside in a unique gene cluster that includes an amber suppressing tRNA (*pylT*), an unusual lysyl-tRNA synthetase (*pylS*), and additional proteins provisionally responsible for converting lysine to pyrrolysine (Srinivasan et al., 2002). The tRNA encoded by *pylT* differs significantly from canonical tRNAs. The anticodon arm has 6 rather than 5 bp, the variable loop has 3 rather than 4 nt, and the nearly universally conserved D-loop GG and T-loop TψC sequences are absent. These features are likely to be important for recognition by the dedicated *pylS* gene product, which resembles the class II tRNA synthetases in its C-terminal catalytic domain. However, the N-terminal domain of the *pylS* lysyl-tRNA synthetase possesses negligible sequence identity with the anticodon binding domains of other class IIb synthetases, which are based on the OB fold. An in-frame amber codon is also found in the *mttB* gene of the gram-positive bacterium *Desulfitobacterium hafniense*, and it is likely decoded as pyrrolysine by a cotranslational mechanism similar to that of selenocysteine. In *D. hafniense*, however, *pylS* is split into two ORFs that separately encode the N- and C-terminal domains. The presence of an unusual LysRS for pyrrolysine suggests that tRNA$^{Lys(Pyl)}$ has diverged beyond the point of recognition by LysRS-I or LysRS-II. Features of the translation machinery that allow context dependent insertion of pyrrolysine remain to be identified, and constitute an interesting follow-up question.

EXCEPTIONS TO THE ADAPTOR HYPOTHESIS: INDIRECT PATHWAYS AND NONCANONICAL tRNA SYNTHETASES

Although the discoveries of selenocysteine and pyrrolysine demonstrate natural examples of expansion of the genetic code, these two unusual amino acids also constitute one of the recent challenges to the Adaptor Hypothesis (Ibba et al., 2000). Historically, the assumption of a universal set of 20 aaRS enzymes was based on studies of *Escherichia coli* and animal cells, but absences of some of the aaRS in bacterial taxa were noted early on (Wilcox & Nirenberg, 1968). Many bacterial and archaeal taxa lack GlnRS, AsnRS, or both, but nonetheless are able to synthesize Gln-tRNA^{Gln} and Asn-tRNA^{Asn} by use of indirect pathways (Ibba & Soll, 2000). In these pathways, tRNA^{Gln} and tRNA^{Asn} are aminoacylated with glutamate and aspartate, respectively, by nondiscriminating GluRS and AspRS enzymes. Conversion of Glu-tRNA^{Gln} and Asp-tRNA^{Asn} to their glutaminylated and asparaginylated versions is subsequently catalyzed by amidotransferases specific for Glu-tRNA^{Gln} and Asp-tRNA^{Asn}. Although the presence of these indirect pathways provides a straightforward explanation of the absence of GlnRS and AsnRS in bacterial and archaeal taxa, the distribution of the amidotransferases is complex, and there are apparent functional duplications that are not easily accounted for. Two different multimeric complexes, GatCAB and GatDE, are present in bacteria and archaea, with the former apparently possessing both GluAdT and AspAdT activity, and the latter possessing only GluAdT activity. The GatCAB may be also responsible for biosynthesis of asparagine in organisms that lack the enzyme asparagine synthetase. For example, the genome of *Deinococcus radiodurans* lacks the gene for asparagine synthetase and the enzyme activity is absent from the cell lysate. This has led to the suggestion that *D. radiodurans* synthesizes asparagine from Asn-tRNA^{Asn} by transamidation of Asp-tRNA^{Asn} (Curnow et al., 1998), providing yet another example of the direct role of the aaRS in amino acid biosynthesis.

The production of mischarged Glu-tRNA^{Gln} and Asp-tRNA^{Asn} poses a significant potential problem for the translation apparatus, because direct participation of these tRNAs in protein synthesis could lead to toxic levels of misincorporation of Glu and Asp. An insight into this problem has arisen in the discovery of a previously unforeseen specificity in EF-Tu for recognition of amino acid–tRNA combinations (LaRiviere et al., 2001). It appears that *Thermus thermophilus* EF-Tu binds with a range of affinities to different tRNAs and amino acids, such that a weak affinity for a given tRNA is balanced by high affinity for its cognate amino acid, and vice versa. Although measurements for the specific mischarged tRNAs described above have not yet been reported, this balanced binding can provide a

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plausible mechanism for preventing mischarged aminoacyl tRNA from participating in translation.

The indirect pathways described above for synthesis of amino acids illustrate that the transfer of the amino acid to the tRNA need not always produce a species ready for direct participation in translation. For these cases, the aminoacylation reaction provides an intermediate that is a substrate for additional amino acid biosynthetic reactions that must occur prior to protein synthesis. Paradoxically, aminoacylation can therefore be seen as a precursor to amino acid biosynthesis. It is therefore remarkable that several enzymes involved in amino acid biosynthesis bear significant structural similarity to aaRS, yet do not catalyze the aminoacylation reaction. Interestingly, such tRNA synthetase-like proteins are apparently more likely to be related to class II than class I aaRS. For instance, the AsnA enzyme, which synthesizes asparagine from aspartate and ATP, is highly homologous to the catalytic domain of aspartyl-tRNA synthetase and utilizes class II conserved residues (Nakatsu et al., 1998). The HisZ protein, the accessory subunit of the first enzyme in histidine biosynthesis (ATP-phosphoribosyl transferase), is homologous to the catalytic domain of HisRS (Sissler et al., 1999), and likely diverged from HisRS early in evolution (Bond & Francklyn, 2000). Also, the BirA enzyme, which catalyzes adenylation of biotin and transfer of the activated biotin to the biotin carrier protein, is a repressor of the biotin operon. Structural analysis of the BirA enzyme shows similarity to the class II SerRS (Wilson et al., 1992).

Several unusual aaRS enzymes also serve as exceptions to the Adaptor Hypothesis. Functional genomics and bioinformatics analysis of recently sequenced genomes indicates that many archaea possess only 16 of the 20 canonical aaRS. In addition to the absence of AsnRS and GlnRS, which are accommodated by the indirect pathways, orthologs to the standard CysRS and LysRS are absent in the archaea. Among most of the archaea and in some bacteria, the canonical class II LysRS has been replaced by a class I version of LysRS (Ibba et al., 1997). Despite their common amino acid substrate and apparent lack of sequence or structural homology, class I and class II LysRS recognize the same recognition elements (namely the anticodon and discriminator base) on $tRNA^{Lys}$ (Ibba et al., 1999a). The structure of the class I LysRS from *Pyrococcus horikoshii* in complex with lysine was reported at Asilomar (Terada et al., 2002). Remarkably, class I LysRS is highly similar in structure to GluRS, sharing in common the Rossmann nucleotide binding fold, the SC-fold domain, and the C-terminal α-helical hemispheric domain; thus, these two enzymes may share a common ancestor. This close relationship, as well as details from the GluRS-$tRNA^{Glu}$ complex (Sekine et al., 2001), were used to construct a docking model of the LysRS-I:tRNA complex. The predicted interactions of LysRS-I and LysRS-II with $tRNA^{Lys}$ are mirror images of each other, consistent with a recently proposed hypothesis concerning the evolution of the two classes of synthetases (Ribas de Pouplana & Schimmel, 2001). Because no organism contains both LysRS-I and LysRS-II, a more extensive sequence analysis of bacterial LysRS-I has suggested that it may be derived from archaea by limited lateral gene transfer (Ambrogelly et al., 2002). The finding of a putative third LysRS involved in pyrrolysine biosynthesis raises additional evolutionary questions.

The search for the missing CysRS in certain Archaea species has also led to an additional unexpected finding. Recent studies show that cysteinylation in *Methanococcus jannaschii*, as well as in other archaea and in a deeply branching eukaryote (*Giardia lamblia*), is catalyzed by a dual function ProRS (Pro-CysRS) capable of aminoacylating both $tRNA^{Pro}$ and $tRNA^{Cys}$ with their appropriate cognate amino acids (Bunjun et al., 2000; Lipman et al., 2000; Stathopoulos et al., 2000). With the exception of misincorporation arising from editing defects (see below), no other tRNA synthetase has been reported to insert two different amino acids into proteins. The sequence of the Pro-CysRS enzyme shares strong homology to the canonical ProRS (Stehlin et al., 1998), but no obvious sequence blocks to account for the unusual dual functionality can be detected. Biochemical studies suggest that binding determinants for proline and cysteine are extensively overlapped (Stathopoulos et al., 2001), and that activation of both cysteine and proline is independent of the cognate tRNA (Lipman et al., 2002). Thus, the two amino acids must compete directly for the overlapping binding site. The crystal structure of the Pro-CysRS from *Methanobacterium thermoautotropicum* has now been determined (Kamtekar, Asilomar), and confirms the expected high degree of structural similarity with the existing ProRS X-ray structure from *T. thermophilus*. However, comparison of this structure with that of the canonical *E. coli* CysRS (Newberry et al., 2002) reveals a dramatic disparity in the molecular nature of the substrate binding cleft. In *E. coli* CysRS, the cysteine thiolate is recognized via ligation to a tightly bound zinc ion, and the binding site possesses a unique array of highly conserved imidazole and other groups that are important to ensuring specificity without the need for editing. By contrast, the amino acid pocket of the Pro-CysRS enzyme is poorly differentiated and suggests no clear mechanism by which specificity for cysteine might be achieved. Clearly, further work will be necessary to clarify how Pro-CysRS carries out the unusual dual aminoacylation.

COMMON FEATURES OF aaRS STRUCTURE AND MECHANISM ARE EMERGING

All efforts to modify the translation system through expansion of the genetic code ultimately rest on a solid

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understanding of aaRS structure and function. Except for AlaRS, representative structures for all families of synthetases have now been determined. Despite the presence of the class-defining conserved catalytic folds and residues, there is remarkable diversity in the strategies employed for amino acid and tRNA recognition (Arnez & Moras, 1997). Nevertheless, several common themes that may ultimately unify all synthetases have emerged. As proposed by Alan Fersht and others, aaRS appear to accelerate the rate of aminoacylation by structuring their active sites to maximize the number of enzyme–substrate contacts in the transition state (Fersht et al., 1985). Achieving this enzyme–substrate transition state complementarity appears to rely heavily on induced-fit conformational change upon substrate binding. For example, induced fit contributes to amino acid recognition for HisRS (Qiu et al., 1999), ProRS (Yaremchuk et al., 2001), LysRS (Onesti et al., 2000), and ArgRS (Cavarelli et al., 1998). In these enzymes, only the correct amino acid is likely to elicit the specific conformational changes required to bring essential catalytic residues into position. Alternatively, the TrpRS and ThrRS systems provide examples of conformational changes induced by ATP binding, typically leading to the ordering of the class-defining conserved KMSK (for class I) or motif 2 loop (class II) peptides (Carter; Sankaranarayanan, Asilomar).

The question of why a subset of aaRS (GlnRS, GluRS, ArgRS, and the class I LysRS) require tRNA to activate amino acid has also been recently addressed. In GlnRS, $tRNA^{Gln}$ binding causes the rearrangement of peptide segments located in the central portion of the structure between the active site and the anticodon binding β-barrel domains. Additionally, the 3′-terminal CCA end stabilizes the configuration of side chains that comprise the amino acid binding pocket (Sherlin, Asilomar). In the closely related GluRS, the apo enzyme binds both Glu and ATP, but tRNA is required to move the ATP from the ground state to a "near attack" configuration (Sekine, Asilomar). In ArgRS, the binding of $tRNA^{Arg}$ causes a conformational change in the ATP binding site, whereas the presence of arginine influences the position of the CCA end (Delagoutte, Asilomar). All of these structural changes underscore the importance of induced fit, and rationalize the role of the tRNA as a true macromolecular cofactor in the first reaction. Detailed comparative analyses of these and other tRNA synthetases, to assess the extent of conservation of these induced-fit changes, should be informative with respect to understanding specificity and to appreciating the complex evolutionary relationships among these enzymes.

The Asilomar conference also addressed additional aspects of tRNA recognition and amino acid editing. Biochemical data and multiple structures of various complexes available for some synthetases, particularly AspRS, suggest that tRNA recognition is likely to be a sequential process involving multiple steps (Eriani & Gangloff, 1999; Moulinier et al., 2001). In the first step, an encounter complex is formed that is driven primarily by interactions with the anticodon (Bovee et al., 1999). This is followed by a subsequent repositioning step in which the acceptor end of the tRNA is moved into the active site, followed by proper orientation of the CCA end. Enzyme and tRNA conformational changes accompany the repositioning process, and the presence of the adenylate serves to order the tRNA acceptor end and enforce tRNA selection (Cusack et al., 1996). Further evidence for the multiple steps of the aminoacylation reaction are also provided by rapid kinetics in other tRNA synthetase systems (Ibba et al., 1999b). In HisRS, for example, some tRNA recognition determinants appear to be specific for ground-state binding, whereas others specifically impact on the transfer step (Hawko, Asilomar). Despite the attractiveness of this general model, individual systems continue to present new surprises. For example, recent determination of the TyrRS–$tRNA^{Tyr}$ complex indicates that this class I synthetase has a class II mode of binding its cognate tRNA (Yaremchuk et al., 2002).

Those aaRS required to select among amino acids that differ by a single methylene group typically require editing mechanisms to achieve sufficient selectivity. According to the "double sieve" model of editing, amino acids larger than the cognate substrate are eliminated at the initial binding step, whereas smaller amino acids require a second catalytic site that hydrolyzes misactivated and/or misacylated tRNAs (Fersht, 1998). Recent work has localized the editing site to the CP-I domain that is common to the class Ia synthetases IleRS (Nureki et al., 1998), ValRS (Lin et al., 1996), and LeuRS (Cusack et al., 2000). All three must discriminate among closely related hydrophobic amino acids. Interestingly, their CP-I sequences share threonine-rich peptide motifs that are likely to participate in hydrolysis of the transiently misacylated tRNA (Nureki et al., 1998; Mursinna et al., 2001). Significantly, the CCA end of the cognate tRNA in the *Staphlococcus aureus* IleRS:tRNAIle complex projects into the putative editing site, and not into the synthetic site (Silvian et al., 1999). Complementary studies on class II enzymes are also in progress, and have indicated that editing occurs in ProRS (Beuning & Musier-Forsyth, 2000), ThrRS (Dock-Bregeon et al., 2000), and based on sequence similarities, AlaRS as well (Sankaranarayanan et al., 1999). Among the numerous issues remaining to be addressed concerning editing are the relative contributions of "pretransfer" and "posttransfer" mechanisms, as well as the importance of conformational changes in the editing domains and the CCA end of tRNAs. Interestingly, the CP-I domains of IleRS and LeuRS exhibit different orientations relative to the catalytic domain (Cusack et al., 2000), and the editing active sites show variability in the regiospecificity of

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deacylation catalyzed at the terminal ribose (Nordin & Schimmel, 2002). Resolving the precise contribution of the tRNA to the editing process is likely to be facilitated by approaches that are capable of strictly isolating the translocation and hydrolysis steps. For example, experiments featuring fluorescence-based transient kinetics suggest that translocation of the tRNA's CCA end to the hydrolysis site may be rate limiting for the editing reaction (Nomanbhoy et al., 1999).

REENGINEERING OF SYNTHETASES AND tRNAs TO ACCOMMODATE UNNATURAL AMINO ACIDS: CREATING A "WILD CARD" IN THE GENETIC CODE

The structural and mechanistic work summarized above also establishes a critical knowledge base for expansion of the genetic code. Incorporation of a nonstandard amino acid into proteins in vivo requires both a dedicated suppressor tRNA and a specialized synthetase that is specific for both the new amino acid and tRNA.[2] Previous experiments showed that misincorporation of amino acid analogs can occur under special conditions. For example, some mutants of PheRS were isolated that exhibited relaxed specificity for para-substituted versions of fluoro-phenylalanine (Ibba et al., 1994), and this has been exploited to explore altered physical-chemical properties of fluorinated proteins (Kirshenbaum et al., 2002). An in vitro approach has also been explored, by which chemically synthesized aminoacyl-tRNA was used to incorporate nonstandard amino acids in β-lactamase (Noren et al., 1989), ribonuclease A (Jackson et al., 1994), and nicotinic acid receptor (Nowak et al., 1995).

Although these approaches have enjoyed limited success, a robust in vivo system is necessary if extensive characterization of the engineered protein is a key objective. The first hurdle toward this technology is the development of a mutually compatible aaRS:tRNA pair that is resistant to challenge by competing interactions with the natural amino acid, and noncognate synthetase or tRNA. Several groups have reported progress in the development of such "orthogonal" aaRS:tRNA pairs that utilize either amber or four base codons for recoding (Liu et al., 1997; Kowal et al., 2001). The second and more technically demanding step is to create a mutant synthetase capable of efficiently aminoacylating the orthogonal tRNA with a nonstandard amino acid. Thus far, the only successful strategy reported involves the *M. jannaschii* TyrRS-tRNA^{Tyr} pair imported into *E. coli* (Wang et al., 2001). A genetic selection employing combinatorial mutagenesis of selected active site residues in *M. jannaschii* TyrRS allowed isolation of mutants that suppress an amber-containing reporter gene, when provided with an unnatural amino acid but not tyrosine. By use of the well-characterized suppression of dihydrofolate reductase, it was demonstrated that the mutant TyRS-tRNA^{Tyr} pair can efficiently insert 2′*O*-methyl tyrosine in response to a stop codon in vivo, although in vitro characterization of the enzyme was limited only to the activation step of the reaction.

Another strategy for incorporating unnatural amino acids into proteins involves the subversion of the editing mechanisms characteristic of selective class I and class II tRNA synthetases. Using the insertion of cysteine into the *thyA* gene as a reporter for decreased editing function, mutants of ValRS were selected that contained substitutions in the editing domain (Doring et al., 2001). In addition to mischarging tRNA^{Val} with threonine, a mutant ValRS (T222P) also brought about a significant level of misincorporation of aminobutyrate (up to 24% of valine) when the unnatural amino acid is present at 0.2 mM in the culture media. This misincorporation by inactivation of the editing site also highlights the less discriminating nature of the synthetic site, and shows how loss of editing can serve as a starting point for selecting synthetases with novel amino acid specificities. However, there may be nonnatural amino acids for which genetic selections are not possible. In these cases, a possible alternative strategy is to extend the repertoire of in vitro selected aminoacylating RNA to include nonnatural amino acids, creating the potential for synthesis of modified proteins through cell-free translation (Bessho et al., 2002). As our foundation knowledge of the aminoacyl-tRNA synthetases increases, it is likely that other aaRS:tRNA pairs will be developed to expand the repertoire of unnatural amino acids inserted into proteins.

THE EXPANDED RANGE OF FUNCTIONS OF tRNA SYNTHETASES AND THEIR RELATIVES IN EUKARYOTIC CELLS

Aminoacyl-tRNA synthetases show a higher degree of structural organization in eukaryotes than prokaryotes. In addition, synthetases and synthetase-like proteins in different compartments of the eukaryotic cell perform a range of diverse functions. For example, tRNAs must undergo an aminoacylation-dependent proofreading step before exit from the nucleus, implying the existence and function of aaRS in the nucleus (Lund & Dahlberg, 1998). Once delivered to the cytoplasm, tRNAs are transported to the protein synthesis apparatus by mechanisms that most likely involve large complexes of aaRS, the EF1A elongation factor, and ribosomes (Stapulionis & Deutscher, 1995). Such organized complexes may also be present in the nucleus (Nathanson & Deutscher, 2000). Indeed, higher eukary-

[2] Devising systems for the "postaminoacylation" transformation of the aminoacyl-tRNA, as is performed naturally by the amido transferases, is an even more ambitious prospect that has not yet been considered seriously.

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otes from flies to humans all contain a multisynthetase complex (MSC) with a molecular mass of 1.5 Mda, which includes nine of the canonical aaRS (Glu-ProRS, IleRS, LeuRS, MetRS, GlnRS, LysRS, ArgRS, and AspRS) and three auxiliary proteins: p43, p38, and p18 (Yang, 1996). The three-dimensional structure of this MSC is beginning to emerge by cryoelectron microscopy, revealing a Y-shape of approximately 20 × 15 nm with a central cavity that is 4 nm in diameter (Norcum & Boisset, 2002). Biochemistry studies show that, in the MSC, interactions among aaRS are mediated by eukaryotic-specific extension domains, which include the lysine-rich K domains, the leucine-rich L domains, the repetitive R domains, and GST-like domains. Frequently repeated, these polypeptide segments typically confer either protein–protein interactions with other members of the complex, or generalized (as opposed to specific) tRNA binding. How the aaRS in the multisynthetase complex work in collaboration to efficiently direct aminoacyl-tRNA to the ribosome remains to be addressed.

The roles of the nonsynthetase partners of the complex are also being defined in recent work. The p18 protein is responsible for the transient interaction of the MSC with the elongation factor EF-1H (Quevillon & Mirande, 1996). The p38 protein is essential for the assembly and stability of the MSC (Kim et al., 2002), and two-hybrid studies suggest that it makes contacts to nearly all components in the complex (Quevillon et al., 1999). The largest nonsynthetase protein in the MSC, p43, appears to possess multiple functions. It contains a nonspecific RNA-binding domain analogous to that of the general tRNA-binding protein, which likely serves to recruit and maintain interactions of tRNA in the complex (Shalak et al., 2001). This general RNA-binding domain may also allow p43 to facilitate nuclear export of tRNA to the cytoplasm (Popenko et al., 1994; Simos et al., 1996). In yeast, which does not have the MSC of higher eukarya, a homolog of p43 is found in the protein Arc1p, which is associated with MetRS and GluRS and promotes their interactions with the cognate tRNAs (Simos et al., 1996). This Arc1p-organized complex formation appears to have a determining effect on the cellular localization of MetRS and GluRS, which upon dissociation from the complex enter the nucleus (Galani et al., 2001). Interestingly, the RNA-binding domain of Arc1p and p43 was recently discovered in the archaeal protein Mj1338, which copurifies with the dual-specific *M. jannaschii* ProRS and is shown to have general tRNA-binding affinity and can interact with several archaeal synthetases. Mj1338 may represent an archaeal homolog of a nonsynthetase protein in an MSC-like structure (Lipman, Asilomar).

An unexpected role of p43 is in cellular physiology, which has also been reported for some of the tRNA synthetases as well. The C-terminal half of p43 is identical to the endothelial monocyte activating protein II (EMAPII), a pro-inflammatory cytokine that stimulates chemotaxis of polymorphonuclear granulocytes and mononuclear phagocytes, and induces tissue factor activity in endothelial cells (Shalak et al., 2001). Notably, EMAPII is released from p43 by caspase-7, one of the key apoptotic proteases, and this cleavage abrogates p43′s RNA-binding function. Thus, the cleavage of p43 may provide the basis for shutdown of protein synthesis during the onset of apoptosis by reducing affinity of the MSC for tRNA. Surprisingly, the EMAPII domain is also found in the C-terminal portion of MetRS (Kaminska et al., 2000) and TyrRS (Kleeman et al., 1997). Although TyrRS has not been found to be associated with the MSC, it too can be cleaved by caspases to release an N-terminal fragment ("mini-TyRS") and an EMAP II-like C-terminal fragment (Wakasugi & Schimmel, 1999), both of which have the cytokine activity. The functions of these cytokines associated with p43 and tRNA synthetases may be to recruit macrophage to the site of cellular apoptosis, attenuating the inflammation that might otherwise occur as a result of secondary necroses. Also unexpected are the findings that several N-terminally truncated forms of human TrpRS exhibit angiostatic activity in vivo and block VEGF-induced proliferation and chemotaxis of endothelial cells in vitro (Otani et al., 2002). These "mini-TrpRS" enzymes may be used in angiostatic therapy, extending the contribution of synthetases to areas of medicine and drug design.

There are other examples of alternative functions of aaRS and aaRS-like proteins in eukaryotic cells. The *E. coli* ThrRS can regulate the translation level of itself by binding to an upstream region of its own mRNA, forming a structure analogous to that of the ThrRS-$tRNA^{Thr}$ complex (Torres-Larios, Asilomar). A similar regulatory role at the translational level has also been reported for yeast AspRS (Frugier, Asilomar). In the mitochondria of certain yeasts and fungi, TyrRS and LeuRS have well-established roles in promoting the splicing of cytochrome genes, most likely by recognizing tRNA-like motifs (Rho & Martinis, 2000; Myers et al., 2002). During packaging of the HIV virus, human LysRS assists $tRNA^{Lys3}$ to enter the virus particle to serve as a primer for viral replication (Javanbakht et al., 2002). The DNA polymerase of mitochondria, Polγ possesses a processivity subunit with a remarkable resemblance to the dimeric glycyl-tRNA synthetases (Carrodeguas et al., 2001). In addition to these special mitochondrial activities, the importance of the aaRS in mitochondrial protein synthesis is receiving increased attention, owing in part to the study of syndromes arising from specific tRNA mutations (Rabilloud et al., 2002). Lastly, recent work shows that the translation control protein GCN2, which appears to regulate translation in eucaryotic cells, possesses a HisRS-like domain that binds tRNA (Dong et al., 2000).

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SUMMARY AND FUTURE PERSPECTIVES

Synthetases have been proposed to be among the earliest proteins emerging in the transition from the RNA to protein world, but their evolution from that juncture has been anything but simple or direct. Although we can only speculate how the translational apparatus developed, the synthetase–tRNA interaction represents an essential feature of protein synthesis that possesses a flexibility and adaptability far greater than what might be implied by the simple textbook picture of translation. This flexibility is reflected in the rich variety of indirect pathways, many of which illustrate a close linkage between synthesis of amino acids and their utilization in protein synthesis, as well as an intricate set of recoding strategies that allow amino acid insertion to be site specific. The success with which extant organisms have exploited this flexibility serves as an inspiration to man-made efforts to expand the repertoire of protein synthesis. As complicated as these recoding strategies are for prokaryotic organisms, an additional emerging challenge for understanding tRNA synthetases is to delineate their complex functions in the eukaryotic cells. Here, the connections between tRNA synthetases and cytokine function, as well as the control of translation, echo the importance of tight regulation of protein synthesis functions when external conditions dictate the apoptotic program. Future work will undoubtedly focus on how tRNA synthetases function in mammalian cells, and on how their cellular locations regulate translation and cellular physiology.

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