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Recoding: Reprogrammed Genetic Decoding with an emphasis on Antizyme regulatory frameshifting.

by

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Thesis in fulfilment for the degree of

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School of Biochemistry and Cell Biology

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Declaration

This thesis is my own work and has not been submitted for another degree, either at University College Cork or elsewhere.

Signed: Martina Yordanova

Abbreviations

Abbreviation	Term
FS	frameshifting
PRF	programmed ribosome frameshifting
ORF	open reading frame
uORF	upstream open reading frame
ribo-seq	ribosome profiling
SD sequence	Shine-Dalgarno sequence
PTC	peptidyl transferase centre
SPD	spermidine
SM	spermine
PUT	putrescine
AZ	antizyme
ODC	ornithine decarboxylase

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Abstract

Recoding embraces mechanisms that augment the rules of standard genetic decoding. The deviations from standard decoding are often purposeful and their realisation provides diverse and flexible regulatory mechanisms. Recoding events such as programed ribosomal frameshifting are especially plentiful in viruses. In most organisms only a few cellular genes are known to employ programed ribosomal frameshifting in their expression. By far the most prominent and therefore well-studied case of cellular +1 frameshifting is in expression of antizyme mRNAs. The protein antizyme is a key regulator of polyamine levels in most eukaryotes with some exceptions such as plants. A +1 frameshifting event is required for the full length protein to be synthesized and this requirement is a conserved feature of antizyme mRNAs from yeast to mammals. The efficiency of the frameshifting event is dependent on the free polyamine levels in the cell. cis-acting elements in antizyme mRNAs such as specific RNA structures are required to stimulate the frameshifting efficiency. Here I describe a novel stimulator of antizyme +1 frameshifting in the Agaricomycotina class of Basidiomycete fungi. It is a nascent peptide that acts from within the ribosome exit tunnel to stimulate frameshifting efficiency in response to polyamines. The interactions of the nascent peptide with components of the peptidyl transferase centre and the protein exit tunnel emerge in our understanding as powerful means which the cell employs for monitoring and tuning the translational process. These interactions can modulate the rate of translation, protein co-translational folding and localization. Some nascent peptides act in concert with small molecules such as polyamines or antibiotics to stall the ribosome. To these known nascent peptide effects we have added that of a stimulatory effect on the +1 frameshifting in antizyme mRNAs. It is becoming evident that nascent peptide involvement in regulation of translation is a much more general phenomenon than previously anticipated.

Chapter 1

Augmented Genetic Decoding: global, local and temporal alterations of decoding processes and codon meanings.

This chapter has been published as a review on Recoding in Nature Reviews Genetics 16, 517–529 (2015)

The non-universality of the genetic code is now widely appreciated. Codes differ between organisms, and certain genes are known to alter the decoding rules in a site-specific manner. Recently discovered examples of the decoding plasticity are particularly spectacular. These include organisms and organelles with disruptions of triplet continuity during translation of many genes, viruses altering the entire genetic code of their hosts and organisms adjusting their genetic code in response to changing environment. In this Review we outline various modes of alternative genetic decoding and introduce some novel terminology to accommodate recently discovered manifestations of this seemingly sophisticated phenomenon.

1.1 Introduction

The famous Jacob Monod phrase *"Anything found to be true of E. coli must also be true of elephants"* reflects the prevalent mindset of biochemists and molecular biologists at the time when the genetic code was being deciphered. Indeed, the chemistry and molecular mechanisms of genetic information inheritance and its decoding from nucleic acids into proteins were seemingly the same for all forms of life. This culminated in Francis Crick's 'frozen accident' hypothesis for the origin of the genetic code (Crick, 1968) according to which the genetic code (Fig. 1.1a) is not only universal, but also unchangeable and unevolvable. Ironically, the time of the

hypothesis formulation also marked the beginning of series of experimental observations of various exceptions from what is known as the standard rules of the genetic decoding, leading to a ‘melting’ in perceptions of the universality of the genetic code (Riyasaty and Atkins, 1968, Weiner and Weber, 1971, Barrell et al., 1979, Yamao et al., 1985, Horowitz and Gorovsky, 1985, Preer et al., 1985, Clare and Farabaugh, 1985, Jacks and Varmus, 1985, Mellor et al., 1985, Chambers et al., 1986, Craigen and Caskey, 1986, Huang et al., 1988, Matsufuji et al., 1995, Keiler et al., 1996, Srinivasan et al., 2002, Jungreis et al., 2011, Prat et al., 2012, Lang et al., 2014) (Fig. 1.1b).

a

```
1         TTTTTTTTTTTTTTTTCCCCCCCCCCCCCCCCAAAAAAAAAAAAAAAAGGGGGGGGGGGGGGGG
2         TTTTCCCCAAAAGGGGTTTTCCCCAAAAGGGGTTTTCCCCAAAAGGGGTTTTCCCCAAAAGGGG
3         TCAGTCAGTCAGTCAGTCAGTCAGTCAGTCAGTCAGTCAGTCAGTCAGTCAGTCAGTCAG
Meaning   FFLLSSSSYY**CC*WLLLLPPPPHHQQRRRRIIIMTTTTNNKKSSRRVVVVAAAADDEEGGGG
```

b

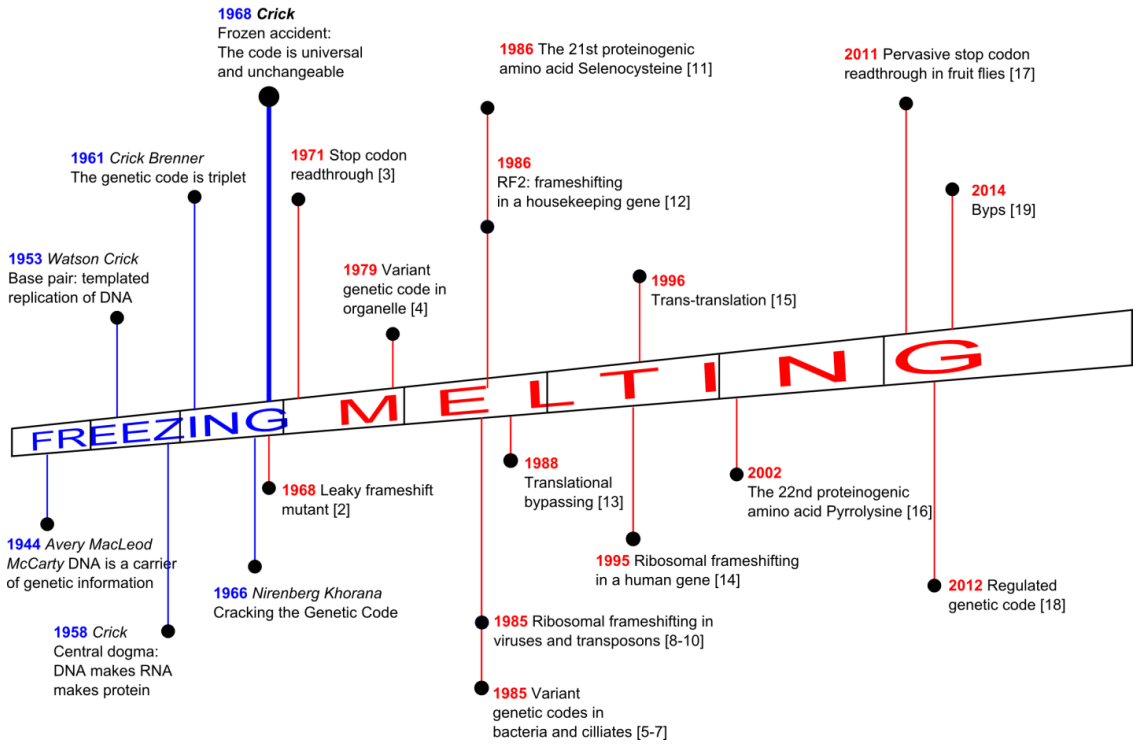


Figure 1.1 *Freezing and melting of genetic decoding. a. The standard genetic code. b. A timeline illustrating major events that shaped current understanding of genetic decoding. The early discoveries that led to the formulation of universal principles of genetic decoding are shown in blue. The more recent findings that revealed evolvability and flexibility of genetic decoding are shown in red.*

By now we are aware of more than twenty natural variant genetic codes (Knight et al., 2001) and it is very likely that there are more to discover. Two non-universal proteinogenic amino acids (Selenocysteine (Chambers et al., 1986) and

Pyrrolysine (Srinivasan et al., 2002)) have been discovered in addition to the 20 in the standard genetic code, and the possibility that more exist cannot be dismissed (Ambrogelly et al., 2007). Numerous mRNAs have evolved special sequence elements to alter the meaning of specific codons or to make ribosomes shift reading frame or even to bypass long untranslated regions (Atkins and Gesteland, 2010). Alterations in genetic decoding are expected to exist in virtually all organisms, bioinformatics screens of bacterial genomes are revealing thousands of genes whose decoding requires ribosomal frameshifting (Sharma et al., 2011, Antonov et al., 2013b, Antonov et al., 2013a) and metagenome analyses of environmental samples point to many organisms with variant genetic codes (Ivanova et al., 2014).

Nature also continues to surprise us with novel types of alternative genetic decoding. The most recent discoveries pose provocative questions and suggest heretical ideas: Could an organism's genetic code change during the organism's lifetime? Could a parasite hijack the genetic code of its host? Could there be genetic codes with non-triplet features? In this Review we will give a brief overview of the known classes of alternative genetic decoding and their implications for entire genomes or individual mRNAs (Table 1.1). We will start with global changes of codon meaning on the scale of the entire genetic code (codon reassignments) and continue with local changes specific to particular sites in particular mRNAs (codon redefinition). Then we will describe processes that disrupt the continuity of triplet decoding, such as ribosomal frameshifting and translational bypassing. Along the way we will expand existing terminology to accommodate the growing complexity of the phenomenon of alternative genetic decoding.

Genomic distribution*		What is affected?‡ (individual codons, protein length or both)	Competes with standard translation in a concurrent or temporal manner?¶	Examples
	Global	Sense codon	No	Codon reassignments that lead to variant genetic codes, see http://www.ncbi.nlm.nih.gov/Taxonomy/Utils/wprintgc.cgi
			Yes	Decoding of CUG as serine and leucine in some *Candida* species (Ohama et al., 1993)
		Stop codon (Protein length is affected)	No	Codon reassignments that lead to variant genetic codes, see http://www.ncbi.nlm.nih.gov/Taxonomy/Utils/wprintgc.cgi
			Yes	The meaning of UAG as a stop or Pyl in the genetic code of *A. arabaticum* is dependent on the energy source (Prat et al., 2012). During infection a phage-encoded RF2 alters the genetic code of its host (Ivanova et al., 2014).
		Protein length	No/Yes	Ribosomal frameshifting in ciliates of the *Euplotes* genus *(Klobutcher and Farabaugh, 2002)* and translation bypassing in mitochondria of *M. capitatus(Lang et al., 2014)* are pervasive. However, it is not yet clear whether these processes are in competition with standard translation.
	Local	Sense codon	No	No known natural examples.
			Yes	Selenocysteine insertion in *Euplotes* species (Turanov et al., 2009).
		Stop codon (Protein length is affected)	No	Selenocysteine insertion at mRNAs encoding selenoproteins with multiple selenocysteine residues is likely to be highly efficient(Hill et al., 1991, Lobanov et al., 2008).
			Yes	Selenocysteine insertion, stop-codon readthrough
		Protein length	No	No known natural examples.
			Yes	Ribosomal frameshifting, translational bypassing

Table 1.1 *Classification of different types of alternative genetic decoding events.*

**Alternative decoding may affect expression of all or most genes within a genome (globally distributed) or only a subset of individual mRNAs that bear specific stimulatory elements (local events).*

‡An alternative decoding event may alter the meaning of a single codon with no effect on downstream codons. It also can alter the length of protein sequence by combining protein sequences encoded in overlapping or disjointed open reading frames (ORFs). A change of codon meaning from stop to sense ultimately creates an elongated proteoform.

¶The alternative decoding event could occur in competition with standard translation (concurrent or temporal) or be a principal process.

1.2 Global codon reassignment

1.2.1 Variant genetic codes resulting from fixed codon reassignments.

At the moment of writing the NCBI Genetic Code database (http://www.ncbi.nlm.nih.gov/Taxonomy/Utils/wprintgc.cgi) provides a list of 21 distinct genetic code tables. Examination of these tables reveals two patterns: high abundance of mitochondrial variant codes and fixed codon reassignment involving stop codons. The only known non-mitochondrial sense codon reassignment is a CUG codon change from Leu to Ser in some yeast of the *Candida* genus (Ohama et al., 1993). The prevalence of mitochondrial reassignments is most likely due to the small number of potentially affected genes, but also due to properties of mitochondria and chloroplasts that are discussed in section 1.2.4. The prevalence of stop codon reassignment is due to the naturally infrequent occurrence of stop codons and their comparatively high evolutionary flexibility, as discussed in section 1.2.3.

1.2.2 Alterations to molecular machineries required for codon reassignment.

The global rules of genetic decoding are defined by the molecular components of the translation machinery. Recognition of sense codons and stop codons differ. Whether a particular sense codon is decoded as a specific amino acid depends on two molecules, a tRNA that recognizes that codon and an aminoacyl-tRNA synthetase (aaRS) that charges the tRNA with the amino acid (Fig. 1.2). Therefore, a change of

sense codon standard meaning should involve either modification of existing tRNAs and/or aaRSs or their loss or gain through gene duplications or horizontal gene transfer.

Stop codons are recognized by class I release factor proteins (RF) that directly interact with mRNA inside the ribosome and trigger hydrolysis and release of the nascent peptide. Thus, which codon is recognized as a stop primarily depends on the structure of the RF proteins, and lack of competition from cognate tRNAs. In most bacteria and organelles, there are two release factors that recognize stop codons semi-specifically: UGA and UAG are each recognized only by one specialized factor while UAA is recognized by both (Duarte et al., 2012). All known cases of codon reassignment in bacteria involve a loss of RF2, which recognizes UGA codons. This may not be accidental: a recent study of a large number of metagenomic sequences revealed many bacterial genes with reassigned stop codons which are exclusively UGA codons, suggesting that RF1 unlike RF2 may be indispensable for bacteria in the wild (Ivanova et al., 2014). In the majority of characterized cases UGA is reassigned to code for Trp (Yamao et al., 1985, Bove, 1993, McCutcheon et al., 2009), but in uncultured SR1 bacteria found in marine and fresh-water environments as well as in human microbiota, UGA was recently shown to encode Gly (Campbell et al., 2013, Ivanova et al., 2014).

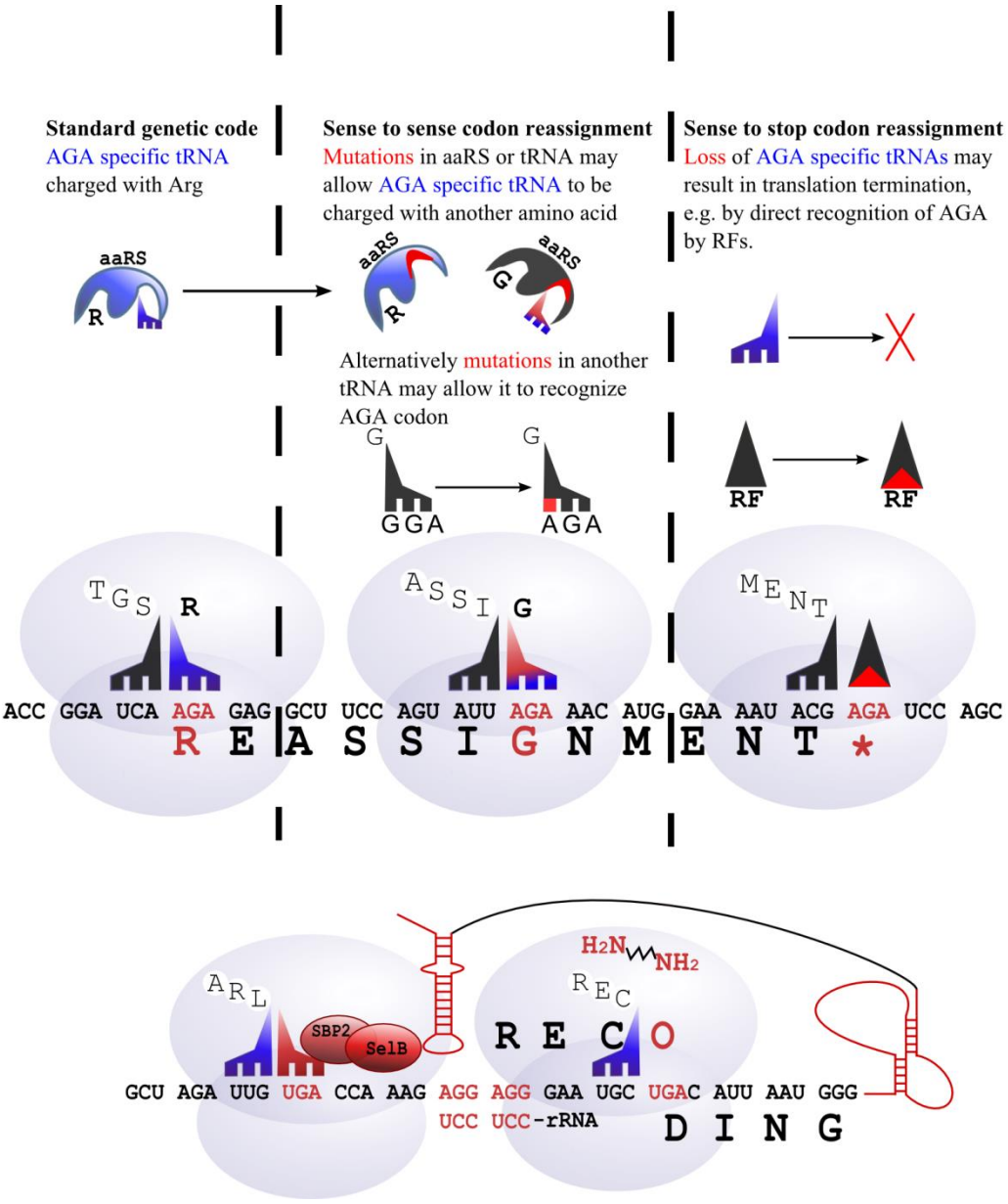


Figure 1.2: *Components that shape alternative genetic decoding. The scheme illustrates a distinction between codon reassignment and recoding which are sometimes confused in the literature. Collective representation (assembled from several examples from different organisms) of stop codon reassignment (part a) and recoding (part b) is shown. Different ways by which codon meaning can be reassigned as exemplified by the AGA codon. It is known to have four different meanings depending on the variant genetic code used in the corresponding organism. It can originate as a result of changes in tRNAs, aaRSs or release factors*

(RFs), though may involve other components as exemplified with AGA reassignment to stop codon in vertebrate mitochondria, see the main text. Codon reassignment affects the expression of all genes in the organism that use the reassigned codon. b | Two recoding events are shown. Codon redefinition (left) is exemplified with a schematic of selenocysteine (one-letter amino acid abbreviation U) insertion at UGA codons in eukaryotes, which requires a selenocysteine insertion sequence (SECIS) element in the 3' UTR, a specialized tRNA, an elongation factor eEFSec and auxiliary protein SECIS-binding protein 2 (SBP2). Ribosomal frameshifting (right) is shown as a collective representation of several frameshifting events. Shine–Dalgarno interactions with ribosomal RNA stimulate frameshifting in bacterial release factor 2 mRNA decoding. A stimulatory downstream RNA pseudoknot structure is present in many eukaryotic antizyme mRNAs. Frameshifting in antizyme is also known to be sensitive to the concentrations of polyamines. These examples illustrate how (in contrast to codon reassignment), recoding events are dependent on favorable sequence contexts that locally alter the interpretation of the codon sequences.

1.2.3 Decoding plasticity in mitochondria.

Global alterations of genetic decoding are frequent in mitochondria. Most known codon reassignments are found in mitochondria (Knight et al., 2001), and pervasive translational bypassing of byps elements was also discovered in mitochondria (Lang et al., 2014). Why are mitochondria so prone to changes of their genetic decoding? There could be several explanations, which are not mutually exclusive.

First, mitochondrial genomes are very small: transfer of genetic material between mitochondrial and nuclear genomes predominantly occurs in one direction (Timmis et al., 2004) leading to extreme mitochondrial genome reduction. The genetic code used for decoding a small genome is easier to modify because a change in a codon meaning would affect fewer codons and fewer genes. Second, endosymbiosis isolated mitochondria from the outer world, making horizontal gene transfer (HGT) to mitochondria an extremely unlikely event. Free-living microorganisms could use HGT to improve their genomes to suit particular environments by obtaining genetic material from other inhabitants of the same

environment. However, to use the foreign genetic material, the recipient needs to use the same genetic language as the donor. This may create evolutionary pressure to maintain a universal genetic code. This pressure is unlikely to exist for organelles that could not use HGT. Third, mitochondrial genome evolution is asexual, thus there is no mechanism for eliminating mildly harmful mutations in contrast to evolution that involves sexual reproduction (Kondrashov, 1988). All these features allow for increased diversity of the molecular processes among mitochondria of different organisms. However, some specific codon reassignments in mitochondria may also be beneficial. AUA reassignment from isoleucine to methionine occurred independently at least twice during eukaryotic evolution (Lang et al., 2014). The resultant increase of methionine content in the mitochondrial proteome may provide mitochondria with protection from its highly oxidative environment (Bender et al., 2008).

1.2.4 Evolutionary routes to codon reassignment.

There are several non-mutually exclusive models for the process of codon reassignment (Knight et al., 2001). The codon capture model (Osawa and Jukes, 1989) involves disappearance of a rare codon accompanied by loss or change of function of its decoder (e.g. UGA and RF2). The next step is reappearance of the codon and its preferential decoding by a different molecule (e.g. near-cognate reading of UGA by tryptophan tRNA, for which the cognate codon is UGG), followed by optimization of the new decoding. This model is supported by the existence of organisms with exceptionally rare codons where expression of the corresponding tRNAs is inessential for growth, e.g. UUA in some *Streptomyces* species (Chater and Chandra, 2008). An example of extreme codon scarcity is the CGG codon that occurs just once in all protein coding genes of *Candidatus Carsonella ruddii* (Nakabachi et al., 2006).

The ambiguous intermediate hypothesis (Schultz and Yarus, 1996) proposes the existence of an intermediate ambiguous state, where a codon has two meanings. A classic example of an ambiguous state is in *Candida albicans* where the CUG-decoding tRNA can be aminoacylated by either seryl-tRNA synthetase or leucyl-tRNA synthetase. As a result Leu and Ser are distributed stochastically in the

Candida proteome at the positions corresponding to CUG codons (Suzuki et al., 1997, Santos et al., 1997). Ambiguous decoding can be beneficial. In response to oxidative stress in mammalian cells, specific phosphorylation of methionyl-tRNA synthetase leads to increased methionylation of non-cognate tRNAs, which increases the amount of methionine in the proteome for protection against reactive oxygen species (Netzer et al., 2009, Lee et al., 2014). A recent example of bacteria that uses two different genetic codes depending on the carbon source is described below. It suggests that a transition from one code to the other does not require a walk through a valley of low fitness as can be imagined in the case of ambiguous decoding leading to the synthesis of presumably harmful aberrant proteins. On the contrary, the organisms could have increased fitness in an environment with oscillating conditions by altering their proteomes to suit the changes. A scenario is plausible in which an organism evolves a regulated genetic code in response to a changing condition and fixes the new variant once the new condition becomes stable.

1.2.5 Regulated codon reassignment.

Pyrrolysine is one of the two non-universal proteinogenic amino acids (the other is selenocysteine) not specified by the standard genetic code. Pyrrolysine is found in methanogenic bacteria and archaea (Srinivasan et al., 2002) and is incorporated at UAG codons. Unlike selenocysteine incorporation (see below), pyrrolysine incorporation uses standard elongation factors and does not require a specialized RNA structure for its incorporation (Namy et al., 2007), although it has been reported that in certain contexts an RNA structure affect the efficiency of pyrrolysine incorporation (Longstaff et al., 2007).

Extreme scarcity of UAG codons and alterations in the mRNA recognition domain of release factors in methanogenic archaea suggest that UAG is not an efficient stop codon and is mostly used for constitutive incorporation of pyrrolysine (Zhang et al., 2005). The frequency of UAG codons in pyrrolysine-utilizing bacteria is much higher and similar to that of other stop codons. Moreover, as both release factors are present, UAG is expected to be recognized as a stop codon.

A recent discovery (Prat et al., 2012) provides a clue to why certain bacteria maintain UAG as a codon for pyrrolysine and as an efficient stop codon.

Acetohalobium arabaticum, which lives in a saline marine environment, can utilize several different sources of energy including trimethylamine (King, 1988). When trimethylamine is available, *A. arabaticum* expresses an operon required for pyrrolysine incorporation, while in the absence of trimethylamine its expression is turned off. Thus, *A. arabaticum* is capable of regulating the meaning of UAG codon in response to environmental conditions (Prat et al., 2012). This marvelous example illustrates the ability of an organism to change its genetic code by using regulated codon reassignment without rewiring its genome.

Another startling example of a regulated genetic code has been proposed in a large metagenomic study of stop codon reassignments, which identified a bacteriophage that encodes RF2 in its genome as well as a tRNA that recognizes UAG codons (Ivanova et al., 2014). These bacteriophages infect bacteria lacking RF2 (with UGA being a sense codon). During the early stage of infection the virus expresses genes using the host genetic code where UGA is sense and UAG specifies a stop). During the later stage of infection when the virus needs to shut off host protein synthesis and redistribute the cell's resources to viral particle production, the virus expresses its RF2 and UAG-recognizing tRNA genes, thus changing the genetic code to one where UGA is a stop and UAG is a sense codon (Ivanova et al., 2014).

1.3 Local codon redefinition

The meaning of a codon can be changed in the context of a specific mRNA or at a specific location within the mRNA. To distinguish it from codon reassignment, this phenomenon is often termed codon redefinition and is considered to be a class of recoding events (Atkins and Baranov, 2010) (see Fig. 1.2 for a visual distinction). Naturally, because codon redefinition takes place in the context of a single or of a subset of mRNAs, these mRNAs should have specific properties or sequence elements that distinguish them from the other mRNAs.

1.3.1 Stop codon readthrough.

Stop codon readthrough is useful when there is a need to synthesize proteoforms with variant C-terminal ends from the same mRNA (Fig. 1.3). Such a situation is highly beneficial for viruses in which stop codon readthrough can be used to economically encode a second product with a C-terminal extension (see refs (Firth and Brierley, 2012, Dreher and Miller, 2006) for reviews). Until recently, stop codon readthrough was reported only in a very small number of cellular chromosomal genes (Steneberg and Samakovlis, 2001, Namy et al., 2003, Namy et al., 2002, Robinson and Cooley, 1997, Klagges et al., 1996). However, emerging evidence indicates that stop codon readthrough can be abundant during cellular gene translation. Phylogenetic analysis of protein coding genes in 12 *Drosophila* species revealed that sequences downstream of annotated stop codons have evolved under constraints of protein-coding selection in almost 300 genes (Jungreis et al., 2011). This indicates that the encoded protein rather than the nucleotide sequences of these regions are important for fruit fly fitness and therefore these sequences are likely to be translated during certain stages of the *Drosophila* life cycle. These predictions were confirmed by ribosome profiling experiments that revealed an even larger number of *Drosophila* genes with detectable stop codon readthrough (Dunn et al., 2013). While frequent stop codon readthrough in these species may be an exception, the number of human genes with documented stop codon readthrough is also growing (Loughran et al., 2014, Eswarappa et al., 2014, Schueren et al., 2014, Stiebler et al., 2014).

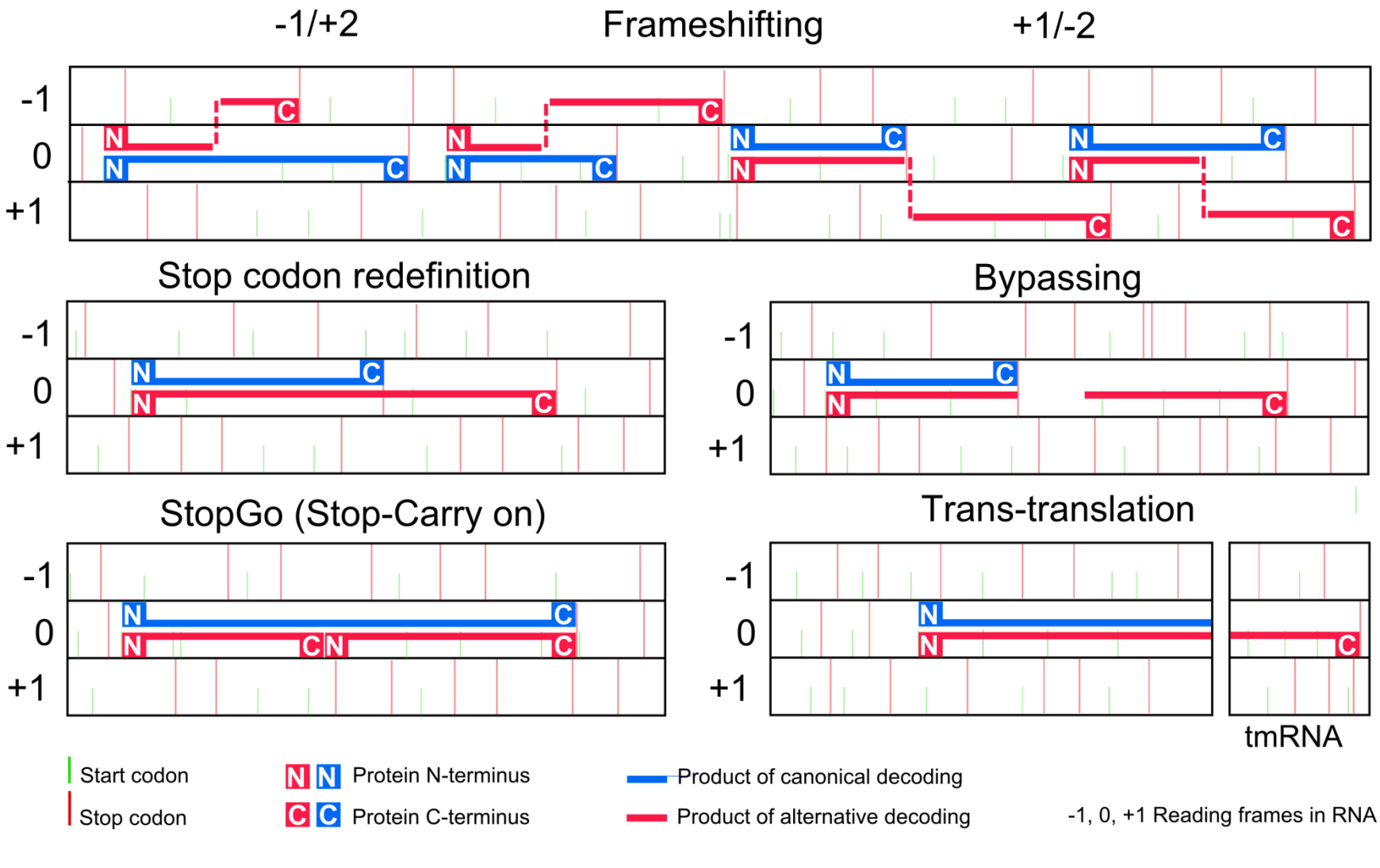

-1/+2
Frameshifting
+1/-2
-1
0
+1
N
C
Stop codon redefinition
Bypassing
StopGo (Stop-Carry on)
Trans-translation
tmRNA
Start codon
Stop codon
Protein N-terminus
Protein C-terminus
Product of canonical decoding
Product of alternative decoding
-1, 0, +1 Reading frames in RNA

Figure 1.3: *Relationship between nucleotide sequences and alternatively decoded proteins. Nucleotide sequences are represented as three horizontal boxes representing three different reading frames. Start and stop codons are shown as vertical lines; green for starts and red for stops. The sequences translated into proteins are shown as horizontal bars with N- and C-terminal ends indicated. The purple bars correspond to standard decoding and orange corresponds to alternative events. Transitions between reading frames are denoted with broken lines.*

Translational termination is slower and less accurate than elongation. Moreover, its efficiency and accuracy are context dependent (Pavlov et al., 1998, Bonetti et al., 1995, Namy et al., 2001). Thus, low-efficiency stop codon readthrough can be achieved in the absence of any sophisticated stimulatory structures. Sequence constraints as short as six nucleotides downstream of a stop codon are sufficient to achieve a readthrough efficiency that is significantly higher than background levels (Skuzeski et al., 1991). Selection for a weak termination context downstream of stop codons is evident among many stop codon readthrough genes; however, a higher efficiency of readthrough often involves additional elements such as RNA secondary structures (Firth et al., 2011).

1.3.2 Selenocysteine incorporation

Selenocysteine (Sec) is one of the two non-universal proteinogenic amino acids and its incorporation into proteins is another type of codon redefinition. Selenocysteine is specified by UGA codons and as with stop codon readthrough this results in synthesis of a protein with a C-terminal extension relative to the corresponding open reading frame (ORF) (Fig. 1.3). However, synthesis of different lengths of alternative proteoforms is not the main purpose of selenocysteine incorporation as only the longest proteoforms are believed to be functional. Rather, selenocysteine is often incorporated into the catalytic centres of certain enzymes to improve their biochemical properties (Lee et al., 2000, Zhong et al., 2000).

The mRNAs of selenoproteins are specified by a special RNA secondary structure, known as a SECIS (SEC Insertion Sequence) element. The structure of

SECIS elements and their location differ between bacteria and eukaryotes. They occur within the coding region of mRNAs in bacteria (Heider et al., 1992), but in the 3' untranslated regions (UTRs) in eukaryotes (Berry et al., 1993), although yet another auxiliary structure (the Sec codon redefinition element (SRE)) occurs within the coding regions of some eukaryotic selenoprotein-encoding mRNAs (Howard et al., 2005, Howard et al., 2007).

The SECIS element is obviously insufficient for this process to take place in any organism. The organism needs to also be able to synthesize Sec and to incorporate it into proteins. These steps involve expression of a number of genes, including those coding for a specialized Sec-tRNA, as well as specialized elongation factors that bring Sec-tRNA to the ribosome and auxiliary factors that allow the recruitment of the Sec-tRNA to only SECIS-containing mRNAs (see refs (Squires and Berry, 2008, Driscoll and Copeland, 2003, Labunskyy et al., 2014, Yoshizawa and Bock, 2009, Rother et al., 2001) for reviews). Acquisition of the selenocysteine machinery in bacteria is conceivably due to frequent horizontal gene transfer from bacteria in which the required genes are organized on a single operon (Gursinsky et al., 2000). In eukaryotes, spontaneous simultaneous transfer of all genes required for selenocysteine specification is unlikely; therefore, the predominant mode for this trait's evolution is its loss, as evident in certain yeast, nematodes (Otero et al., 2014) and insects (Chapple and Guigo, 2008).

The decoding of UGA as a selenocysteine is not 100% efficient, although it is expected to be highly efficient in selenoprotein P mRNA where selenocysteine is incorporated at multiple locations (Hill et al., 1991). For example, selenoprotein P in sea urchin has 28 selenocysteine residues (Lobanov et al., 2008). If we assume that the efficiency of selenocysteine incorporation at each UGA is 90%, only about 5% (0.9^{28}) of the ribosomes translating selenoprotein P mRNA would be able to synthesize the full length protein, while the rest would terminate on one of the 28 UGA codons. Therefore, it has been hypothesized that Sec incorporation is inefficient only at the first UGA codon. Sec incorporation at this codon might serve as a checkpoint for the availability of factors required for selenocysteine incorporation. The efficiency of all subsequent Sec incorporations would be close to 100% (Berry and Howard, 2010).

The sensitivity of Sec incorporation to the availability of selenium varies among different selenoprotein mRNAs (Howard et al., 2005). This allows the synthesis of particularly important selenoproteins at the expense of less important ones under conditions of selenium deficiency. Interestingly, the same selenocysteine insertion machinery can be used to incorporate standard cysteine at UGA codons, albeit inefficiently (Xu et al., 2010). This mechanism provides the possibility to synthesize some levels of a full-length but Sec-depleted variant of a 'selenoprotein' even in the absence of selenium. Although less active, enzymes with a cysteine instead of selenocysteine retain some activity (Lee et al., 2000, Zhong et al., 2000). An inefficient enzyme is better than no enzyme at all.

Selenocysteine is not always specified by a stop codon. In ciliates of the genus *Euplotes*, which possess the selenocysteine machinery, the UGA stop codon is reassigned to code for cysteine (Hoffman et al., 1995). Despite this reassignment, the SECIS structure in the 3' UTR of *Euplotes* mRNA can, at UGA codons in specific locations in mRNAs, direct a proportion of the ribosomes to insert selenocysteine instead of cysteine in the growing polypeptide (Turanov et al., 2009).

Clearly, UGA did not evolve to encode selenocysteine after being globally reassigned to a sense codon in *Euplotes*. In an ancestor of *Euplotes* with UGA specifying a stop, UGA was redefined to specify selenocysteine and only then UGA-stop was reassigned to UGA-Cysteine in the entire genetic code. Therefore, even this seeming sense-to-sense codon redefinition is a product of two evolutionary events, both involving a modification of stop codon meaning (BOX 2). Yet it provokes a question whether other sense-to-sense codon redefinition may exist. It is comparatively simple to find codon redefinition events that involve stop codons due to their dramatic effect on the decoded products (Fig. 1.3). It is easier to detect protein products for which size is substantially altered; it is also possible to use phylogenetic approaches for finding cases of stop codon redefinition as the nucleotide sequence downstream of the redefined stop codons is likely to exhibit signatures of protein-coding evolution (Jungreis et al., 2011). Sense-to-sense codon redefinition in *Euplotes* was discovered primarily to satisfy scientific curiosity regarding how selenocysteine is incorporated in *Euplotes* where UGA is not a stop codon, hence the investigators knew exactly where to look (Turanov et al., 2009). If there are other cases of sense-to-sense codon redefinition, we do not know where to

look for them. It is unclear what would be the benefit of redefining the meaning of a codon from one standard amino acid to another standard amino acid at a single position of a specific mRNA if this could be accomplished with often less than three point mutations. The benefit of sense codon redefinition is obvious only in case of incorporation of non-universal proteinogenic amino acids. While we cannot exclude the possibility that there is a 23rd proteinogenic amino acid (Ambrogelly et al., 2007), so far it has not been found (Lobanov et al., 2006, Fujita et al., 2007).

1.4 Ribosomal frameshifting

Irrespective of the meaning of individual codons in a variant genetic code, most proteins in all modern organisms are expected to be decoded as uninterrupted sequences of nucleotide triplets, with no gaps or overlaps between the codons. Ribosomal frameshifting (Fig. 1.3) is often described as programmed ribosome frameshifting (PRF) when it occurs at a specific location of mRNA, implying that the sequence of mRNA "programs" frameshifting to occur. There is a minority of genes whose expression requires PRF for the synthesis of encoded proteins. Frameshifting may also affect expression of other genes that encode proteins in the standard triplet manner. In this case the synthesis of encoded full-length protein would be abolished. Because of the opposing effects of frameshifting on gene expression, we propose that productive PRF and abortive PRF need to be distinguished and separately defined, therefore we describe them in different subsections below.

1.4.1 Productive PRF

The requirement for ribosomal frameshifting is especially common in viral decoding where it is sometimes used for the same purpose as stop codon readthrough, i.e. to generate proteoforms with alternative C-termini and different functional properties.

Due to the sensitivity of ribosomal frameshifting to cellular conditions, often it is used for regulatory purposes. In this case, synthesis of a functional product depends on ribosomal frameshifting, whereby the product of standard translation is usually dysfunctional and the efficiency of frameshifting is regulated. One such example is decoding of bacterial release factor 2 (RF2) mRNA, for which the

product of its translation, the RF2 protein, inhibits frameshifting and thus downregulates its own expression through a negative feedback loop (Craigen and Caskey, 1986). This mechanism is highly conserved and operates in the vast majority of bacteria (Baranov et al., 2002b, Bekaert et al., 2006). In eukaryotes, frameshifting in antizyme mRNAs is dependent on the concentration of polyamines (Matsufuji et al., 1995) whose synthesis and uptake is regulated by antizyme proteins, and the mechanism operates in all sequenced antizyme genes with the exception of antizyme in *Tetrahymena* (Ivanov and Atkins, 2007). Recently it was discovered that in some bacteria the mechanism of the response to antibiotics also involves frameshifting (Gupta et al., 2013).

Thus far, numerous examples of productive PRF are well documented and extensively studied and, perhaps, can be found in all or almost all organisms (see refs (Cobucci-Ponzano et al., 2012, Firth and Brierley, 2012, Dinman, 2012, Baranov et al., 2002a, Baranov et al., 2006, Atkins and Gesteland, 2010, Farabaugh, 1997, Namy et al., 2004) for selected comprehensive reviews). However, recent years have been particularly fruitful for discovering novel cases of productive PRF due to the abundance of sequenced genomes, which allowed for powerful comparative sequence analyses. These comparative analyses can provide clear evidence of frameshifting functionality by detecting purifying evolutionary selection acting on either the sequence that is required for efficient ribosomal frameshifting or the protein-coding region that requires upstream frameshifting for its expression (Firth et al., 2010a). This approach permitted the identification of a number of novel 'hidden' genes in viruses that require a frameshifting event for their expression (Jiang et al., 2014, Fang et al., 2012, Loughran et al., 2011, Firth et al., 2010b, Melian et al., 2010, Firth and Atkins, 2009, Firth et al., 2008) including one in influenza A (Firth and Brierley, 2012, Jagger et al., 2012).

Similar analyses of bacterial genomes revealed numerous genes that are under the control of ribosomal frameshifting; these genes occur predominantly in transposable elements and prophages (Antonov et al., 2013b, Antonov et al., 2013a, Sharma et al., 2011). As in bacteria, the majority of eukaryotic nuclear genes that are known to utilize frameshifting in their expression are in transposable elements, or their derivatives (Gao et al., 2003). In humans, for example, only five genes that require frameshifting for their expression are currently known: three antizyme

paralogues (*OAZ1*, *OAZ2* and *OAZ3*) (Ivanov and Atkins, 2007) and two transposon-derived genes *PEG10* (Shigemoto et al., 2001) and *PNMA3* (Wills et al., 2006). While it is likely that many cases of productive PRF are undiscovered, it is also likely that productive PRF is very rare among non-mobile chromosomal genes in most organisms (although see below for a noticeable exception involving pervasive frameshifting in *Euplotes*). For instance, comparative sequence analysis of insect genomic sequences revealed only four genes with overlapping coding regions that might be expressed through PRF (Lin et al., 2007). Only one of these genes, encoding a homologue of the human tumour suppressor adenomatous polyposis coli (APC), was confirmed to use ribosomal frameshifting for the expression of an alternative proteoform (Baranov et al., 2011). Ribosome profiling technique (ribo-seq) (Ingolia, 2014) allows determination of the positions of the translating ribosomes on mRNA *in vivo* and with sub-codon resolution. Thus, it can be used for detecting what reading frame is translated in mRNA. Ribo-seq data obtained in yeast and in human cultured cells did not reveal abundant occurrence of ribosomal frameshifting, although it confirmed some of the previously identified cases and revealed a number of sequences that are translated in more than one frame (Michel et al., 2012). Low sequence coverage in early ribo-seq data limited the predictive power of this work; perhaps future applications of similar methods will become more fruitful at identifying novel instances of ribosomal frameshifting.

1.4.2 Abortive PRF

Ribosomal frameshifting does not necessarily lead to the synthesis of functional products. When frameshifting occurs due to limitations of translation accuracy it leads to the synthesis of aberrant products. Thus, it is expected that frameshift-prone sequences should be avoided in most coding regions. Indeed a strong evolutionary selection is observed that eliminates sequences that are strongly prone to frameshifting in protein-coding genes (Shah et al., 2002). Such selection is also observed for some (Gurvich et al., 2003), but not all, weak frameshift-prone sequences (Jacobs et al., 2007, Sharma et al., 2014, Belew et al., 2008), and in either case they are frequently found among coding regions (Gurvich et al., 2003, Jacobs et al., 2007, Sharma et al., 2014, Belew et al., 2008). These occurrence patterns suggest

that abortive frameshifting at low frequency might also be beneficial for regulatory purposes under certain conditions. Such frameshifting has been shown to reduce the stability of those mRNAs where it occurs, thus downregulating expression of the encoded proteins (Belew et al., 2011). Moreover, a human gene, the HIV-1 co-repressor CCR5, where such abortive frameshifting is regulated by miRNAs has been reported recently (Belew et al., 2014).

1.4.3 Frameshifting sites, stimulators and attenuators.

Perhaps one of the reasons why ribosomal frameshifting is widespread as a local decoding alteration is its responsiveness to the sequence elements within an mRNA. Most cases of ribosomal frameshifting require a combination of a frameshifting site (a specific mRNA sequence where frameshifting takes place) and a stimulatory element (a sequence in the same mRNA that increases the efficiency of frameshifting).

With the exception of those cases described later, frameshifting sites alone are insufficient to trigger efficient frameshifting and require one or more stimulatory elements embedded in mRNA. Stimulatory elements can be of diverse types. In bacteria, mRNA complementary to ribosomal RNA may facilitate frameshifting in the +1 as well as in -1 directions (Weiss et al., 1988, Larsen et al., 1994, Prere et al., 2011). Nascent peptides are also known to modulate ribosomal frameshifting (Gurvich et al., 2011, Yordanova et al., 2015). The largest class of stimulators are RNA secondary structures: stem-loops (Kim et al., 2014, Yu et al., 2011, Mazauric et al., 2008), simple (Brierley et al., 1989) and relatively complex RNA pseudoknots containing extra stems (Baranov et al., 2005, Plant et al., 2005, Su et al., 2005) or triple helices (Chou and Chang, 2010, Chen et al., 2009), kissing loops (Herold and Siddell, 1993), G-quadruplexes (Endoh and Sugimoto, 2013, Yu et al., 2014) or long-range interactions (Tajima et al., 2011, Barry and Miller, 2002) (see refs (Giedroc and Cornish, 2009, Chung et al., 2010, Brierley et al., 2008, Atkins and Gesteland, 2010) for reviews). mRNA interacts with various cellular components, and these interactions may alter the stimulatory properties of particular structures or sequences. Both protein molecules (Li et al., 2014) and nucleic acid molecules (Howard et al., 2004, Olsthoorn et al., 2004, Belew et al., 2014) have been shown to

modulate frameshifting in *trans*. The dependence of frameshifting on stimulatory elements, as well as the responsiveness to cellular conditions, provides translation with a powerful regulatory mechanism. Fine regulation can be achieved through the balance between positive and negative regulators; therefore it is reasonable, though nonetheless surprising, that in addition to stimulators, there are also attenuators of frameshifting (Kurian et al., 2011, Su et al., 2005, Plant et al., 2010). The remarkable way in which such an attenuator operates was described in the *S. cerevisiae OAZ1* antizyme gene, where it has been proposed that in the presence of high polyamine levels a nascent peptide in the exit tunnel promotes ribosome stalling at the end of antizyme ORF2. It was proposed that the resultant pile up of ribosomes promotes termination of ribosomes at the ORF1 stop codon, thus reducing the frameshifting efficiency (Kurian et al., 2011).

1.4.4 Frameshifting at triplet repeats.

The frameshifting observed during translation of certain triplet repeats (Stochmanski et al., 2012, Wills and Atkins, 2006, Toulouse et al., 2005, Girstmair et al., 2013) can be described as a combination of frameshifting site and its stimulator. In this case it is difficult to clearly distinguish what is the stimulator and what is the frameshifting site because frameshifting can take place at more than one location in the sequence, which also serves as a stimulator. Decoding a long sequence of consecutive codons corresponding to the same tRNA could lead to depletion of the charged tRNA in the vicinity of the ribosome making the frameshifting on such repeats sensitive to tRNA concentrations (Girstmair et al., 2013). This is consistent with a frameshifting role as a sensor of cellular conditions.

1.4.5 Universal frameshifting.

In some circumstances, stimulators are not needed for efficient frameshifting. In *S. cerevisiae*, up to 40% efficient +1 frameshifting can occur at C.UU_A.GG_C (underscores denote codon boundaries in the initial reading frame and dots denote codon boundaries in the shifted reading frame) (Belcourt and Farabaugh, 1990). The

high efficiency of frameshifting can be explained by a simple kinetic model: upon tRNA slippage from CUU to UUA at the P-site, translation could quickly continue in the new frame due to a high concentration of tRNA cognate for the +1 frame GGC, while translation in the original frame is slowed due to the scarcity of tRNA cognate to zero frame AGG (Baranov et al., 2004). Frameshifting at C.UU_A.GG_C was initially found to be used in transposon Ty1 (Clare et al., 1988) and later was also found to be required for synthesis of ABP140 (Asakura et al., 1998). A few codons other than CUU were found to promote unusually efficient frameshifting when in the P-site (Vimaladithan and Farabaugh, 1994). For all these codons tRNAs with optimal codon–anticodon base pairing are missing in *S. cerevisiae* and there is evidence that frameshifting is strongly promoted by incorporation of near-cognate isoacceptor tRNAs at corresponding codons (Sundararajan et al., 1999).

Therefore +1 ribosomal frameshifting at these heptameric high-efficiency frameshift sequences is a specific feature of *S. cerevisiae* and could be regarded as a feature of its genetic code. This feature affects the evolution of all genes in the genome because occurrence of strong frameshift-prone heptameric sequences would be highly detrimental for accurate protein synthesis of the genes that do not require frameshifting. As a result, these frameshift-prone heptamer sequences are the rarest heptamers in coding regions of *S. cerevisiae* (Shah et al., 2002).

Another case for frameshifting as universal feature of the genetic code has been proposed for vertebrate mitochondria where there are no tRNAs that recognize AGA and AGG codons. These were believed to be stop codons in the genetic code of vertebrate mitochondria. Temperley et al (Temperley et al., 2010) suggested that AGG and AGA codons are not recognized by termination factors, but instead promote -1 frameshifting at N_NN.U_AG.G and N_NN.U_AG.A (where N is any nucleotide). This places the A-site of the ribosome at the overlapping UAG stop codons. If this is true, the vertebrate mitochondrial genome could be described as non-triplet, with AGG and AGA codons as signals for -1 frameshifting rather than amino acid or stops. However, evidence was also provided that AGG and AGA may be recognized as stop codons by mitochondrial release factors (Young et al., 2010).

More recently the frameshifting based model was challenged by a study which suggested that the nascent peptide chain is released from the stalled ribosome with the aid of ICT1, a general rescue factor of stalled ribosomes in mammalian

mitochondria (Akabane et al., 2014). Irrespective of whether Temperley's elegant hypothesis is right, it provides an intriguing scenario for the existence of genetic codes with non-triplet features.

1.4.6 Pervasive frameshifting.

Normally ribosomal frameshifting is very infrequent. This is even true in organisms with universally strong frameshifting patterns such as C.UU_A.GG_C in *S. cerevisiae* because despite their high frameshifting efficiency these heptamers occur only rarely in the genome. By contrast, ribosomal frameshifting in *Euplotes* ciliates is widespread. Analysis of the sequence and expression of several *Euplotes* genes revealed the frequent presence of frameshifting strongly associated with A.AA_U.AA_N and its minor variant A.AA_U.AG_N. It has been estimated that frameshifting is likely to occur in the decoding of 10% of all *Euplotes* genes (Klobutcher and Farabaugh, 2002). As described above the *Euplotes* genetic code is a variant one with the UGA stop codon reassigned to cysteine. The current explanation for the high frequency of frameshifting in *Euplotes* is that the alteration of the mRNA-recognition properties of the *Euplotes* release factors required for the reassignment also weakened recognition of UAG and UAA codons. Inefficient termination at these codons could favour an alternative event, ribosomal frameshifting at AAA codons (Vallabhaneni et al., 2009). It remains unclear, however, whether all occurrences of A.AA_U.AA_N and A.AA_U.AG_N sequences lead to frameshifting in *Euplotes* and with what efficiency. It is also unknown whether these sequences are the only ones that promote frameshifting in *Euplotes*.

1.5 The multitude of stop codon meanings.

Stop codons are clearly the most versatile codons. Nearly all codon reassignments in non-mitochondrial genomes involve stop codons. Stop codons are frequently found in +1 frameshifting sites, a stop codon is required for translational bypassing in T4 bacteriophage gene 60 and all known codon redefinitions involve stop codons (with

the conspicuous exception of selenocysteine insertion in *Euplotes* species (Turanov et al., 2009). See Fig. 1.4 for various examples of altered stop codon meanings.

What makes stop codons so flexible? First, stop codons are the rarest codons in coding sequences. Genes that do not use alternative decoding in their expression need only a single stop codon. As a result stop codons are less frequent than most sense codons by an order of a magnitude. Furthermore, stop codon usage is often skewed (Korkmaz et al., 2014) and as a result a particular stop codon can be exceptionally rare. Thus a codon reassignment of a stop codon may affect only a few codons in the genome. In addition alteration of stop codon meaning is unlikely to dramatically change the properties of protein products. Stop codons are as frequent outside of coding regions as any other triplet, thus if a stop codon is decoded as a sense codon a corresponding protein will be extended only by a few amino acids, as another stop codon is likely to be found downstream.

The other important difference in decoding of stop codons is that they are recognized by release factor proteins rather than by RNA molecules. In fact, the reason why they are recognized by proteins could also be due to their comparatively high evolvability. Release factors in eukaryotes and archaea do not share a common ancestor with those in bacteria, suggesting that the protein-based termination of translation is a relatively recent invention and the last universal common ancestor (LUCA) used a different mechanism (perhaps RNA based) for termination of protein synthesis. Because termination of translation relies on protein factors, the mechanism of mRNA recognition is substantially different. While release factors still recognize stop codons as triplets, they interact with larger region of mRNA and thus efficiency and accuracy of termination is more sensitive to the sequence downstream than in the case of strictly triplet tRNA decoding. As a result, the strength of stop codons varies substantially depending on the sequence downstream of stop codons (Pavlov et al., 1998, Bonetti et al., 1995, Namy et al., 2001). Particularly weak codons are often used for stop codon readthrough or ribosomal frameshifting because the weak termination provides latitude for a competing process.

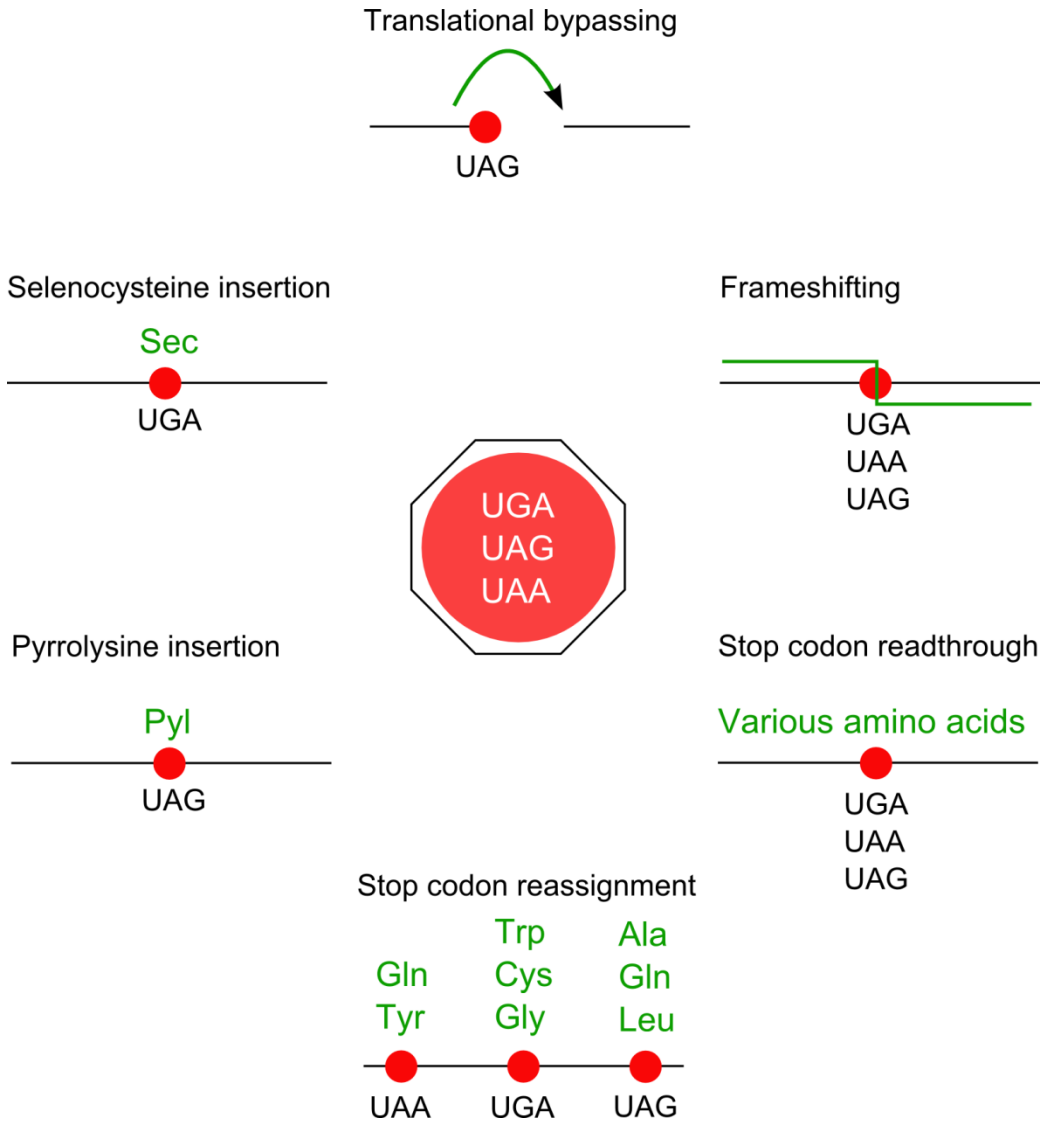


Figure 1.4: *The multitude of stop codon meanings. Stop codons are shown as red circles, and the types of altered meanings are shown in green (for amino acid incorporations or translational bypassing) and grey (for frameshifting).*

1.6 Unusual disruptions of triplet continuity

1.6.1 Translational bypassing.

What happens during translation of bacteriophage T4 gene *60* could be described as a +50 frameshifting, as two codons encoding a pair of adjacent amino acids in its protein product are separated by a 50 nucleotide-long non-coding gap (Fig. 1.3). Ribosomes suspend translation at a specific glycine codon and resume translating at another glycine codon 50 nucleotides downstream. This case was discovered in 1988 (Huang et al., 1988) (Fig. 1.1b), and until recently was the only known example of translational bypassing (also known as ribosomal hopping). The intensive research of the molecular mechanism of translational bypassing revealed a complex nexus of stimulatory elements within the mRNA that includes an enigmatic requirement for a stop codon at the start of the non-coding gap to be dynamically folded into an RNA secondary structure and a role for the specific sequence of the nascent peptide (Wills et al., 2008, Herr et al., 2000a, Herr et al., 2000b, Weiss et al., 1990, Samatova et al., 2014).

The uniqueness of this case, combined with the complexity of the sequence elements involved, seeded doubt about the existence of other examples of translational bypassing. However, recently likely candidates have been identified in *Streptomyces* phages (Smith et al., 2013), with most striking examples of translational bypassing being found in mitochondria of the yeast *Magnusiomyces capitatus* (Lang et al., 2014). The transcriptome of these mitochondria contains dozens of untranslated regions of various sizes which were termed byps to indicate their relationship to the translational bypassing in decoding bacteriophage T4 gene *60*. The exact sequences of byps vary, although they share certain features of primary as well as of secondary RNA structure (Lang et al., 2014). Bypassing at *M. capitatus* byps does not occur when the corresponding sequences are expressed in *E. coli* and hence is likely to require specific features of the *M. capitatus* translational machinery (Lang et al., 2014). Byps are probably mobile genetic elements and it is likely that the *M. capitatus* translational machinery evolved the ability to avoid them

during translation so that insertion of byps into a coding region would not be deleterious (Lang et al., 2014).

1.6.2 Trans-translation

The term trans-translation describes the translation of two messenger RNAs into a single polypeptide (Fig. 1.3). Most bacteria use trans-translation to deal with truncated mRNAs that lack stop codons. Ribosomes that stall at the end of truncated mRNAs are recognized by elongation factor EF-Tu in a complex with a molecule called tmRNA (for transfer and messenger RNA). tmRNA functions as tRNA, as it is charged with an amino acid which is then transferred onto the nascent peptide in the stalled ribosome. It also functions as mRNA, as it contains a short ORF that is decoded after its incorporation into the ribosome. The tmRNA ORF encodes a signal for protein degradation, thus allowing the cell to destroy potentially toxic products of truncated mRNAs and also to recycle the stalled ribosomes (see refs (Keiler and Ramadoss, 2011, Himeno et al., 2014) for reviews and the database of tmRNA sequences (Hudson and Williams, 2014)). This system is remarkably conserved in the bacterial world and has been lost only in a few highly reduced genomes (Hudson et al., 2014). Therefore, trans-translation may be described as a global feature of genetic decoding in organisms with tmRNA, since it universally adds a particular peptide sequence to any protein product of translated ORF that lacks a stop codon.

1.6.3 StopGo.

StopGo (also known as Stop-Carry on) allows two separate peptides to be produced from the same ORF (Fig. 1.3). It was first characterized in an aphthovirus, foot-and-mouth disease virus (FMDV), where it occurs during decoding of a polyprotein-encoding mRNA (Donnelly et al., 2001a, Donnelly et al., 2001b). In this case, ribosomes decode codons according to the standard genetic code and in a triplet manner. However, the continuity of decoding is disrupted by a break at a specific location. The function of this event is parallel to the proteolytic cleavage at specific

sites in other viral polyproteins, but with StopGo there is no requirement for a protease.

1.7 Transcriptional recoding and alternative initiation of translation.

The focus of this Review is on alternative decoding events that take place during translation elongation or termination. However, there are many pre-translational events (occurring co-transcriptionally or post-transcriptionally) that alter genetic decoding in similar ways. C-to-U and A-to-I RNA editing may change the meaning of a codon template in DNA (see refs (Maas, 2012, Kiran et al., 2011, Mallela and Nishikura, 2012) for reviews). Pseudouridinilization of mRNA (Carlile et al., 2014) affects decoding in a complex way and could result in readthrough of stop codons containing a pseudouridine (Karijolich and Yu, 2011).

Programmed transcriptional realignment (PTR) affects decoding similarly to programmed ribosomal frameshifting (PRF) and these two mechanisms are easily interchangeable (Fig. 1.5a). *dnaX* was the first example where the use of PRF and PTR in orthologous genes was revealed (Larsen et al., 2000).

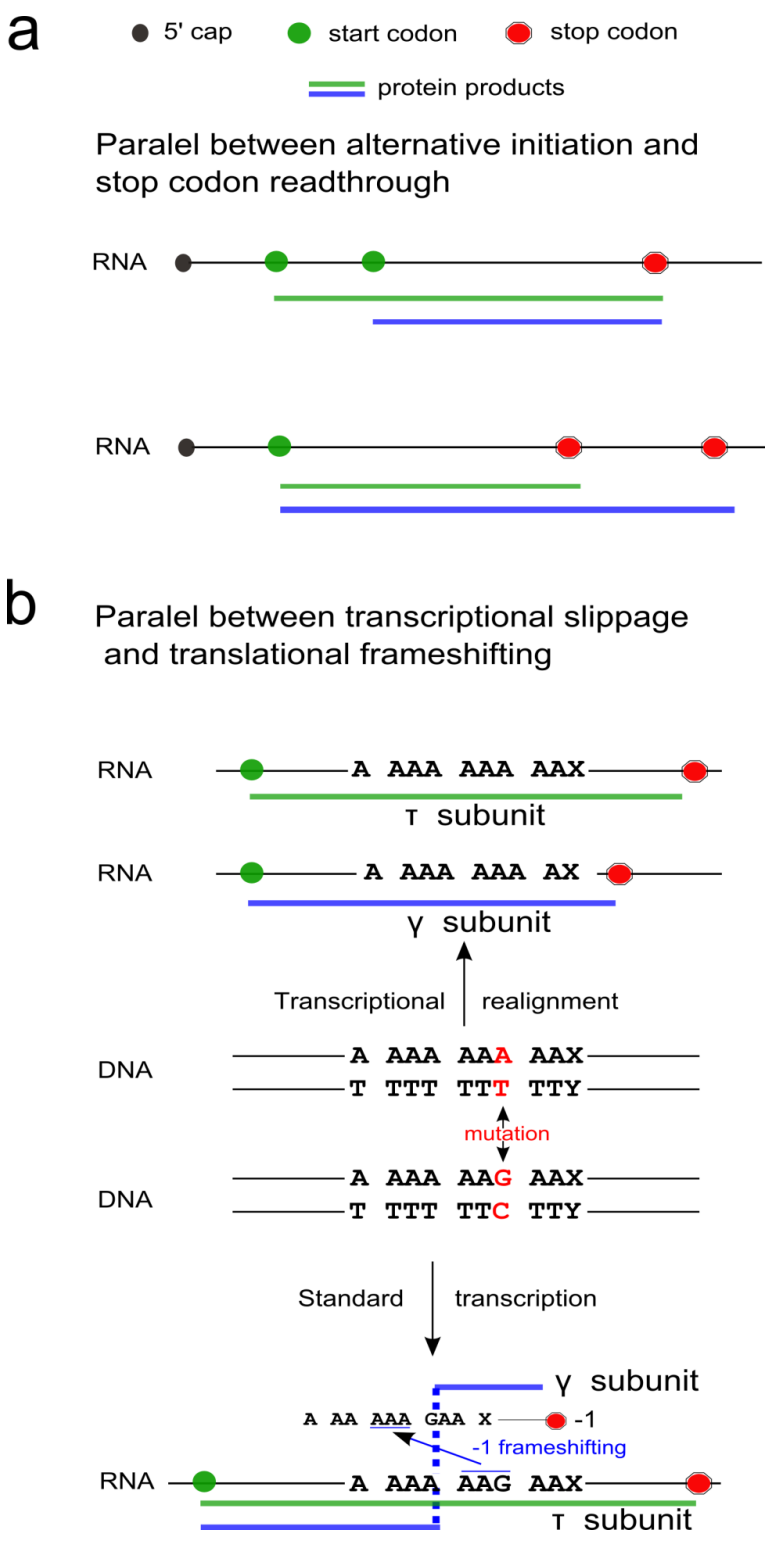


Figure 1.5 *Schematic representation of the parallel between alternative initiation and stop codon readthrough (a) and between transcriptional slippage and translational frameshifting (b).*

Alternative initiation of translation is analogous to stop codon readthrough in the sense that both generate multiple proteoforms, but with variant N-termini in case of alternative initiation and C-termini in case of stop codon readthrough (Fig. 1.5b). Alternative translational initiation is a widespread phenomenon with important implications for gene expression. Many human genes contain evolutionarily conserved protein-coding non-AUG initiated extensions in their 5' leaders (Ivanov et al., 2011) and repeat-associated non-AUG initiation is implicated in human neurodegenerative disorders (Wojciechowska et al., 2014, Kearse and Todd, 2014, Cleary and Ranum, 2013).

1.8 Conclusions and future perspectives

In this Review we focused on alternative genetic decoding occurring during translation elongation. Parallel mechanisms can also be employed during transcription or translation initiation, and we have only briefly discussed them. However, the remarkable flexibility of genetic decoding is evident from the examples discussed. Genetic decoding can be altered globally as a result of modifications to the translation machinery or locally in specific mRNAs that evolve special sequence elements to alter their decoding and to regulate their own translation. A crucial aspect of alternative genetic decoding is relevance to synthetic biologists who can take advantage of genetic decoding plasticity to construct regulatory and sensory genetic modules. Synthetic organisms with genetic codes not found in nature are also being made.

It is unlikely that we are aware of all the ways that genetic decoding can be altered in nature, and future discoveries will undoubtedly continue to surprise and inspire us. Comparative sequence analysis may help us to reveal alternatively decoded genes that are currently hidden from our sight because they do not fit standard gene models used for identification of protein coding genes. However, it is unlikely to reveal the entire spectrum of recoded genes. The power of comparative sequence analysis is limited by the range of species. With higher eukaryotes the problem also lies in deconvolution of signatures of evolution in protein-coding regions in the presence of alternative splicing. Complementary experimental techniques for genome-wide analysis of protein synthesis such as ribosome profiling

have emerged. Although, for many recoded genes it may be difficult to find conditions in which they are expressed, combinations of genome-wide phylogenetic and biochemical approaches are already accelerating the discovery of new cases. The growing repertoire of alternative genetic decoding events will ultimately challenge the way we annotate genes and genomes and how we represent protein coding information in sequence databases.

1.9 Glossary

A-site

The ribosomal site that accommodates either the aminoacyl-tRNA carrying the next amino acid to be added to the growing polypeptide chain or a release factor.

Abortive PRF

Programmed ribosomal frameshifting (PRF) that results in synthesis of dysfunctional protein products or in downregulation of functional protein synthesis.

Ambiguous intermediate

An evolutionary state in the history of an organism evolving a variant genetic code in which a particular codon has two standard meanings.

Byps

Noncoding gaps in mRNAs of mitochondria (in *M. capitatus* and related species) that escape decoding through frequent translational bypassing.

Codon capture

An evolutionary event in which a codon that disappears from a genome, reappears in its descendant and acquires a different standard meaning, thus leading to a variant genetic code.

Codon redefinition

A local change of a codon meaning which is dependent on the context in which it occurs.

Fixed codon reassignment

A complete unconditional change of the standard meaning of a codon.

Frameshifting site

(Also known as frameshift site or shift site). A sequence where ribosomal frameshifting takes place. It includes codons in the A- and P- sites of the ribosomes just before and after the frameshifting. It is useful to describe the sequence of the frameshifting site denoting codons in the original and new frames, e.g. C.UU_A.GG_C. Such representation unambiguously reflects the direction (minus or plus) as well as the mechanism of frameshifting (+1, +2, etc)

Genetic code

A correspondence between 64 triplet combinations of four nucleotides and their standard amino acid or stop meanings.

P-site

The ribosomal site which accommodates the peptidyl-tRNA carrying the growing polypeptide chain.

Productive PRF

A type of programmed ribosomal frameshifting (PRF) that is required for the production of a functional protein product.

Programmed ribosomal frameshifting (PRF)

Ribosomal frameshifting that is programmed (by a sequence context) to occur at a specific mRNA location.

Proteinogenic amino acids

Amino acids that are incorporated into proteins co-translationally.

Proteoforms

Groups of sequence-related proteins arising from the same mRNA.

Purifying evolutionary selection

The removal of disadvantageous traits. In the case of protein-coding sequences it results in a higher rate of synonymous substitutions relative to non-synonymous substitutions.

Recoding

A process of context- or condition-specific alteration of the genetic decoding.

Regulated codon reassignment

A conditional change of the standard meaning of a codon.

Ribosomal frameshifting

A process in which a ribosome changes its reading frame.

SECIS element

(Also known as Sec insertion sequence). An mRNA secondary structure that functions as a stimulatory element for selenocysteine incorporation.

Standard meaning

The way the translational machinery interprets a codon (coding for a proteinogenic amino acid or a signal for translation termination) unless it occurs in a special context.

Stimulatory element

An mRNA element that is required for the efficient local alteration of genetic decoding.

StopGo

(Also known as Stop-Carry on). A process in which production of a polypeptide chain is interrupted at a specific place while triplet mRNA decoding continues. This results in production of two protein products from a single open reading frame.

Stop codon readthrough

A redefinition of a stop codon to a sense codon irrespective of functional implications of the identity of incorporated amino acid.

Isoacceptors

Different tRNA species carrying the same amino acids but with different anticodon sequences

Trans-translation

A process in which a single protein is translated from two mRNA molecules as templates.

Translational bypassing

A process in which ribosomes skip three or more nucleotides without decoding.

Variant genetic codes

Genetic codes that differ from the standard genetic code shown in Fig. 1.1a.

Acknowledgements

This review was inspired by the discussions during EMBO Workshop “Recoding: Reprogramming genetic decoding” that took place in Killarney, Ireland during 13-18 of May 2014. Therefore we are grateful to all participants who shared their ideas and experimental data during this meeting. We apologize to our colleagues whose relevant works we did not cite in this review as it has not been intended as a comprehensive review and we aimed to keep it concise.

Chapter 2

Regulatory nascent peptides.

2.1 The ribosomal exit tunnel

The formation of the peptide bond during protein synthesis occurs in a cleft of the large subunit of the ribosome near the interface between the two subunits , at a site referred to as the peptidyl transferase centre (PTC) (Simonovic and Steitz, 2009). Following the peptide bond formation, the growing peptide chain leaves the ribosome through the exit tunnel in the large ribosomal subunit (Bischoff et al., 2014).

The tunnel is about 100 Å long and 20 Å wide and accommodates a 30 to 40 amino acid long poly-peptide chain in its extended conformation. The tunnel can be separated into several areas – the upper tunnel is the closest to PTC; the constriction area is the narrowest part of the tunnel where residues of the ribosomal proteins L4 and L22 (naming as in bacteria; in eukaryotes the protein L17 is the homolog of the bacterial L22) protrude; the lower tunnel, and the vestibule next to the tunnel exit. Owing to its confined volume, the tunnel cannot accommodate bulk domains with tertiary structure. However, alpha-helix formation was observed in the lower tunnel (Wilson and Beckmann, 2011) and Nilsson et al., have demonstrated that small domains such as ADR1a domain can fold within the vestibule of the exit tunnel (Nilsson et al., 2015). The authors have suggested that similarly to chaperones, that bind to the emerging nascent polypeptide chain and facilitate its proper folding, the interaction of the nascent polypeptide with exit tunnel components might be promoting the folding of some small domains (Nilsson et al., 2015).

The ribosomal tunnel is predominantly composed of large ribosomal subunit rRNA. Amino acid residues of large ribosomal subunit proteins also participate in the shaping of the tunnel, such as residues of the proteins L4 and L22 (L17 in eukaryotes) which form the tunnel constriction. The dominant occurrence of rRNA

results in an overall negative environment of the tunnel (Lu et al., 2007, Lu and Deutsch, 2008). The negative potential is the least in the area just next to the PTC and the greatest in the area of the constriction (Lu et al., 2007). Consistent with the negative charge of the tunnel, a stretch of positively charged amino acids while in the tunnel has been shown to cause the translating ribosomes to pause (Lu et al., 2007). The negative potential however is not static – as it moves through the tunnel, the nascent chain can alter the tunnel environment locally (Lu et al., 2007). This is accomplished in part by the charged residues of the nascent chain. In addition, the bulkiness of the amino acids side chains and the local conformations of the polypeptide chain within the tunnel determine the access of water and ions that shield the tunnel charges.

Originally the tunnel was considered to be immune to interaction with the growing peptide chain. However further discoveries have shown that the tunnel is not simply a passive conduit that contains the nascent polypeptide chain until it emerges at the surface of the large subunit. The interactions of the nascent polypeptide within the tunnel of the ribosome may pause or even stall the ribosome. A class of stalling nascent peptides employ this feature for regulatory purposes. Such peptide sequences can stall the ribosome during translation elongation or termination. In bacteria, several cases are known where stalling at regulatory leader peptides promotes the reorganisation of RNA secondary structure, resulting in exposure of the Shine-Dalgarno sequence of the downstream cistron, upregulating its translation (Murakami et al., 2004, Gong and Yanofsky, 2003). Stalling at uORFs in eukaryotes reduces the number of ribosomes reaching the main ORF start codon and thus inhibiting translation of the main ORF. The stalling may be achieved with polypeptides that are shorter than the length of the ribosome tunnel and is often dependent on the presence of small molecules like antibiotics or amino acids.

Several stalling peptides are well described, surprisingly however they display very low sequence similarity. Despite the lack of consensus sequence, a prolyl-tRNA is often found in the A-site. Proline is an imino acid because the nitrogen of the amino group involved in peptide bond formation is in addition bound to the carbonyl of the side chain. This results in a reduced reactivity of the amino

group and accounts for the slow kinetics of peptide bond formation with prolyl-tRNA in the A-site (Pavlov et al., 2009). It has been suggested that the slow kinetics of peptide bond formation with proline as a substrate may provide a time interval for the formation of specific contacts of the nascent chain within the tunnel, resulting in ribosome stalling. In bacteria, where it has been shown that runs of prolines cause ribosome stalling, the translation factor EFP was found to alleviate the stall at polyproline stretches. A recent study demonstrated that the major determinant of the slow kinetics of the reaction is the suboptimal orientation of Pro-tRNA in the PTC. It was established that EFP facilitates the peptidyl transferase reaction by promoting catalytically productive orientation of the Pro-tRNA in the PTC (Doerfel et al., 2015).

Regulatory nascent peptides, alone or in concert with other molecules (e.g. antibiotics or small metabolites) establish contacts with tunnel components. These interactions are transformed into instructions for the ribosome to alter its behaviour in response to environmental cues. Mechanistically this is achieved by relaying allosteric changes, initiated by the interactions of the nascent peptide with tunnel components, to the residues in the PTC resultant in an impaired PTC function.

The growing polypeptide chain is attached through an ester bond to the 3′ hydroxyl of the 3’ terminal ribose of the P-site tRNA. In the absence of an A-site substrate, residues in the PTC in particular U2585 (see Table 2.1) shield the peptidyl ester linkage, protecting it from hydrolysis by water molecules. Upon binding of an A-site substrate (aminoacyl-tRNA or a release factor) a rearrangement of the residues in the PTC is observed (Voorhees et al., 2009) making the peptidyl ester available for nucleophilic attack. The precise positioning of the peptidyl ester linkage in the P-site and the nucleophilic amino group of the aminoacyl-tRNA in the A-site is crucial for peptide bond formation and it is maintained by the specific orientation of the 23S residues in the PTC. Similarly, the correct positioning of the release factor and in particular that of its GGQ domain, is crucial for efficient termination to occur, and it is mediated by rRNA residues (Brown et al., 2015). The dynamic orientation of PTC residues is therefore reflective of the ribosome functional state. This is why even the slightest change in the orientation of the PTC

residues can be inhibitory on the peptide bond formation or hydrolysis (Wilson and Beckmann, 2011).

There are two main areas where the nascent peptide chain communicates with tunnel components; the constriction area in the ribosome tunnel and the area of the upper tunnel close to the PTC where rRNA residues can directly transmit the signal to the PTC. The rRNA residues of the PTC are the final receivers of the signal initiated by the contact between the nascent polypeptide chain with tunnel components. Most rRNA residues in the PTC are universally conserved and mutating some of them was shown to be lethal. Naturally occurring substitutions account for functional differences. Such is the case with adenine at position 2058 (A2058) in bacteria which has been naturally substituted for guanosine in eukaryotes and archaea. This is considered to be a major factor contributing to erythromycin resistance exhibited by eukaryotes and archaea and not by bacteria (Dunkle et al., 2010). However, some bacteria can temporarily modify A2058 in response to erythromycin and other similar antibiotics. The inducible methyltransferases add a methyl group to A2058 which alteration is considered as the main factor for the acquired antibiotic resistance of these bacteria.

Owing to their importance, mutating many of the rRNA residues in the PTC is lethal. Hence, direct testing of the effects of specific residues on translation was not possible till now. Recent studies described the creation of the first fully orthogonal ribosome–messenger RNA system (Orelle et al., 2015, Fried et al., 2015). It allows for testing the effect of various alterations of the ribosome, including mutations of the PTC residues known to be otherwise lethal. Indeed using this system generated the first direct evidence for the role of a specific 23S rRNA nucleotide, namely A2451, in SecM programmed ribosome stalling (Orelle et al., 2015).

A2058	-part of the binding site of macrolide antibiotics such as erythromycin	
A2059	-part of the binding site of macrolide antibiotics such as erythromycin	
U2585	-catalysis of peptide bond formation -The second most flexible residue in the PTC -conformational state reflects the functional state of the ribosome	(Schmeing et al., 2005), (Voorhees et al., 2009)
A2602	-The most flexible residue in the PTC -conformational state reflects the functional state of the ribosome -crucial for the proper peptidyl hydrolysis activity -mutations strongly reduce SecM and ErmCL-mediated stalling but have no effect on TnaC- stalling	(Vazquez-Laslop et al., 2010) (Wilson and Beckmann, 2011)
A2053	-a crucial component of the tunnel wall, that together with the nearby A2062 transmit signal from the tunnel to the PTC -mutations strongly reduce SecM and ErmCL-mediated stalling but have no effect on TnaC- stalling	(Vazquez-Laslop et al., 2010). (Wilson and Beckmann, 2011)
Residues L4 and L22	-binding site for some antibiotics. -mutations in these residues alleviate the effect of nascent peptide mediated stalling	(Tu et al., 2005)

Table 2.1: *Role of some prominent components of the tunnel wall and the PTC.*

2.2 Regulatory peptides in Bacteria

2.2.1 SecM

In bacteria the export of proteins through the Sec translocon is powered by the motor protein SecA, an ATPase (Denks et al., 2014). SecA expression is regulated in accordance with the secretion needs of the cell (Oliver et al., 1998). Monitoring the secretory status of the cell relies on a regulatory mechanism that takes place during the translation of SecM (secretion monitor) protein (Nakatogawa and Ito, 2001). In its N terminus the protein SecM carries a secretory signal which guides its export through the membrane into the periplasmic space where SecM is quickly degraded. The 170 codon long SecM ORF is located upstream of SecA in the same operon. The amino acid sequence starting at Phenylalanine (Phe) at position 150 and ending at Proline (Pro) at position 166 acts from within the ribosome exit tunnel to stall the translating ribosome with SecM1-165-tRNA^{Gly} in the P-site and Prolyl166- tRNA^{Pro} in the A-site (Nakatogawa and Ito, 2002). As the arrest sequence is close to the C terminus of the protein, at the moment of stalling, most of the protein has already emerged at the surface of the ribosome. This newly synthesised polypeptide chain is bound by the components of the translocation machinery. The ribosome stalling is released upon the 'pulling' of the protein chain exerted by the co-translationally occurring translocation process (Butkus et al., 2003). Ribosome stalling at the SecM arrest sequence, allows for rearrangement of an mRNA secondary structure that exposes the Shine-Dalgarno sequence of the downstream SecA. In conditions where SecM is efficiently translocated through the membrane, the stalling at the arrest peptide is transient. Therefore normally, SecA is constitutively expressed at low levels (Murakami et al., 2004). In case of secretion deficiency the duration of the stall is prolonged and this results in an increase in SecA expression (Murakami et al., 2004) (Fig.2.1).

Mutational analysis has identified 9 key residues in the SecM arrest peptide sequence from *E. coli* (shown in bold) ^{150} **F**STPV**WI**SQAQ**GIRAGP**^{166}. Mutating

these residues reduced or completely alleviated the stalling (Nakatogawa and Ito, 2002). However, a more extensive analysis has revealed an unexpected flexibility regarding the identity of residues in the arrest peptide. Only Arg163 was indispensable (Yap and Bernstein, 2009). Therefore, it was proposed that the role of the non-essential residues was to promote the formation of specific SecM nascent peptide conformation in the tunnel. Perhaps different combinations of amino acid residues can fold the nascent peptide in the tunnel in a way to bring the essential Arg163 residue in contact with specific tunnel components. Such interactions could initiate allosteric rearrangements that are relayed to the PTC and result in interfering with peptide bond formation to promote stalling. The authors have suggested that the presence of essential and context-specific residues might be a general feature of arrest peptides that could explain their sequence diversity (Yap and Bernstein, 2009).

Several mutations in tunnel components that disrupt stalling at SecM were identified. One such was a mutation of the 23S rRNA nucleotide A2058 which faces the interior in the constriction area of the tunnel. Mutation at that position alleviated the stalling (Nakatogawa and Ito, 2002). Similarly the stalling was alleviated by a nucleotide insertion within the A749–A753 region, also located in the constriction area however at the opposite tunnel wall. Mutations of several residues of the large ribosome protein L22 that protrudes into the constriction area were also among those that alleviated SecM mediated stalling. The mutation of A2053, which was identified as an important component of the relay system that connects the tunnel and the PTC, also abolishes stalling with ErmCL as well as with SecM (Vazquez-Laslop et al., 2010). It is likely that A2053 acts together with the closely positioned A2062. A2053 and A2062 are proximal to PTC and their interaction with the indispensable Arg residue has been revealed by CryoEM (Gumbart et al., 2012)

The translation pause at the SecM arrest sequence is released by the pulling force exerted by the association with the translocon. It was demonstrated that a similar pulling force could also be generated by the co-translational folding of the newly produced polypeptide chain at the surface of the ribosome (Goldman et al., 2015). When sequence encoding protein domain with a known folding potential was connected by a linker upstream to the arrest peptide encoding sequence, the force

generated from the folding of the domain at the ribosome surface could rescue the stalled ribosome and increase the translation rate. This opens the possibility that such coordination between co-translational folding and elongation rate might be a more general feature of the translation process. For example, pausing at certain sequences during elongation can provide the means for monitoring the folding status of the polypeptide molecule that already has emerged from the exit tunnel. The pulling force generated by proper folding of the chain would rescue the stalled ribosome and signal translation to continue. In case of a folding defect, the stalled ribosome could not be rescued and prolonged stalling may result in triggering different rescue mechanisms.

The binding of protein partners to the emerging chain could be another means for generating force to alleviate stalling and hence modulate the translation elongation rate (Goldman et al., 2015).

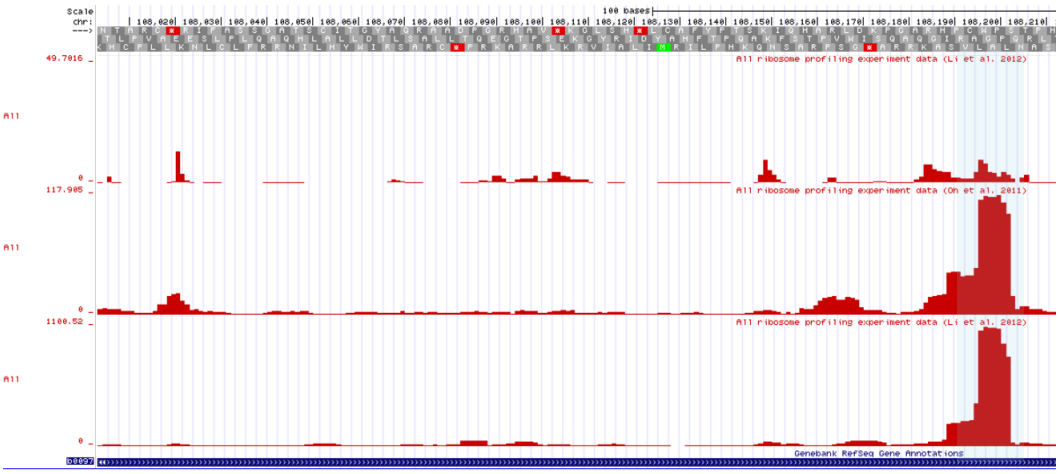


Figure 2.1: *Ribo-seq (red) coverage in a portion of the SecM transcript from three different studies. The light blue panel indicates the position of the peak corresponding to the site of nascent peptide mediated stalling during SecM translation elongation. First row illustrates data generated by (Liu et al., 2013), second row (Oh et al., 2011), and third row (Li et al., 2012). The peak at the end of*

SecM is not noticeable in the first row where data was obtained in the conditions of bacteriophage lambda infection. The image was obtained from GWIPS Viz.

2.2.2 TnaC

The TnaC gene in the E. coli tryptophanase operon encodes a regulatory leader peptide that 'senses' tryptophan (Trp) levels (Gong and Yanofsky, 2002, Gong et al., 2001). It regulates the expression of 2 structural genes further downstream in the operon; TnaA and TnaB which encode enzymes for Trp degradation (tryptophanase), and export (permease) respectively (Deeley and Yanofsky, 1981, Edwards and Yudkin, 1982).

TnaC is an example of a mechanistic coupling of transcription and translation (Ito and Chiba, 2013, Gong and Yanofsky, 2003). The translation of TnaC regulates the transcription of TnaAB in the Tna operon. Observe that this is possible in bacteria because their transcription and translation are not separated either spatially or temporally. The RNA polymerase transcribing the Tna operon stalls at the beginning of the TnaA gene. In order to proceed, it needs a 'push' from the ribosome translating TnaA. With high Trp the translating ribosomes stall at the end of TnaC, preventing the reformation of a secondary mRNA structure and in this way exposing the TnaA Shine-Dalgarno sequence. Ribosomes can now initiate translation of TnaA and catch up with the paused RNA polymerase. The latter can then proceed transcribing the TnaAB operon (Gong and Yanofsky, 2003). The stalling is seen with ribosome profiling data (Fig. 2.2). In low Trp levels, the ribosomes translating TnaC terminate at the stop codon and dissociate, allowing reformation of the mRNA structure that hides the Shine-Dalgarno sequence. In addition, when there is no a stalled ribosome to hinder its binding sites, the transcription termination factor Rho binds to the mRNA sequence between TnaC and TnaAB. Transcription termination then occurs prior to TnaAB transcription (Konan and Yanofsky, 2000, Gong and Yanofsky, 2003).

The ribosome translating TnaC stalls with peptidyl-$tRNA^{pro}$ in the P-site and the UGA stop codon in the A-site (Gong et al., 2001). Extensive mutagenesis of the

24 amino acid long TnaC polypeptide has revealed the identity of critical residues as well as their spacing relating to the P-site. A recent CryoEM study has provided a detailed view of the nascent TnaC peptide within the exit tunnel (Bischoff et al., 2014). The authors revealed that the nascent peptide acquires specific conformation which allows for the formation of two hydrophobic pockets, in each pocket a Trp molecule was found to be bound.

One of the Trp molecules binds between nucleotides A2058 and A2059 on one side of the tunnel and U2609 and A752 on the other (Bischoff et al., 2014). The nascent peptide interacts with L4 and L22 residues of the constriction area of the tunnel. In particular, the indispensable TnaC residues D16 and W12 were shown to interact with residues of L22. These and other interactions relay the signal to the PTC where residues U2585 and A2602 acquire conformations that hinder proper RF2 binding. This causes the observed ribosome stalling with the stop codon in the A site (Bischoff et al., 2014). Most of the rRNA nucleotides and the protein residues that were identified to interact with the nascent peptide chain, were also already established in mutational analysis as crucial participants required for the stalling effect in response to Trp (Cruz-Vera et al., 2007).

In the model derived with the CryoEM data, the TnaC nascent peptide forms a specific conformation within the exit tunnel accomplished via interactions between peptide residues with components of the tunnel. In this specific conformation two hydrophobic pockets form in the tunnel. When high levels of Trp are available two Trp molecules are accommodated in the hydrophobic pockets. The bound Trp molecules interact with specific 23S rRNA nucleotides as well as with TnaC residues further stabilising a particular nascent peptide conformation within the tunnel. This conformation triggers allosteric rearrangements resulting in the positioning of nucleotides from the PTC in such a way as to impair the proper binding of the release factor which results in ribosome stalling.

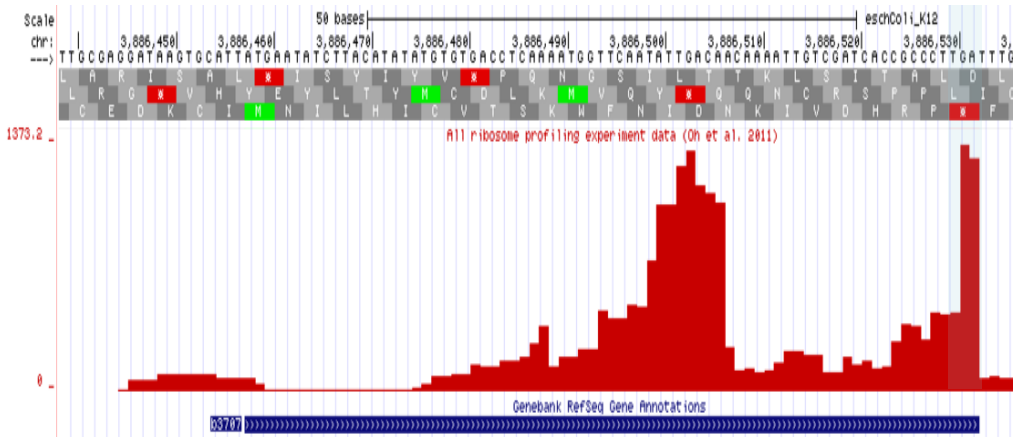


Figure 2.2: *Ribo-seq (red) data coverage in the TnaC gene from the tryptophanase operon in E.coli. The light blue panel indicates the position of the peak corresponding to the site of TnaC mediated stalling. The peak to the left may represent the second stalled ribosome.*

2.2.3 ErmCL

Inducible erythromycin resistance methylase (erm) genes in bacteria render resistance to macrolide antibiotics such as erythromycin. The expression of the erm genes is regulated at the level of translation by a programmed ribosome stalling event, mediated by arrest leader peptides.

Upon entering the ribosome exit tunnel, erythromycin establishes contacts with specific rRNA and protein components of the tunnel. The bound antibiotic limits the conformational freedom of the nascent chain in the tunnel stabilizing specific interactions of the latter with tunnel components. Together the interactions of the macrolide and the nascent chain with tunnel components result in PTC rearrangements that inhibit protein synthesis. These rearrangements can impede peptide bond formation between particular donor and acceptor amino acids, or preclude the accommodation of the A-site tRNA as in the case of ermCL (Ramu et al., 2011, Kannan et al., 2014). Interestingly erythromycin inhibits peptide bond formation of different combinations of donor and acceptor amino acids with different efficiency, thus its inhibition of protein synthesis is selective to a certain degree (Kannan et al., 2014).

The inducible erm genes, such as ermC, have 'learnt' how to take advantage of the erythromycin inhibitory mechanism for induction of their own expression (and for providing bacteria expressing erm cassettes with resistance to antibiotics.) A regulatory region in the ermC 5'leader encodes a 19 amino acid long leader peptide, ErmCL. In the absence of antibiotic, the expression of the main cistron is inhibited due to the unavailability of its Shine-Dalgarno sequence and start codon, sequestered in an RNA secondary structure. Erythromycin causes the translating ribosome to stall at codon 9 of ErmCL, with the peptidyl-$tRNA^{Ile}$ in the P site. Following this, RNA is refolded and the Shine-Dalgarno sequence becomes available for ribosome binding, which results in translation of the main cistron.

The ermC product is an rRNA methylase that adds methyl groups to N^6 of the 23S rRNA residue A2058. This methylation is considered to be a major factor for the acquired resistance to erythromycin and similar antibiotics, due to the resultant conformational changes of the ribosome, interfering with the accommodation of the antibiotic.

Unlike other stalling peptides such as SecM or TnaC, ErmCL is only 9 amino acids long and thus it does not span the entire ribosome tunnel. The 9 residue long nascent chain has the sequence ^{1}MGIFSIFVI^{9}. Mutations have revealed that the residues of the C terminus, Ile6, Phe7, Val8, and Ile9 are crucial for the stalling to occur, while Met1, Gly2, Ile3, Phe4 and Ser5 at the N terminus are dispensable, although they are required for an optimal efficiency. These results are consistent with studies of other nascent peptides that have stalling effect in the presence of an antibiotic, for which only the sequence at the very C terminus is critical for the effect. It has been proposed that the antibiotic predisposes the ribosome for stalling even with very short arrest peptides in the tunnel (Sothiselvam et al., 2014).

The antibiotic bound in the exit tunnel narrows the tunnels space and promotes a nascent peptide conformation resultant in contacts with the tunnel and PTC. Specifically valine at position 8 interacts with U2506 and isoleucine at position 6 – with U2586, supporting previous studies where mutations of these amino acids to alanine have greatly reduced ribosome stalling (Johansson et al., 2014, Vazquez-Laslop et al., 2008).

Mutations of the rRNA nucleotides A2053 and A2062 alleviated the erythromycin induced stalling at ermCL and identified these nucleotides as crucial component of the relay system sending signal to the PTC. A2053 is located at the tunnel wall close to PTC, and it has been demonstrated that changes of its conformation or methylation status directly induce changes in PTC. It has been identified as a crucial component of the tunnel wall, which together with the nearby A2062 transmit signal from the tunnel to the PTC (Vazquez-Laslop et al., 2010).

Eventually the interactions within the tunnel result in conformational change in A2585 in the PTC. As discussed above, U2585 is located in the PTC active site and is critically involved in the catalysis of peptide bond formation (Schmeing et al., 2005, Voorhees et al., 2009). This prevents the correct positioning of the A site serine tRNA and hence the formation of a peptide bond is inhibited.

Mutations of specific ErmCL amino acid residues also alleviated the stall (Johansson et al., 2014).

2.3 Regulatory peptides in Eukaryotes

2.3.1 CGS1

The CGS1 gene expressed in plants encodes cystathionine γ-synthase, an enzyme of the methionine biosynthetic pathway. Its expression is regulated with a feedback loop where a derivative of methionine, S-adenosyl-methionine (also known as SAM or AdoMet), has been shown to downregulate the translation of CGS1.

Toe-print analysis of *in vitro* translation reactions in wheat germ extract (WGE) has revealed that ribosomes transating CGS1 mRNA stall at the Ser codon at position 94 in the presence of AdoMet (Onouchi et al., 2005). Mutational analysis of the amino acid sequence preceding the stalling site has identified that a core peptide of 11 amino acids with the sequence RRNCSNIGVAQ, was essential for inducing the stalling (Ominato et al., 2002). Located 6 residues N' terminal to Ser-94, at the time of the stall, this peptide most likely occupies the tunnel constriction. Although

there are no structural studies of the CGS1 nascent peptide in the ribosome tunnel, it is conceivable that similarly to other regulatory schemes involving nascent peptides such as TnaC in *E. coli,* when in high concentration AdoMet interacts with the nascent peptide in the tunnel. This putative interaction may stabilise specific contacts with the tunnel components that trigger allosteric changes, resulting in rearrangements in the PTC. Therefore, the translating ribosome with CGS1 nascent peptide in the tunnel could act as a sensor for AdoMet levels.

It was demonstrated that at least two ribosomes are tightly stacked behind the one stalled at Ser codon at position 94 (Yamashita et al., 2014). In addition, a variety of 5' degradation intermediates were identified for CGS1 mRNA. These and other findings are supportive of ribosome stacking inducing RNA degradation (Yamashita et al., 2014). The translation profile of CGS1 mRNA shown in figure 3, confirms the strong pause at Ser-94, already demonstrated by different techniques.

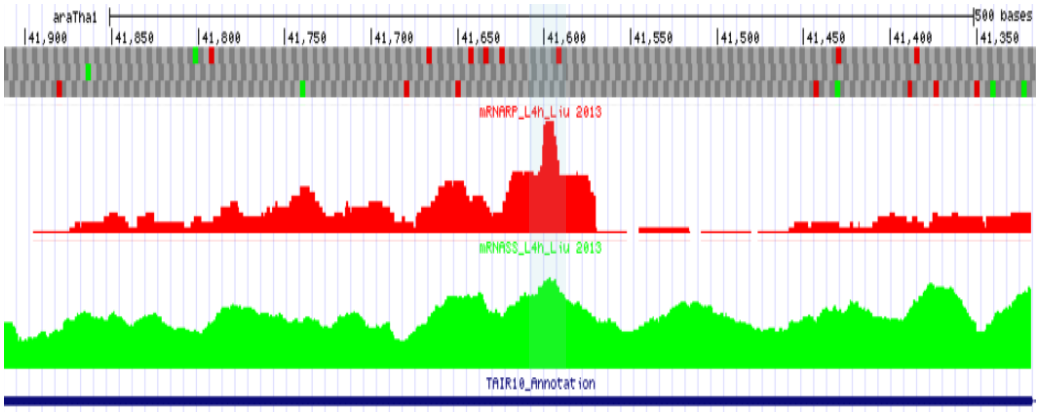


Figure 2.3: *Ribo-seq (red) and mRNA-seq (green) coverage in the first part of the first exon of CGS1 gene in Arabidopsis thaliana. The light blue panel indicates the stalling site that takes place during translation elongation at serine at position 94.*

2.3.2 MAGDIS

S-adenosylmethionine decarboxylase (AdoMet DC) is one of the key enzymes in polyamine biosynthesis (Pegg, 2009b). AdoMet DC catalyses the decarboxylation of

S-adenosyl methionine (AdoMet or SAM). The decarboxylated S-adenosyl methionine (dcAdoMet) has a single known function in the cell, which is of a donor of aminopropyl groups for the synthesis of spermidine from putrescine and that of spermine from spermidine. The reactions are catalysed by spermidine and spermine synthases respectively (Pegg, 2009b).

dcAdoMet is committed to polyamine synthesis and its production is a rate limiting step for this process. Therefore, AdoMet DC expression levels are finely tuned. Regulation of transcription and protein half-life are important aspects. The translation of AdoMet DC mRNA provides an additional layer of regulation that employs a nascent peptide responsive to polyamine levels (Hill and Morris, 1993, Ruan et al., 1996).

The nascent peptide occurs in a six codon ORF upstream of the main coding ORF. This encodes the peptide sequence MAGDIS, which is used to refer to the nascent peptide. Mutational analysis of MAGDIS has identified the amino acid residues essential for stalling (Mize et al., 1998). Aspartate (D) and Isoleucine (I) at positions 4 and 5 are almost obligatory for stalling; altering the aspartate residue to any other amino acid disables stalling. Isoleucine at position 5 may only be substituted with a valine residue. Many amino acid residues were tolerated at position 6 (Mize et al., 1998). The strict requirement for particular amino acid residues at those two positions and the fact that these are the two most conserved residues, suggests that these in particular might be involved in interactions with components of the translational machinery in the vicinity of PTC, resulting in ribosome stalling (Fig.2.4). The stalling is enhanced in the presence of high polyamine levels suggesting that polyamines can stabilise the putative interactions of the nascent peptide and the ribosome.

Just six residues long, MAGDIS occupies only the upper portion of the exit tunnel. By comparison to other stalling peptides for which structural data exists, the conserved MAGDIS residues at positions 4 and 5 should be located in the vicinity of U2585 and A2062 at the time of stalling. It is conceivable that polyamines when in high levels in the tunnel, facilitate or promote a specific conformation of the nascent

peptide such that would stabilise specific contacts with tunnel residues which then result in stalling.

The regulatory uORF's initiation codon is positioned unusually close to the mRNA cap - only about 14 nucleotides from it. Initiation at AUG codons located very close to the 5'cap is known to be less efficient (Kozak, 1991a, Kozak, 1991b) owing to ribosomes failing to recognise the initiation codon. It has been tempting to speculate that the position of the uORF starting so close to the cap is a component of the polyamine regulation. However, it has been shown to be used independently from the polyamine response and is a mechanism for tuning mRNA expression in cell specific manner. In lymphoid T cells, ribosomes easily recognise, and initiate at, the uORF AUG which results in strong suppression of the main ORF translation. AdoMet DC mRNA from these cells was found primarily in the monosome fraction (Ruan et al., 1994). In non-lymphoid cells, where a large proportion of ribosomes fail to initiate at the uORF start due to the close proximity to the cap, and therefore reach the main coding region, AdoMet DC mRNA is found to be associated with polysomes (Ruan et al., 1994).

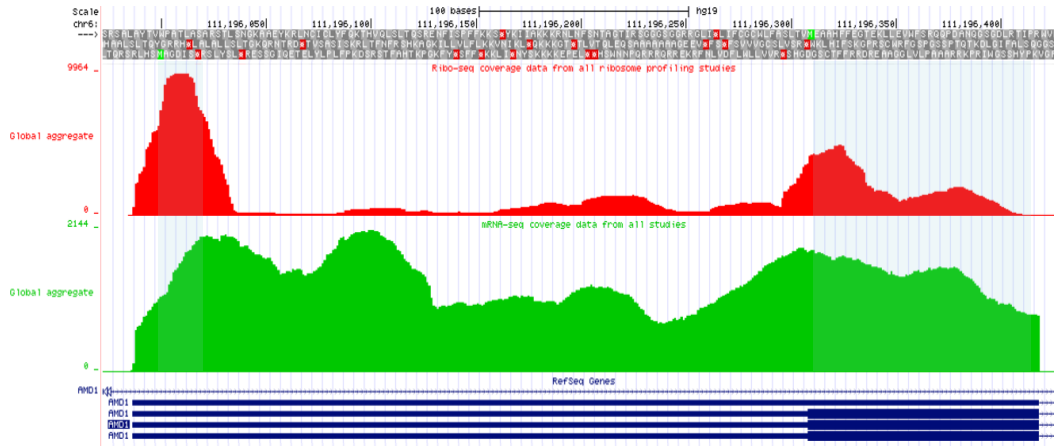


Figure 2.4: *Ribo-seq and mRNA-seq coverage in the first exon of the gene encoding AdoMet DC. The two blue panels indicate translation of the uORF encoding the peptide MAGDIS and the main ORF respectively. Ribosome coverage representative of an aggregate of multiple experiments is shown in red. The corresponding mRNA-seq coverage is in green.*

2.3.3 AAP

Carbamoyl phosphate synthetase catalyses the first step committed to arginine (Arg) biosynthesis. In fungi e.g. *Neurospora crassa*, the translation efficiency of the small subunit of the heterodimer enzyme is regulated by the translation of an uORF in the 5' leader region. The uORF encoded arginine attenuator peptide (AAP) functions from within the ribosome tunnel to stall the ribosome in the presence of high levels of free Arg (Wang and Sachs, 1997) (Fig.2.5). Ribosomes stalled at the end of the AAP coding sequence block the following ribosomes from reaching the main ORF AUG downstream (Wang and Sachs, 1997) thus reducing the expression of the main ORF.

The AAP encoding uORF has been found to be conserved among fungi but not outside of the group (Hood et al., 2007). In the presence of high Arg levels, ribosomes stall (Spevak et al., 2010) with the uORF stop codon in the A-site (Wang and Sachs, 1997). However AAP preserved its stalling potential even when the AAP encoding sequence was fused to a reporter gene or placed internally (Wang et al., 1998b), suggesting that impairment of the peptidyl hydrolysis reaction may not be the reason for the observed stalling. A puromycin release assay suggested that, translation of AAP in high Arg conditions results in either inhibition of the peptidyl transferase activity of the PTC or in restricted access to the A-site.

The *N. crassa* AAP is 24 amino acids long and has the sequence: ^{1}MNGRPSVFTSQ**DYL**SDHL**W**RALNA^{24}, where crucial residues are in bold. Mutational analysis revealed that the minimum domain needed for the regulatory response to Arg levels is comprised of AAP residues 9-20 (Spevak et al., 2010). Mutating Asp-12, Tyr-13, Lys-14 and Trp-19 has been shown to abolish the regulation in response to high Arg (Spevak et al., 2010). Interestingly AAP variants extended by one amino acid or shortened by one or two amino acids also had an inhibitory effect on PTC activity. Truncated or extended AAP variants were also capable of stalling the ribosome, but less efficiently. Such flexibility is unusual since the general understanding is that for stalling to occur, key residues of the nascent peptide contact specific tunnel components. Hence the spacing between the key residues and the P-site is considered to be critical for nascent peptide function.

A possible explanation of such flexibility may come from structural studies of the AAP nascent peptide in the ribosome tunnel. Two prominent areas of contacts between tunnel components and AAP residues were identified. In the constriction area, Asp-12 and its neighbour residues form contacts with L17 and L4 residues as well as with rRNA nucleotides such as A751. The second prominent area is further in the upper tunnel where U2609 and A2062 contact Trp-19 and its neighbour residues. Extensive contacts between a large number of AAP residues with the tunnel components in the constriction and the upper tunnel areas may explain why shifting of the nascent peptide by one or two amino acid residues does not fully abolish stalling.

In addition, structural data of the AAP nascent peptide within the ribosome exit tunnel revealed that the nascent peptide communicates with the upper tunnel nucleotides U2585 and A2062 (Bhushan et al., 2010).

Similar to other eukaryote arrest peptides such as CMV as well as the bacterial TnaC and SecM, mutations in L22 or insertions in the A751 region abolished peptide mediated stalling. This is consistent with an important role of the constriction area for nascent peptide mediated ribosome stalling.

Structural analysis of the AAP nascent peptide within the exit tunnel has revealed that D12 forms contacts with residues and L17 (eukaryote homolog of L22) as well as with the nucleotide A751 (bacterial numbering) in the constriction area. Structural studies of the AAP nascent peptide within the exit tunnel in the presence of high levels of Arg are still not available. However, it is conceivable that similar to the bacterial TnaC stalling system AAP forms Arg binding pockets within the tunnel. With high Arg concentration, binding of Arg in the pockets would result in stabilisation of nascent peptide contacts resulting in stalling.

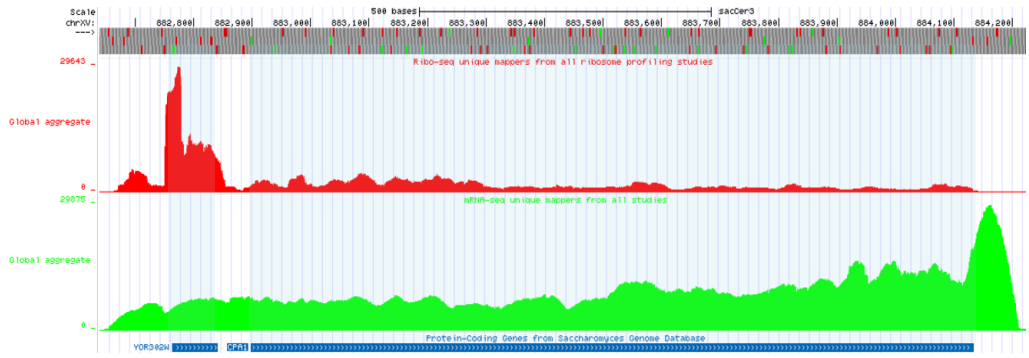


Figure 2.5: *Translation of the AAP encoding uORF in CPA1 gene of S. cerevisiae is detectable with ribo-seq data. Location of uORF (upstream) and main ORF (downstream) regions is indicated by the light blue panels. Red depicts ribosome coverage representative of an aggregate of multiple experiments. In green is the corresponding mRNA-seq coverage.*

2.3.4 Antimicrobial peptides

Proline rich antimicrobial peptides are a class of small peptides that plants and animals have evolved as part of their natural defence against microbes. These are currently being extensively studied due to the potential use of antimicrobial peptides as an alternative to antibiotics. Recent studies have revealed the structure of oncocin Onc112 in complex with the bacterial ribosome. Onc112 was found to be positioned within the protein exit tunnel similarly to the nascent polypeptide chain during translation. However Onc112 orientation in the tunnel was reverse, its N-terminus was localised in the PTC near the A-site while its C-terminus was positioned at the vicinity of the tunnel constriction. Similarly to some regulatory nascent peptides discussed earlier as well as to antibiotics that bind within the ribosome tunnel, Onc112 establishes various interactions with components of the PTC and the exit tunnel. These interactions result in disturbance of the PTC function and prevent the proper positioning of the upcoming aminoacyl-tRNAs (Roy et al., 2015).

2.4 Conclusion

The nascent peptide within the ribosome exit tunnel can alter ribosome behaviour. This feature is employed by the translation machinery to fine tune specific mRNA expression according to cellular needs. The interactions of various molecules such as amino acids and antibiotics with the nascent peptide in the tunnel, are transformed into instructions that navigate the ribosome to alter its behaviour in response to changes in the environment. In this way the translating ribosome with specific nascent peptide sequences in the tunnel, functions as a sensory machine which integrates environmental cues into regulatory response to fine tune the translation of specific mRNAs. Not only is this feature of the translating ribosome shedding light on a dynamic regulatory mechanism that takes place during protein translation but also it generates a better mechanistic understanding of how the ribosome itself functions.

Chapter 3

Polyamines and their regulation

3.1 Introduction

In 1678 Antoni van Leeuwenhoek looked under his primitive microscope and described the occurrence of crystalline structures in human semen. These were composed of what would later be identified as a phosphate of the polyamine spermine. One can easily imagine Leeuwenhoek's astonishment if he were to know that the structures he observed were made of a molecule that belongs to the group of polyamines, now known to be indispensable regulatory molecules in the living cell. Even more surprising however is the fact that over 300 years after Leeuwenhoek's observation and almost 100 years since polyamines structure was uncovered (Rosenheim, 1924), it is still not known precisely how polyamines exert their functions.

Polyamines are a class of molecules with aliphatic chains and more than two amino groups. Diamines such as putrescine and cadaverine are often referred to as polyamines which is misleading because they have only two amino groups. At physiological pH the amino groups are mainly protonated and therefore polyamines can be described as organic polycations. The polyamines spermidine and spermine and their precursor the diamine putrescine, are present in mammalian tissues (Pegg and Casero, 2011). Spermidine and spermine are also the most common polyamines in higher plants (Gill and Tuteja, 2010). Fungi such as *Ustillago maydis* contain spermidine but not spermine (Valdes-Santiago et al., 2012). Thermophile bacteria contain a wide variety of polyamines, including the longer caldopentamine and caldohexamine, and the more unusual, branched polyamines such as tetrakis (3-aminopropyl) ammonium (Oshima, 2007). Various combinations in different ratios of these and other naturally occurring diamines and polyamines are present in virtually all living cells.

Polyamines are involved in multiple cellular processes (Pegg, 2009a). Due to their positive charges, polyamines interact with macromolecules with acidic nature such as nucleic acids, membrane phospholipids and some proteins. Indeed, polyamines are found mostly bound to DNA and RNA. Due to their polycationic nature, polyamines bind DNA and neutralise the phosphate charges, with polyamine levels altering the condensation state of DNA (Raspaud et al., 1998). It has been shown that polyamines can promote transition between A, B and Z double helical structures of DNA by stabilizing one of them over the others (Ali and Ali, 1996, Bryson and Greenall, 2000, Thomas et al., 1991). In thermophilic bacteria polyamines serve to stabilize nucleic acids in conditions of high temperature (Oshima, 2007). Polyamines were also found to affect chromatin condensation and remodelling (Sarkar et al., 2009, Fredericq et al., 1991, Hobbs et al., 2002). These functions are suggestive of a general involvement of polyamines in modulation of gene expression by altering the efficiency of gene transcription through changes in DNA structure and chromatin condensation. Therefore transcription of many genes is anticipated to be, and indeed is, affected by polyamines (Wallace et al., 2003, Childs et al., 2003, Janne et al., 2004).

RNA is similarly bound and stabilised by polyamines and this could have an effect on protein translation through alteration of mRNA secondary structure and/or ribosome function (Pegg, 2009a). Specific cases of polyamine regulation of translation, mostly of genes connected with polyamine metabolism, have been identified and well-studied (Ruan et al., 1996, Ivanov et al., 2000). However, it is becoming apparent that polyamines have a much more general effect on translation. The ‘polyamine modulon’ in bacteria refers to genes whose expression was affected by polyamines at the level of translation (Yoshida et al., 2004). In fact, the ‘polyamine modulon’ concept could be naturally extended to yeast and mammals.

The ubiquitous distribution and extensive interaction of polyamines with nucleic acids and other macromolecules and their participation at every level of gene expression makes the identification of the exact biochemical roles a very difficult task. Our understanding of the precise mechanism of polyamine action lags behind that of other important regulatory molecules.

One of the ways polyamines can influence translation is through the translation factor eIF5A (Dever et al., 2014). As its name reveals, it was first thought to be an initiation factor that stimulated the f-Met-puromycin formation and the transition from late initiation to elongation (Benne and Hershey, 1978). Later on it was revealed that eIF5A was actually acting to facilitate elongation. Therefore it was recently proposed that the factor be renamed to eEF5 which stands for eukaryote elongation factor 5 (Dever et al., 2014). eEF5 is the sole known protein to utilise the posttranslational modification namely hypusination, the substrate for which is the polyamine spermidine. Despite many years of research, mystery still surrounds this translation factor and its functions. Various functions have been assigned to eEF5 and for all of them the unusual spermidine derived hypusine modification appears to be crucial. In *S. cerevisiae* polyamine auxotrophs, the effect of spermidine supplementation on cellular growth was primarily mediated by the hypusine modification of eEF5 (Chattopadhyay et al., 2008). Increased levels of the two isoforms of eEF5 were identified in many cancers and were promoted as tumour markers (Mathews and Hershey, 2015). In a recent study, sulphated eEF5 was found to function as a humoral apoptosis promoting factor in conditions of oxidative stress (Seko et al., 2015). In addition, studies have suggested that similarly to its bacterial homolog EFP, eEF5 is required for efficient translation of polyproline stretches (Gutierrez et al., 2013, Doerfel et al., 2013, Ude et al., 2013).

Early studies revealed that polyamines have a stimulatory effect on the growth of bacteria (Herbst and Snell, 1948, Martin et al., 1952, Kihara and Snell, 1957). In *E. coli* hundreds of genes were upregulated in response to polyamines, many of them at the level of transcription. Those upregulated at the translation level were assigned to the so called ‘polyamine modulon’ (Yoshida et al., 2004). Among these, there were genes with weak or not optimally positioned Shine-Dalgarno sequence, for which enhanced polyamine levels increased translation efficiency. Optimisation of SD sequence and its position, resulted in a loss of polyamine regulation. This is consistent with a model for polyamines altering RNA structure to overcome the suboptimal positioning of SD and stimulating the initiation efficiency (Yoshida et al., 2004).

In eukaryotes polyamines are absolutely required for cell growth and proliferation, as revealed by knockouts of the main polyamine biosynthetic enzymes in mice which had lethal effect in early days of embryonic development (Pegg and Casero, 2011). However too much of a good thing can be harmful and increased levels of polyamines and/or of polyamine biosynthetic enzymes such as ornithine decarboxylase (ODC) were found in many tumours. These findings stimulated an extensive research aiming to identify the connections between polyamine pathways and cancer (Bachrach, 2004). Anti-cancer compounds were developed that target important enzymes in the polyamine biosynthesis (Babbar and Gerner, 2011). Such is the case of D,L-alpha-difluoromethylornithine (DFMO) which was developed for cancer treatment in the late 1970s. DFMO is an analogue of the substrate for ODC and acts as an irreversible inhibitor of this enzyme. However DFMO proved to have only modest therapeutic effect and induced side toxicity effects. Despite this, the interest in DFMO in regard to cancer has not ceased although it has shifted from its therapeutic potential toward that of a chemoprevention agent, and the latter is being extensively explored (Meyskens and Gerner, 1999, Bailey et al., 2010). DFMO, also known as Eflornithine was shown to be a potent drug against Human African trypanosomiasis (HAT), also known as sleeping disease (Bacchi, 2009, Bacchi et al., 1980).

Recent study has revealed the interdependence of polyamine metabolism and circadian rhythmicity in mammals (Zwighaft et al., 2015). In cultured cells, polyamine content had an effect on the circadian period with low polyamine levels found to correlate with longer circadian period. The observed effect was reverted upon polyamine supplementation. Such longer circadian period was observed in older mice where it correlates with reduced polyamine levels in older animals. Key enzymes from the polyamine metabolism such as ODC, SPD SYN, and AMD1 and their products, mainly putrescine and spermidine, were found to be produced in a cyclic manner daily. This daily oscillation of the enzymes and the corresponding products was affected by key regulators of the circadian rhythmicity (Zwighaft et al., 2015).

Polyamine biosynthesis begins with arginine. Arginine is transformed

through ornithine into putrescine and the second half of this reaction is catalysed by ornithine decarboxylase (ODC) (Fig.3.1). In an alternative route utilised by plants, arginine is decarboxylated by arginine decarboxylase to produce agmatine. The latter is then converted to putrescine by the enzyme agmatinase.

Putrescine is then utilised as a substrate for the synthesis of the polyamines spermidine and spermine (Fig.3.1). The main route for polyamine biosynthesis utilised by mammals, plants, yeast, archaea and many bacteria was identified in Celia and Herbert Tabor's laboratory (Tabor et al., 1958). In this route, putrescine was identified as the source of the diaminobutane moiety in spermidine and spermine, while decarboxylated S-adenosylmethionine (dcSAM or dcAdoMet) was identified as the source of the terminal aminopropane moiety in these compounds (Tabor et al., 1958) (Fig.3.1). Two aminopropyl transferases, spermidine and spermine synthases, catalyse the transfer of aminopropyl groups from dcAdoMet to putrescine or spermidine for the production of spermidine and spermine respectively. A second route for polyamine biosynthesis was later described to occur in many other bacteria (Tait, 1976). It does not rely on dcAdoMet as an aminopropyl substrate and the species that utilise this route lack the enzyme catalysing the reaction of AdoMet decarboxylation, namely AdoMet decarboxylase (AdoMetDC).

Reducing the levels of polyamines includes degradation and export promoted by enzymes of the polyamine catabolism. This begins with the acetylation of spermidine and spermine by spermidine/spermine N1-acetyltransferase (SSAT), a major catabolic enzyme of the polyamine pathway (Casero and Pegg, 2009) (Fig.3.1). The products of the acetylation reaction, N^1-acetylspermidine, N^1-acetylspermine and N^1, N^{12}-diacetylspermine, are exported. In addition, N^1-acetylspermidine and N^1-acetylspermine are converted to putrescine and spermidine respectively by N^1-acetylpolyamine oxidase (APAO). Another catabolic enzyme, spermine oxidase (SMO) efficiently converts spermine to spermidine, which can then be acetylated by SSAT and converted to putrescine by APAO. Putrescine is a smaller molecule which is easier to export. In addition, putrescine can be degraded by diamine oxidase (Fig.3.1).

The enzymes for polyamine synthesis and catabolism are very well regulated

at the levels of transcription, translation, and protein turnover. A plethora of regulatory events that takes place during translation includes the involvement of uORFs, stalling nascent peptides, and even a programmed recoding event. The function and regulation of the main enzymes in the polyamine pathway will be further reviewed in more detail.

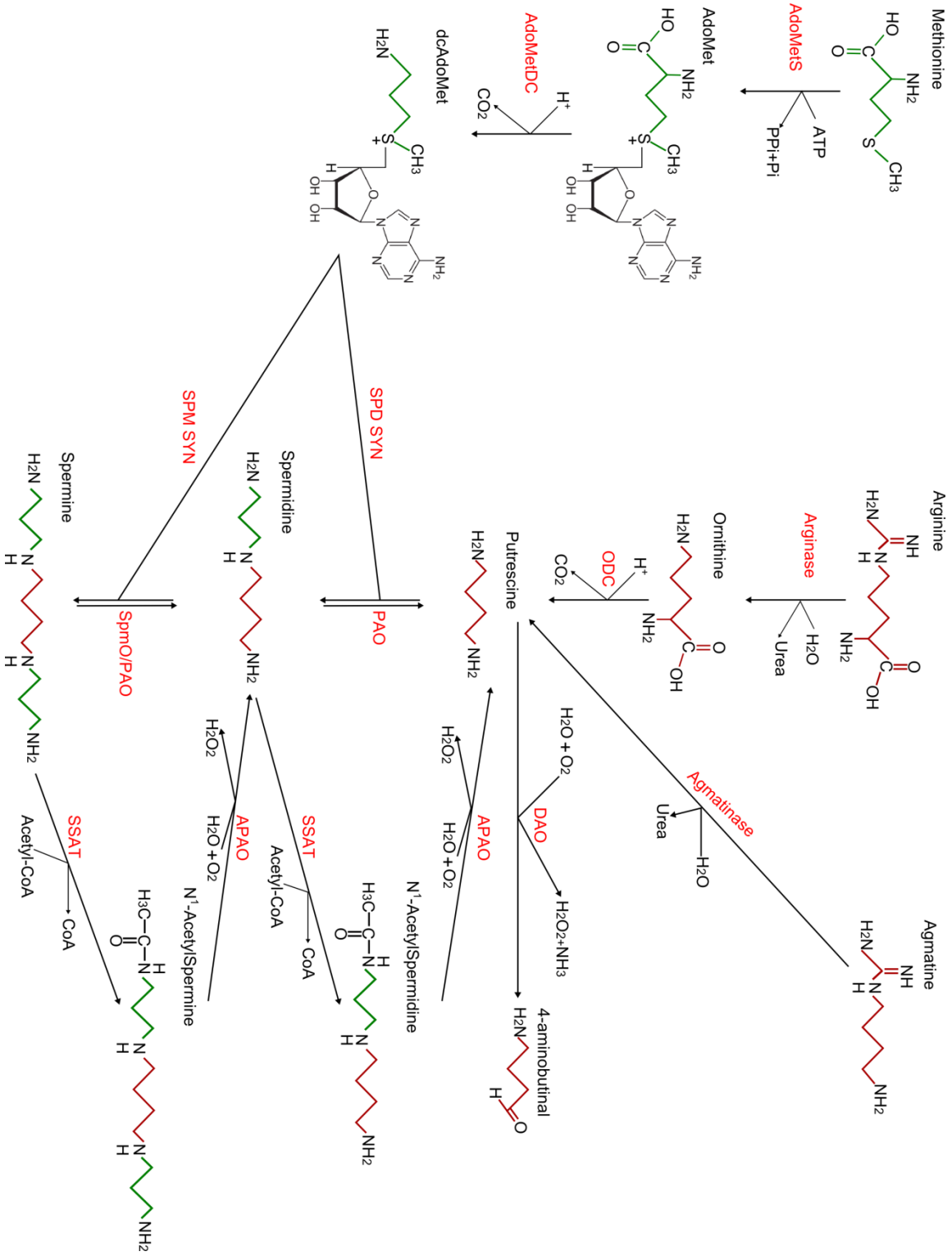


Figure 3.1: *Polyamine metabolism*

3.2 Enzymes of the polyamine metabolism and their regulation

3.2.1 Ornithine decarboxylase (ODC)

ODC is a pyridoxal phosphate dependent amino acid decarboxylase that catalyses ornithine decarboxylation to produce putrescine (Pegg, 2006) (Fig.3.1). Since putrescine is a substrate for the synthesis of the polyamines spermidine and spermine, the ODC catalysed reaction is a critical step in polyamine biosynthesis. Therefore, and not surprisingly, ODC synthesis and degradation are highly regulated.

ODC degradation is very rapid, with protein half-life in mammals of less than one hour (Pegg, 2006). The active form of the enzyme is a homodimer. A key point of ODC regulation in response to polyamine levels is its ubiquitin independent proteasomal degradation, directed by the protein antizyme (Murakami et al., 1992). In high polyamine levels antizyme binds to ODC monomers and exposes a degradation signal, recognised by the proteasome, resulting in proteasomal degradation without the need for ubiquitination (Wu et al., 2015, Takeuchi et al., 2008, Kahana, 2007). The ODC degradation signal was found to occur at the C-terminus in mammals (Ghoda et al., 1989) but at the N-terminus in *S.cerevisiae* (Godderz et al., 2011). Antizyme translation is regulated by polyamine concentrations via an unusual translational event featuring frameshifting which will be described in more detail in a section covering antizyme. In addition, in low polyamine levels, antizyme in its turn is downregulated by antizyme inhibitor, an ODC homolog, which binds with strong affinity to antizyme and sequesters it (Lopez-Contreras et al., 2006) (Fig.3.2).

ODC transcription levels are altered in response to a wide range of stimuli such as growth factors, hormones, oncogenes and others (Pegg, 2006, Miller-Fleming et al., 2015). The ODC gene promoter contains various sequence elements that allow for binding and response to different factors. For example the ODC promoter has two CACGTG E-boxes to which the oncogene product c-Myc binds in complex with its partner Max (Wagner et al., 1993, Nilsson et al., 2004). In addition,

the heterodimeric transcriptional activator, BMAL1:CLOCK, a key component of the circadian rhythmicity in mammals (Huang et al., 2012), was found to also bind to E-box motifs in the ODC gene. This promotes the rhythmic transcription of ODC, shown to be a component of the circadian clock control (Zwighaft et al., 2015)

ODC mRNA has a long and highly structured 5'UTR that was shown to have an inhibitory effect on translation (Pegg, 2009a). A conserved uORF was found in the 5' leaders of ODC in animals other than mammals and in some fungi (Ivanov et al., 2008). In most cases it was initiated by AUU or by an AUG in a week Kozak context. This conserved uORF may provide an additional layer of regulation in response to polyamine levels, just as it does for antyzime inhibitor, as discussed below.

3.2.2 Antizyme inhibitor (AZIN)

AZIN is an ODC homolog that has lost its decarboxylase activity (Murakami et al., 1996) but has a greater affinity for binding AZ, compared to ODC. AZIN binds and sequesters AZ, disrupting or preventing the formation of complexes with ODC, hence limiting AZ inhibitory effect on polyamine synthesis (Fig.3.2). In mammals there are two AZIN paralogs namely AZIN1 which is present in all tissues and AZIN2 which is present in brain and testis (Lopez-Contreras et al., 2010). Deletion of AZIN1 in mice was shown to lead to ODC degradation and critically reduced levels of putrescine and spermidine and has a lethal effect (Tang et al., 2009).

Polyamine levels have similar inhibiting effects on AZIN and ODC. In the presence of elevated polyamine levels, the AZIN synthesis is downregulated and AZ becomes available for binding ODC and targeting it for degradation.

Sequence analysis of vertebrate AZIN1 5' leaders revealed the occurrence of a conserved uORF of about 50 codons, starting with an AUU codon positioned in a good Kozak context (Ivanov et al., 2008). The C-terminal end of the uORF encoded polypeptide is highly conserved with two prominent sequential proline residues followed by a tryptophan.

The regulatory potential of the conserved uORF was experimentally tested

for the mouse AZIN1 sequence (Ivanov et al., 2008). In the presence of elevated polyamine levels, the uORF significantly reduced translation of the main ORF. The repression was relieved upon substitution of the AUU initiator codon with a non-initiator codon, UUU. Likewise, substitution of the start codon AUU with AUG in an optimal Kozak context precluded downstream main ORF translation. Importantly, the inhibitory effect in the presence of high polyamine levels was lost when the sequence encoding for the last 10 amino acids was placed out-of-frame (Ivanov et al., 2008). These observations are consistent with a model in which the conserved nascent peptide acts from within the ribosome protein exit tunnel in concert with polyamines to stall ribosomes translating the uORF. The stalled ribosomes would present a physical barrier for scanning ribosomes to reach the downstream main ORF start codon, consistent with the observed reduction of main ORF translation (Ivanov et al., 2008).

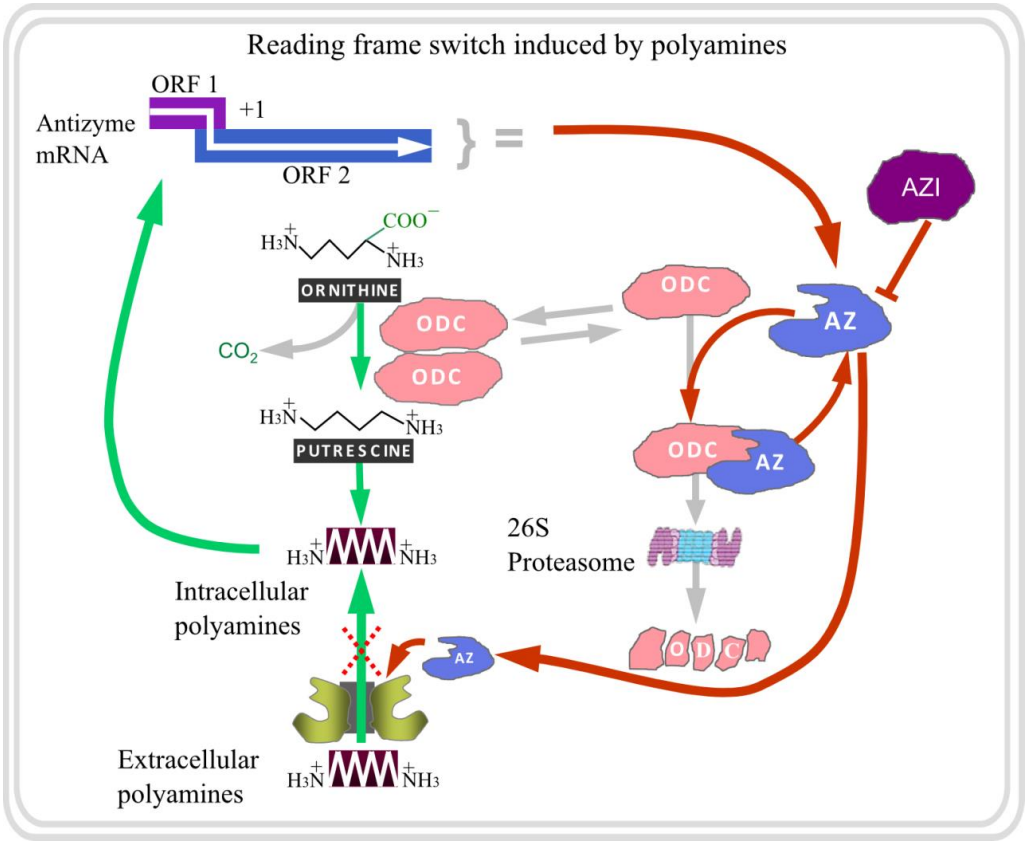


Figure 3.2: *Schematic representation of the interactions between ODC, AZ and AZIN, crucial for polyamine homeostasis. Antizyme binds ODC monomers and targets them for ubiquitin independent degradation by the 26S proteasome. Antizyme also regulates the polyamine membrane transporter by inhibiting polyamine uptake and stimulating polyamine excretion. AZI binds and sequesters AZ. Antizyme protein synthesis requires translational frameshifting which is stimulated by free intracellular polyamines. This event completes an auto regulatory circuit.*

3.2.3 S-Adenosylmethionine decarboxylase (AdometDC)

The aminopropyl transferases, spermidine and spermine synthase, utilize decarboxylated S-adenosylmethionine (dcAdoMet) as a donor of aminopropyl groups to produce the polyamines spermidine and spermine respectively from putrescine (Fig.3.1). S-Adenosylmethionine decarboxylase (AdoMetDC) is thus a rate limiting enzyme in polyamine synthesis, since it catalyses the production of

dcAdoMet, which is committed to polyamine synthesis (Pegg, 2009b) (Fig.3.1).

The translation of AdoMetDC mRNA yields an AdoMetDC proenzyme which undergoes a spontaneous cleavage at a conserved serine residue (Bale and Ealick, 2010). As a result of the internal serinolysis reaction, a large α subunit with a pyruvoyl group at the N-terminus and a small β subunit are produced (Bale and Ealick, 2010). The pyruvoyl group in the active site serves as a prosthetic group and provides for the decarboxylase activity of the enzyme, which belongs to the pyruvoyl type decarboxylases (Pegg, 2009b). The active form of the enzyme is comprised of two (αβ) heterodimers. In mammals and yeast putrescine has an activating effect on AdoMetDC as it stimulates the proenzyme cleavage as well the activity of the mature enzyme (Bale et al., 2008). In other organisms where putrescine is highly abundant, it does not have the same stimulatory effect upon AdoMetDC (Pegg, 2009b). Structural studies have revealed that putrescine binds AdoMetDC within a pocket distanced from the active site (Bale et al., 2008). There is one putrescine binding site per (αβ) heterodimer. Upon binding, putrescine triggers rearrangements of the residues connecting the putrescine binding pocket and the active site. The activation effect is thus a result of triggering the favourable positioning of the catalytic residues in the active site.

AdoMetDC is another enzyme of the polyamine pathway that is very well regulated. Since dcAdoMet is utilised almost entirely for polyamine synthesis, while its precursor AdoMet is used as a methyl group donor in numerous reactions, the levels of AdoMetDC are kept low and decarboxylated AdoMet corresponds to only about 1% of the AdoMet cellular pool (Pegg, 2009a).

Probably the best studied aspect of AdoMetDC regulation involves a uORF encoded nascent peptide with the amino acid sequence MAGDIS. The ribosome translating this uORF in the presence of high polyamine levels is stalled and as in the case of AZIN1, this results in inhibition of the translation of the downstream main ORF. The MAGDIS mediated regulation of AdoMetDC was discussed in more detail in the previous chapter covering regulatory nascent peptides.

3.2.4 Spermidine-spermine-N1-acetyltransferase (SSAT)

SSAT is the main catabolic enzyme in polyamine metabolism (Pegg, 2008). Along with ODC and AdoMetDC, SSAT is another enzyme crucial for maintaining polyamine homeostasis. SSAT acetylates spermidine and spermine thus reducing their charges and facilitating their excretion. In addition to being excreted, these acetylated products are substrates for oxidation by APAO (Pegg, 2008) (Fig.3.1).

SSAT mRNA levels are normally kept low in the cell with low constitutive expression of SSAT. In the presence of high polyamines, SSAT transcription is elevated through the interaction of transcription factors with a *cis*-acting polyamine response element (PRE) found upstream of the transcription start site in the SSAT gene (Wang et al., 1998a, Wang et al., 1998b, Wang et al., 2001).

SSAT levels are highly regulated. In addition to transcription other levels of regulation include mRNA stability and protein turnover as well as translation (Pegg, 2008). Studies revealed an intriguing control mechanism involving a repressor protein bound to a stem loop in the 5' coding region in SSAT mRNA (Perez-Leal et al., 2012). With high polyamine levels the protein dissociates and translation can resume.

It was suggested that translation regulation did not depend on the presence of the 5' or 3'UTRs. However, two uORFs were identified to occur in vertebrates (Perez-Leal et al., 2012, Ivanov et al., 2010). The longer one is in +1 frame and overlaps the main ORF. The second one which is further upstream and is only 4 codons long seems to be highly translated as illustrated with ribo-seq data obtained from Gwips-viz (Fig.3.3).

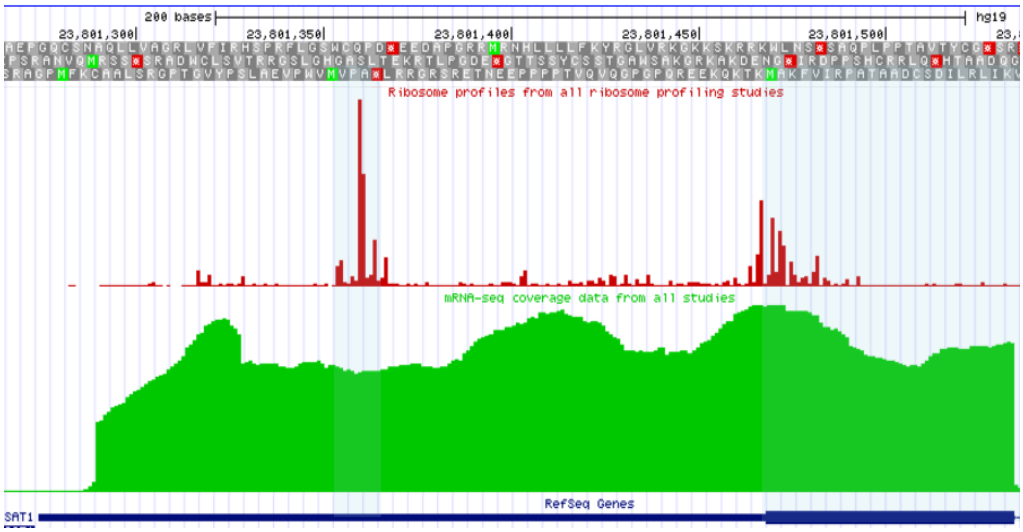


Figure 3.3: *Ribo-seq (red) and mRNA-seq (green) coverage in the 5' UTR and the first part of the main ORF of ssat1 gene. The left blue panel indicates the short uORF and the blue panel to the right indicates the first part of the main ORF.*

3.2.5 Antizyme (AZ)

The protein antizyme occurs in cells from yeast to mammals. It targets specific proteins for ubiquitin-independent proteasome-mediated degradation. Its best known interaction is with ODC and with the exception of plants, where it is not present, antizyme is a key regulator of ODC activity and as such it is a very important regulator of cellular polyamine homeostasis (Ivanov and Atkins, 2007). In addition to its effect on ODC, antizyme inhibits the import, and stimulates the export, of polyamines through the cellular membrane (Sakata et al., 2000).

A remarkable mechanism for sensing cellular free polyamine levels features translation of antizyme mRNA. Antizyme mRNA contains two partially overlapping open reading frames (ORFs). Expression of biochemically active antizyme requires translation of ORF2 (Miyazaki et al., 1992, Ichiba et al., 1994). In cells this is accomplished via translational frameshifting occurring at the end of ORF1 (Matsufuji et al., 1995). The rate of the frameshifting event is determined by the levels of free polyamines in the cell. High levels of free polyamines stimulate the frameshifting efficiency in decoding antizyme mRNA resulting in an increased

synthesis of antizyme protein. Through its inhibition of ODC, antizyme triggers a decrease of free polyamine levels in the cell. Likewise, low concentrations of free polyamines result in low antizyme mRNA frameshifting efficiency, allowing ODC and transporter activities to restore free polyamine levels (Fig.3.2). Thus the frameshifting is a sensor and effector in a polyamine induced auto regulatory circuit involved in maintaining the homeostasis of the free polyamines in the cell (Ivanov and Matsufuji, 2010, Coffino, 2001) (Fig.3.2).

As a negative regulator of cellular polyamine levels, antizyme is an inhibitor of cell proliferation and transformation. It was demonstrated that antizyme induces the ubiquitin-independent degradation of the anti-apoptotic protein DeltaNp73 which is very relevant to resistance of tumor cells to chemotherapeutic drugs. Degradation of DeltaNp73 through the antizyme induced ubiquitin independent pathway is stimulated by transcription factors such as c-Jun by a mechanism that was only recently unravelled (Bunjobpol et al., 2014). It features C-Jun mediated inhibition of acetylpolyamine oxidase (PAOX), an enzyme of the polyamine catabolism.

Given the importance of the small organic polycations, polyamines, for a wide range of cellular processes, and probably other roles of antizyme, it is not surprising that antizyme synthesis is itself highly regulated. The level of antizyme is governed in several ways including sequestration by AZIN, whose own synthesis is polyamine regulated (Fujita et al., 1982, Kahana, 2009), by C-Jun effects on polyamine catabolism (Bunjobpol et al., 2014), and by the level of its own synthesis being responsive to polyamine levels. This response is mediated by the relative efficiency of a programmed shift of ribosomal reading frame necessary to synthesize functional antizyme since it is encoded in two different, and partially overlapping, reading frames (Fig. 3.2) (Matsufuji et al., 1995). The frameshifting involved is a crucial sensor and effector of an autoregulatory circuit with elevated polyamines resulting in the synthesis of more antizyme which dampens both the synthesis and uptake of polyamines. *cis*-acting elements in antizyme mRNA are required for optimal frameshifting efficiencies. Curiously, a recent study has revealed that

frameshifting efficiency in *C. elegans* antizyme was induced in stress conditions such as starvation, and independent of polyamine levels (Stegehake et al., 2015)

Chapter 4

A nascent peptide signal responsive to endogenous levels of polyamines acts to stimulate regulatory frameshifting on antizyme mRNA

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The protein antizyme is a negative regulator of cellular polyamine concentrations in eukaryotes. Synthesis of functional antizyme requires programmed +1 ribosomal frameshifting at the 3' end of the first of two partially overlapping ORFs. The frameshift is the sensor and effector in an auto-regulatory circuit. Although the frameshift site alone only supports low levels of frameshifting, the high levels usually observed depend on the presence of *cis*-acting stimulatory elements located 5' and 3' of the frameshift site. Antizyme genes from different evolutionary branches have evolved different stimulatory elements. Prior and new multiple alignments of fungal antizyme mRNA sequences from the Agaricomycetes class of Basidiomycota show a distinct pattern of conservation 5' of the frameshift site consistent with a function at the amino acid level. As shown here when tested in *S. pombe* and mammalian HEK293T cells, the 5' part of this conserved sequence acts at the nascent peptide level to stimulate the frameshifting, without involving stalling detectable by toe-printing. Yet the peptide is only part of the signal. The 3' part of the stimulator functions largely independently, and acts at least mostly at the nucleotide level. When polyamine levels were varied, the stimulatory effect was seen to be especially responsive in the endogenous polyamine concentration range. A conserved RNA secondary structure 3' of the frameshift site has weaker stimulatory and polyamine sensitizing effects on frameshifting.

4.1 Introduction

Notwithstanding some early antibiotic studies, shortly after determination of the first structure of the "tube" through which the nascent peptide passes from the internal ribosome site of its synthesis to the ribosome's exterior, the interior of this exit tunnel was thought to behave like "molecular Teflon" and not interact with nascent peptide sequence thereby allowing unimpeded peptide egress (Nissen et al., 2000). However, interactions do occur with certain specific amino acid sequences, and evolution has exploited this for diverse and important functions. Here we explore the possibility that acting as a stimulatory signal for ribosomal frameshifting utilized positively for gene expression, can be added to this list. The work focuses on antizyme frameshifting.

Recoding signals serve to potentiate high levels of frameshifting at the relatively inefficient frameshift site at the end of ORF1 in most antizyme mRNAs. An exception is the budding yeast *Saccharomyces cerevisiae*, and presumably closely related species, in which the recoding signals serve to reduce, in a polyamine dependent manner, the inherently very high level of frameshifting at the shift site in budding yeast (Kurian et al., 2011). Effects of the nascent peptide within the exit tunnel of the ribosome that just synthesized it, are an important component of the negatively acting recoding signals in *S. cerevisiae* antizyme frameshifting (Kurian et al., 2011).

In contrast to the nascent peptide effects in *S. cerevisiae*, all the experimentally investigated antizyme frameshifting signals from *S. pombe* to humans act at the RNA level (Ivanov et al., 2000). These recoding signals are both 5' and 3' of the shift site. Different mRNA pseudoknots are strongly represented in the 3' signals (Matsufuji et al., 1995, Ivanov et al., 2004). As judged by deletion analyses, the 5' stimulatory sequence in rats involves 50 nucleotides immediately upstream of the frameshift site. Though all is required for optimal levels of frameshifting, the sequence of the 3 codons just 5'of the ORF1 stop codon have the greatest effect (Matsufuji et al., 1995). Comparative sequence analysis has revealed

that the 5’ stimulatory sequence has a modular structure with the different modules evolving independently in the different evolutionary clades with the closest module to the shift site, module A, being the most highly conserved (Ivanov et al., 2000). In contrast to the results from the deletion analysis, single nucleotide substitutions of what is now called module A of mammalian antizyme 1, showed only a modest effect on frameshift efficiency (Matsufuji et al., 1995). Without further experimental testing, it was considered possible that it may act via interaction with rRNA of the mRNA exit tunnel, based on the precedent of 5’ stimulators for other cases of frameshifting being known to act in this manner (Huang et al., 1988, Larsen et al., 1994).

With the exception of one high level case of incidental bacterial frameshifting (Gurvich et al., 2011), all non-antizyme investigated viral and chromosomal cases of programmed frameshifting involve recoding signals that act at the RNA level. Nevertheless, prior phylogenetic analysis of one case of positively utilized frameshifting where stimulatory signals are required to boost frameshifting efficiency indicated a recoding signal that acts at the nascent peptide level. This analysis was of the antizyme mRNA sequence from the 10 species then known from the Agaricomycotina sub-phylum of Basidiomycota fungi. One of these species was *Coprinopsis cinerea*. A highly conserved region of about 40 nucleotides was identified and its 3’ boundary is approximately at the 5’ boundary of module A and so 5’ of the frameshift site. The pattern of conservation in this region suggested that it functions at the peptide level and not at the nucleotide level (Ivanov and Atkins, 2007).

The putative 3’ stimulatory element is a potential RNA secondary structure starting 19 nucleotides downstream of the frameshift site. The structure consists of two directly adjacent stem-loops suggesting that they may co-axially stack on each other (Fig. 4.1 B,C and Fig. 4.2). Its position relative to the frameshift site suggests that it would be just outside the ribosome mRNA tunnel during the frameshift event (Ivanov and Atkins, 2007). With the exception of two recently described cases (Kiran et al., 2011, Li et al., 2014), this is different from the other previously described stimulatory structures that are located closer to the frameshift site and

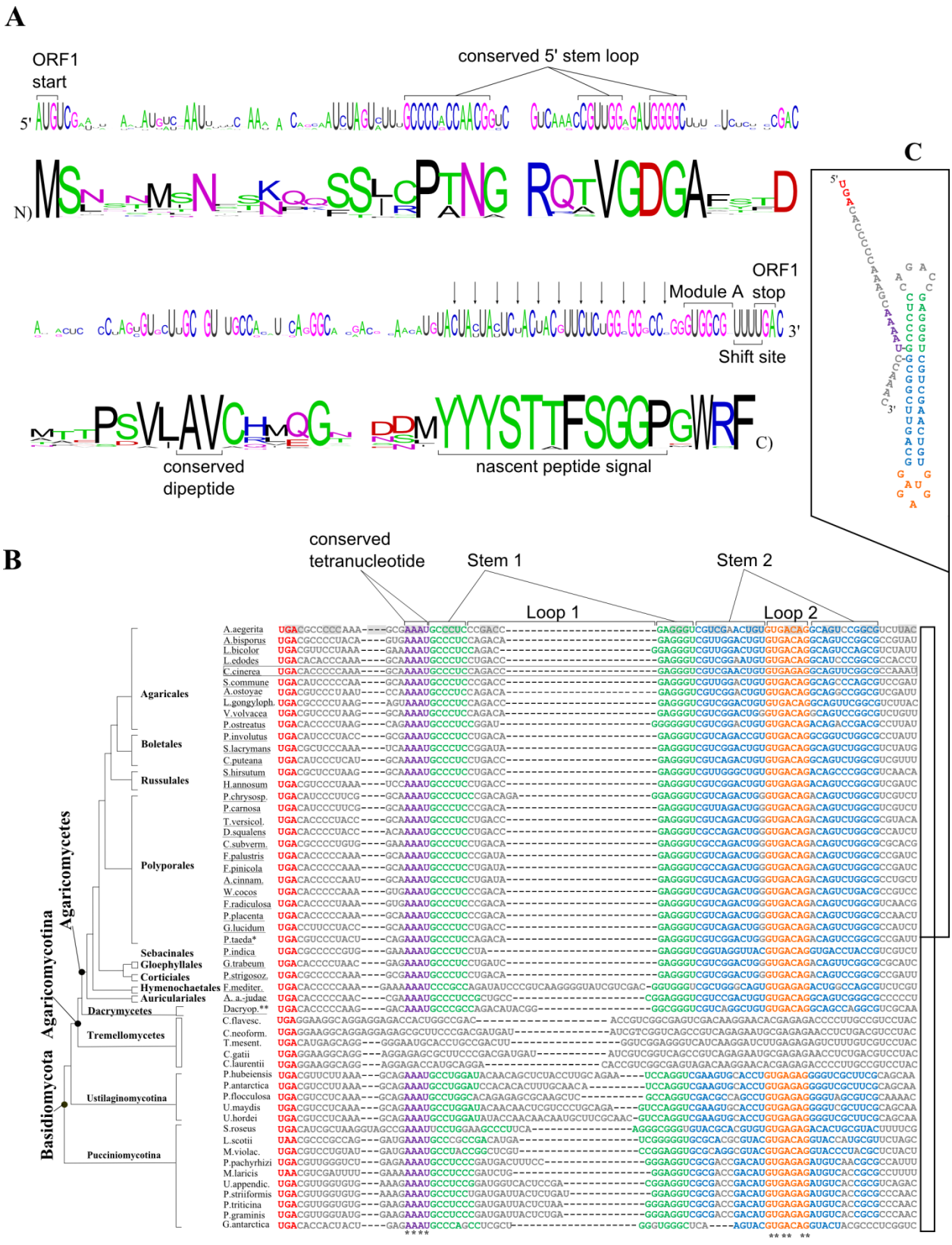

A
ORF1
start
conserved 5' stem loop
5'
N)
ORF1
Module A
stop
3'
Shift site
conserved
dipeptide
nascent peptide signal
C)
C
5'
3'
B
conserved
tetranucleotide
Stem 1
Stem 2
Loop 1
Loop 2
Agaricomycetes
Agaricomycotina
Basidiomycota
Agaricales
Boletales
Russulales
Polyporales
Sebacinales
Gloephyllales
Corticiales
Hymenochaetales
Auriculariales
Dacrymycetes
Tremellomycetes
Ustilaginomycotina
Pucciniomycotina
A.aegerita
A.bisporus
L.bicolor
L.edodes
C.cinerea
S.commune
A.ostoyae
L.gongyloph.
V.volvacea
P.ostreatus
P.involutus
S.lacrymans
C.puteana
S.hirsutum
H.annosum
P.chrysosp.
P.carnosa
T.versicol.
D.squalens
C.subverm.
F.palustris
F.pinicola
A.cinnam.
W.cocos
F.radiculosa
P.placenta
G.lucidum
P.taeda*
P.indica
G.trabeum
P.strigosoz.
F.mediter.
A. a.-judae
Dacryop.**
C.flavesc.
C.neoform.
T.mesent.
C.gatii
C.laurentii
P.hubeiensis
P.antarctica
P.flocculosa
U.maydis
U.hordei
S.roseus
L.scotii
M.violac.
P.pachyrhizi
M.laricis
U.appendic.
P.striiformis
P.triticina
P.graminis
G.antarctica

Figure 4.1: *Conservation of antizyme mRNA sequences from Basidiomycota, and their encoded products. A. An alignment of 35 ORF1 antizyme mRNA sequences was used to generate the nucleotide and amino acid logos shown here. 34 of these sequences belong to Agaricomycetes and 1 belongs to Dacrymycetes. Starting from the 3' end of the nucleotide logo indicated are the ORF1 stop codon, the shift site, the sequence of module A and the conserved peptide encoding region. Other conserved features more distal to the shift site such as a conserved dipeptide and a conserved 5' stem loop are also indicated. The gaps in the logos indicate that each nucleotide or amino acid residue occur with equal probability at the corresponding position. B. Alignment of 54 Basidiomycotal antizyme mRNA sequences 3' of the shift site. The region shown here contains the conserved 3'RNA structure. The ORF1 stop codon is in red, nucleotides of the predicted stem 1are in green, those of stem 2 are in blue and the nucleotides of loop are in orange. Nucleotide variations in loop 2 are highlighted in green. The correct reading frame is indicated in the first sequence of the alignment. The absolutely conserved four nucleotide sequence abutting stem 1 are shown in magenta. Completely conserved nucleotides are indicated by an asterisk below the alignment. The four sequences of the Tremellomycetes class that lack the potential RNA structure were left uncolored and were not taken into account when analyzing the alignment. C. The predicted secondary structure of the putative RNA stimulator, inferred from the alignment, featuring the Coprinopsis cinerea mRNA sequence; the coloring corresponds to that of the alignment. The underlined names are of the sequences used for the alignments in Fig. 2A. The scheme of the phylogenetic tree was adapted from Floudas et al. (29) and is not intended to indicate precise evolutionary distances and scales. P.taeda* refers to an EST derived from a Pinus taeda library that is in fact an Agaricomycetes contaminant of unknown identity. Dacryop.** stands for Dacryopinax sp.*

presumed to act from within the mRNA entrance tunnel at the mRNA unwinding site (Matsufuji et al., 1995).

As some nascent peptides pass through the ribosome peptide exit tunnel, specific residues interact with tunnel components which are mostly RNA. These interactions can modulate translation downstream from sequence encoding the interacting segment of the nascent peptide (Wilson and Beckmann, 2011). To what extent such consequences are influenced by ribosome conformational changes associated with the interactions, and to what extent by stalling of nascent peptide progression, is likely case specific.

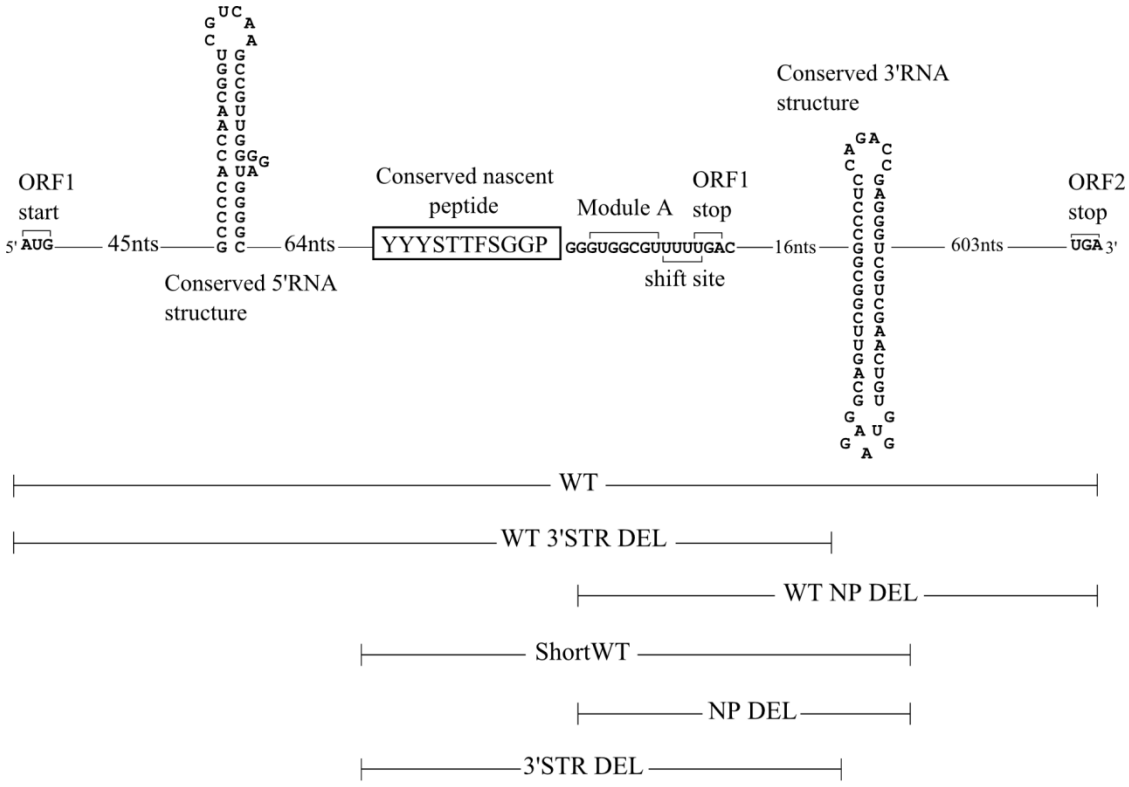


Figure 4.2: *Schematic representation of Agaricomycotina antizyme mRNA with conserved regions. The sequence used in the scheme is that of C.cinerea antizyme mRNA. Shown from the left are the ORF1 start codon, a conserved putative RNA structure occurring 106 nts 5' of the shift site, the putative nascent peptide signal shown in a box, the module A sequence, shift site and ORF1 stop codon. 19 nts 3' of the shift site, a conserved putative 3'RNA structure was previously identified. Below: Extent of the sequence present in 6 antizyme constructs used in this study.*

Antizyme homologs are present in diverse eukaryotic evolutionary branches, including in animals, fungi, and protists (Ivanov and Atkins, 2007, Ivanov et al., 2000, Ivanov et al., 1998). However, there is no indication that antizyme is present in plants. Fungal antizymes sequences have been identified in six separate phyla -

Ascomycota, Basidiomycota, Glomeromycota, Zygomycota, Neocallimastigomycota and Blastocladiomycota (Ivanov and Atkins, 2007); (I.P. Ivanov and JFA unpublished results). The Basidiomycota phylum contains three subphyla: Pucciniomycotina, Ustilaginomycotina and Agaricomycotina. Agaricomycotina includes organisms like the white rot fungi that are the only organisms capable of substantial lignin degradation (Floudas et al., 2012), the Lingzhi mushroom (*Ganoderma lucidum*) used in the traditional Chinese medicine for more than 2000 years (Chen et al., 2012), tree root pathogens such as the major forest pathogen annosum root rot (*Heterobasidion annosum*) and *Armillaria bulbosa* – one of the largest and oldest of all organisms (Aanen, 2014), also mushrooms and others. Pucciniomycotina and Ustilaginomycotina contain plant pathogens such as cereal rusts (*Puccinia graminis*) and Corn smut (*Ustilago maydis*).

Prior work showed that antizyme frameshifting is reproducible in heterologous systems from *S. pombe* to mammalian cells (Ivanov et al., 1998) [though not in *S. cerevisiae* (Hayashi et al., 1996)]. The FS stimulatory pseudoknot present in a subset of invertebrates was confirmed by testing the oyster antizyme mRNA in mammalian cells. Polyamine levels can be manipulated in a refined manner in mammalian cells (Howard et al., 2001). The present study examines the frameshift stimulators in a subset of fungal antizymes. Currently there is no homologous system available for testing the fungal antizyme mRNAs being investigated here. Because the polyamine levels can not readily be manipulated so effectively in the closer related *S. pombe*, the experiments involving manipulation of the polyamine levels were performed in mammalian cells.

The ancient origin of frameshifting in the antizyme gene and its evolution are reflected in the large diversity of its *cis*-acting stimulatory elements. Exploration of antizyme sequences from the different branches provides insights to the means by which the evolution preserves the essential traits while it navigates through diverse possibilities.

4.2 Experimental procedures

Sequence Assembly and Analysis.

Sequences were obtained and processed as described previously (Ivanov et al., 2008). Alignments were generated using Clustal Omega (Sievers and Higgins, 2014). Nucleotide and amino acid logos were generated using WebLogo (Crooks et al., 2004).

Plasmids

Oligonucleotides were synthesized at IDT, UK. A synthesized antizyme gene with the sequence from *Coprinopsis cinerea* was purchased from Gen Script Corporation, New Jersey where it was cloned in the vector pUC57 using an EcoRV cloning strategy. This sequence was used as template to generate the WT sequence (WT), which in turn was used as template for generating the other clones. The amplicons were generated by standard one-step or two step PCR. All amplicons were digested with BglII and XhoI and cloned into BglII/XhoI-digested vector pDluc (Fixsen and Howard, 2010, Grentzmann et al., 1998). The vector pDluc contains *Renilla* and firefly luciferase genes, separated by a short cloning site. Both luciferases are under the control of an upstream SV40 promoter. The antizyme cassettes were inserted between the two luciferase genes such that the upstream *Renilla* luciferase is in-frame with ORF1 of antizyme, while the downstream firefly luciferase is in-frame with ORF2. All constructs were transformed in *E. coli* strain DH5-α and were verified by sequencing with the primer PD1550. The in-frame (IF) controls were generated by using a template wherein the U of the ORF1 UGA stop codon was deleted. The antizyme construct from the Alanine scan series wherein the glycine codon at position -4 was substituted with an alanine codon was compared with the ShortWT IF control instead. For testing antizyme frameshifting in *S.pombe*, antizyme cassettes were designed using primers which introduced the restriction sites for BstE II and Kpn I at the 5’ and 3’ end of the amplicon respectively.

Following digestion the antizyme cassettes were cloned into BstE II/Kpn I-digested vector PIU-LAC between GST and *lacZ* (Ivanov et al., 1998).

Cell culture and transfection.

Human HEK293T cells were maintained as monolayer cultures, grown in DMEM supplemented with 10% FBS, 1mM L-glutamine and antibiotics at 37C^{o} in an atmosphere of 5% CO_2. For dual luciferase assays $4x10^6$ HEK293T cells were plated in 10 cm tissue culture dishes. After 24 hours the cells were detached with trypsin, suspended in fresh media and transfected in triplicate with Lipofectamine 2000 reagent (Invitrogen), using the 1-day protocol in which suspended cells are added directly to the DNA complexes in half-area 96-well plates. For each transfection the following was added to each well: 25 ng plasmid DNA, 0.2 µl lipofectamine 2000 in 25 µl OptiMem (Gibco). $4x10^4$ cells in 50 µl DMEM, were added to the transfecting DNA complexes in each well. Transfected cells were incubated at 37 C in 5% CO_2 for 20 hrs and assayed using the dual luciferase assay. For polyamine manipulation protocol, the cells were grown in the presence of 2.5 mM difluoromethylornithine (DFMO) for 5 days prior to transfection. After the incubation with DFMO, the cells were transfected using the protocol described above. $4x10^4$ cells in 25 µl DMEM, grown in the presence of 2.5 mM DFMO, were added to the transfecting DNA complexes in each well. Transfected cells were incubated at 37 C in 5% CO_2 for 24 hrs. After 24 hrs, 50 µl fresh media was added containing 1 mM aminoguanidine, 2.5mM DFMO, and either no polyamines, or polyamines to achieve final concentrations specified by experimental requirements. The cells were incubated for an additional 20h before being assayed.

Dual luciferase assay.

To measure the frameshifting efficiency of antizyme mRNA cassettes, a dual luciferase assay was employed. Relative light units were measured on a Veritas Microplate Luminometer fitted with two injectors (Turner Biosystems). The firefly and *Renilla* luciferase assay buffers were prepared as described (Dyer et al., 2000). Transfected cells were washed once with 1× PBS and then lysed in 12.6 µl of 1× passive lysis buffer (PLB; Promega), and light emission was measured following

injection of 50µl of each luciferase substrate buffer. The product of the upstream *Renilla* luciferase gene reflects zero-frame translation. Synthesis of the downstream firefly luciferase is dependent on frameshifting when the ORF1 stop codon is in the ribosomal A-site. Therefore, for each data point firefly translation activity was normalized relative to the *Renilla* activity. Data was obtained from three independent transfection experiments each performed in triplicate. The 9 data points for each construct were averaged and standard deviations calculated. The frameshifting efficiency (calculated as a percentage) is obtained by comparing firefly to *Renilla* activity ratio of each antizyme cassette to its corresponding in-frame control cassette.

S. pombe strains and culture.

S. pombe WT strain h-s leu1-32 ura4 ade6-210 was used in these experiments. Plasmids were prepared from *Escherichia coli* strain DH5-α and were transformed by a standard electroporation protocol. Edinburgh Minimal Medium (EMM), Complete Medium Supplement (CSM) and CSM-uracil were all purchased from Sunrise Science Products, San Diego, USA.

Assay of β-galactosidase activity in _S. pombe_.

The method described by Guarente (Guarente, 1983) was essentially used to assay the *β-galactosidase* activity in *S.pombe*. For each experiment three single colonies of each construct were analyzed. The experiments were performed in two or three independent replicas. As for the dual luciferase assay, the frameshift efficiencies were calculated as percentage by comparing the activities of the antizyme cassettes to the activities of their corresponding in-frame controls.

In vitro transcription

Capped and polyadenylated mRNAs were transcribed *in vitro* by T7 RNA polymerase (Wu et al., 2007) using DNA fragments generated from 2 rounds of PCR (see list of PCR primers). The yield of RNA was quantified using ImageQuantTL by comparison to known amounts of standard markers using ethidium bromide-stained agarose gels imaged with a GE Typhoon Trio

phosphorimager. Plasmid pPR301 was linearized with *Eco*RI to use as template for mRNA synthesis (Wu et al., 2007).

Primer-extension inhibition (toe-print) assays.

For toe-printing *N. crassa* ribosomes in cell-free extracts that were synthesizing nascent peptides containing the partial antizyme domain, translation reaction mixtures (10 µl) supplemented with 0, 10, or 50 µM of spermidine were programmed with 60 ng of mRNA and incubated at 26°C for 10 min. As controls, Luc mRNA containing wild-type AAP uORF was translated in the presence of 10 µM or 2 mM Arg. Cycloheximide was added to translation reactions at a final concentration of 0.5µg/µl before the incubation started or at the end of the incubation. Toe-print assays were performed as described (Wei et al., 2012).

4.3 Results

4.3.1 Comparative sequence analysis.

Intending to probe putative regulatory *cis*-acting sequences using a comparative genomics approach, we assembled sequences from additional Basidiomycota species (see Methods). The current present set contains 55 such antizyme mRNA sequences (Fig. 4.1). One of the sequences was partial and was used only for the analysis of the 5'conserved region.

Comparative sequence analysis of the 5'conserved region

Comparison of the 55 Basidiomycota sequences from the current set revealed that the 5' conserved element was present in all 34 sequences from Agaricomycetes class as well as in one sequence from class Dacrymycetes of Agaricomycotina. However, antizyme sequences from the class Tremellomycetes of Agaricomycotina, as well as all the sequences of species outside of Agaricomycotina subphylum, did not show similarity at the amino acid level in the vicinity of the shift site and were excluded from further analysis of the 5' sequence. Consequently, the 35 antizyme sequences

were employed for the current analysis of the 5' conserved element. The sequence from *Dacryopinax sp*. class Dacrymycetes represents the maximum sequence divergence. Nucleotide and amino acid sequence alignments were used to generate respective sequence logos for ORF1, (Fig. 4.1A). All Agaricomycetes antizyme mRNA sequences analyzed plus the sequence from Dacrymycetes have the frameshift site UUU-UGA.

Analysis of the 35 sequences confirms the previous observation that within the region 5' adjacent to module A, there is high conservation at the peptide level (Fig. 4.1A) accompanied by synonymous substitutions in the corresponding codons at their third positions (note downward pointing arrows in Fig. 4.1A). The situation with module A itself is considered below. Additional conservation, previously unnoticed, is observed further upstream. Prominent among the other conserved features in ORF1 are two adjacent codons, encoding absolutely conserved Ala and Val, which show a high rate of synonymous substitutions. Another feature contained within the first half of ORF1 is a region of approximately 50 nucleotides, 29 of which are absolutely conserved. This region appears conserved at the nucleotide level and the most conserved nucleotides potentially form an RNA stem with 14 base-pairs and predicted stability of at least 30 kcal/mol (Fig. 4.1A).

Comparative analysis of the 3' RNA putative stimulatory structure

The new analysis revealed that a 3' structure that is homologous to the one identified in Agaricomycotina can be formed in a number of antizyme mRNA sequences belonging to two other subphyla of Basidiomycota. The broad distribution of this putative stimulator suggests its importance in many antizymes from Basidiomycota. 54 antizyme sequences having the first 72 nucleotides of ORF2 were aligned to show the conservation in the region encoding the putative secondary structure (Fig. 4.1B, C)

Antizyme sequences from the class Tremellomycetes of Agaricomycotina (e.g. the "yeast-like" human pathogen *Cryptococcus neoformans*) did not seem to possess similar structure folding potential and were excluded from further analysis of the 3' putative structure.

The 3’ structure in sub-phyla Ustilaginomycotina and Pucciniomycotina is similar to the one already published for Agaricomycotina (Fig. 4.1B, C). There are extensive co-variant changes in stem 2. Stem 2, especially in antizyme mRNAs from Ustilaginomycotina and Pucciniomycotina, has several bulged nucleotides or nucleotide mismatches. No mismatches and very few co-variations are present in stem 1 from Agaricomycetes but the sequences from non-Agaricomycotina species show numerous co-variations. In all the sequences but one, there is no break in base-pairing at the junction between stem 1 and stem 2 which is consistent with the previous suggestion that they may co-axially stack on each other.

All examined Basidiomycota sequences, possessing the putative RNA structure, contain an absolutely conserved four nucleotide sequence AAAU abutting the 5’ end of stem 1. The corresponding region appears to be unstructured but might be part of unconventional ternary interactions.

The sequence of loop 1 is highly variable, both in composition and length. It can be as short as 3 nucleotides, as in *Piriformospora indica*, or as long as 28 nucleotides, as in *Fomitiporia mediterranea*. By contrast, the sequence of loop 2 is the most highly conserved region within the potential 3’ structure. Six out of the seven nucleotides that comprise the loop are absolutely conserved. The loop has features suggesting it may exist in something other than a single-stranded state (Ivanov and Matsufuji, 2010, Ivanov and Atkins, 2007) similar to previously published RNA triloop structures (Mitrasinovic, 2006).

4.3.2 Experimental analysis: Polyamine levels and degree of relevance of sequence flanking the putative nascent peptide encoding signal

Previously our lab developed a protocol for a fine manipulation of the polyamine levels in mammalian HEK293T cells (Howard et al., 2001). The lowest polyamine levels were achieved by pre-treatment of HEK293T cells with D,L-alpha-difluoromethylornithine (DFMO) which is an irreversible inhibitor of ODC (Pegg, 2006). DFMO pre-treatment, combined with adding polyamines to the media, allows controllable manipulation of free polyamine levels (Howard et al., 2001). Based on

preliminary titration experiments with spermidine (SPD) (data not shown) three data points were chosen for most experiments presented here: DFMO treatment alone, DFMO plus 50 μM SPD and DFMO plus 2 mM SPD.

We designed frameshift cassettes based on the sequence of *Coprinopsis cinerea* antizyme mRNA which were cloned between Renilla and Firefly luciferase with antizyme ORF1 in-frame with Renilla and Firefly in-frame with antizyme ORF2 (Figs.4.2 and 4.3). The WT cassette (WT) contains the full sequence of both ORFs. The antizyme sequence in a construct termed ShortWT starts at the 5' end of the putative nascent peptide encoding sequence and extends to the 3' end of the conserved RNA structure 3' of the frameshift site (Figs.4.2 and 4.3)

In the context of the WT cassette, deletion of sequence from the 3' end of ORF2 up to that specifying the 5' end of the structure 3' of the shift site (WT 3'STR DEL) yielded 19.7% frameshifting efficiency with DFMO plus 50 μM SPD treatment. This resulted in 1.9 fold reduction compared to WT levels (38%) (Fig.4.3). Also in the context of the WT cassette, a deletion from the 5' end of ORF1 to the 3' nt of the region encoding the putative nascent peptide signal (WT NP DEL) yielded 3.2% frameshifting efficiency with DFMO plus 50 μM SPD treatment. This is a 12 fold reduction compared to WT.

In the context of ShortWT, the corresponding deletion of the structure 3' of the shift site (3'STR DEL), or of the putative nascent peptide encoding sequence (NP DEL), were tested with DFMO plus 50 μM SPD treatment, yielding frameshifting levels of 13% and 1.8% respectively. Compared to their control, in this case ShortWT, these levels involve the same reduction, 1.7 and 12 fold, as their counterparts in full length WT context (Figs.4.2 and 4.3). (The frameshifting efficiency of ShortWT is 22%.) The results suggest that the region specifying both the putative nascent peptide signal and the 3'RNA structure, has the same capacity to enhance frameshifting levels in the context of full length WT and ShortWT.

However, the absolute frameshifting efficiency values of WT and its derivative constructs WT 3'STR DEL and WT NP DEL, were higher compared to those of ShortWT and its derivative constructs 3'STR DEL and NP DEL respectively. In addition the fold difference was dependant on polyamine

concentrations. When comparing WT and ShortWT FS efficiencies, with DFMO treatment alone a reduction of 3.1 fold was observed; with DFMO plus 50 μM SPD treatment - 1.7 fold and DFMO plus 2 mM SPD - 1.2 fold (Fig.4.3, compare WT and ShortWT). The results suggest that there are additional stimulatory elements other than the regions encoding the putative nascent peptide signal and the conserved 3'RNA structure which are not present in ShortWT. A detailed exploration of proximal and distal stimulatory elements is outside the scope of the present study which focuses mainly on the effect of the putative nascent peptide signal on frameshifting in the context of ShortWT.

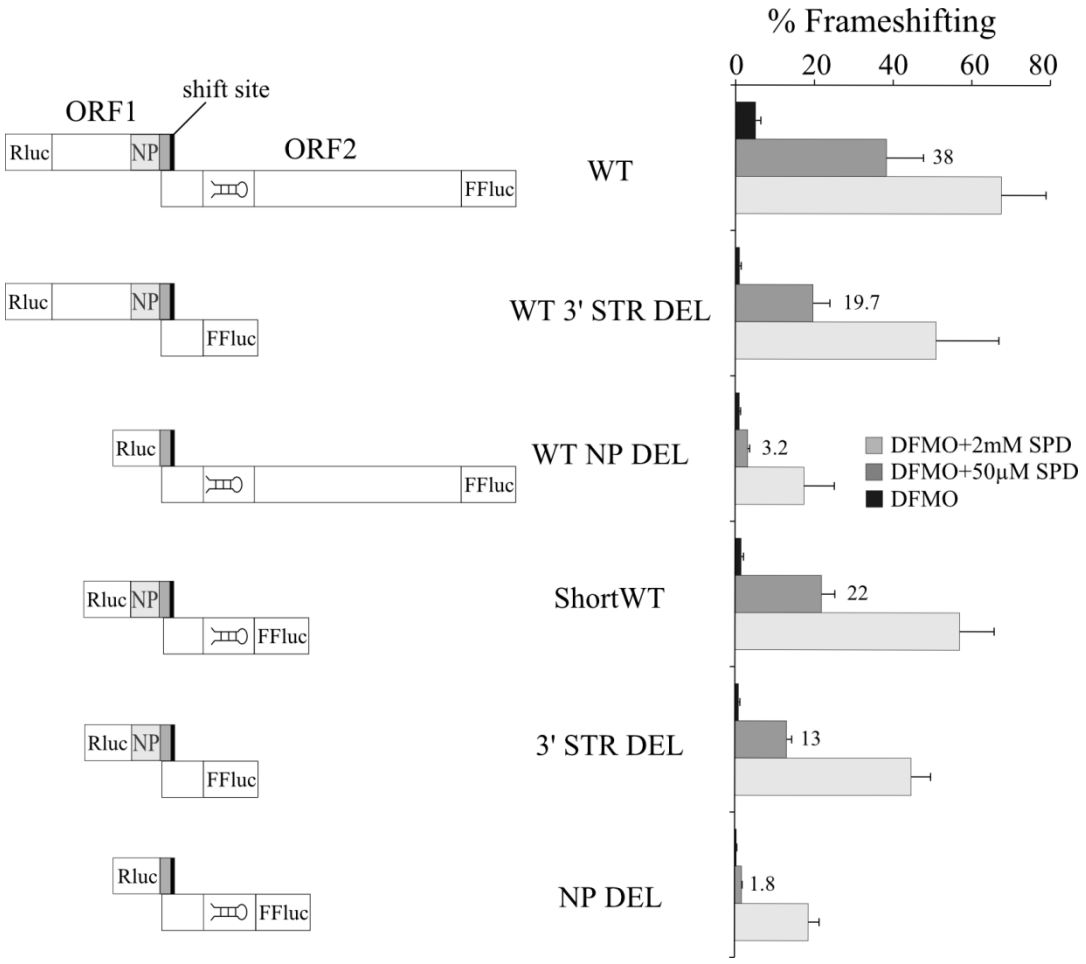


Figure 4.3: *Establishing the boundaries of the 5' and 3' stimulatory elements of C. cinerea antizyme mRNA from tests in HEK293T cells. The WT construct contains the full length ORF1 and ORF2 of the C. cinerea antizyme mRNA. The WT 3'STR DEL construct contains full length ORF1 but has a deletion starting 19 nucleotides 3' of the shift site that includes the putative 3'RNA structure. The WT NP DEL construct has a full length ORF2 but the sequence encoding the conserved nascent peptide was deleted, leaving intact the sequence of module A and the shift site. The ShortWT construct contains the last 42 nucleotides of ORF1, including the sequence coding for the conserved nascent peptide, the sequence of module A and the shift site, and the first 64 nucleotides of ORF2 including the putative 3'RNA structure. 3'STR DEL construct is a derivative of ShortWT with the region encoding the putative 3'RNA structure deleted. NP DEL construct is another derivative of ShortWT wherein the sequence encoding the putative nascent peptide signal was deleted. All constructs*

were tested in three conditions: DFMO treatment (black bars), DFMO treatment plus 50 μM SPD (grey bars) and DFMO treatment plus 2 mM SPD (light grey bars).

One of the additional frameshift cassettes based on ShortWT had both regions encoding the putative nascent peptide signal and the 3'RNA structure deleted but retained module A (5' of the shift site), the shift site and 19 nts 3' of the shift site. This yielded 0.4% frameshifting with DFMO + 50 μM SPD treatment which is a 57 fold reduction compared to ShortWT (Fig. 4.4A, NP+STR DEL). With the same treatment, a derivative of ShortWT in which the sequence of module A was substituted with its complement, yielded 0.6% frameshifting, a 38 fold reduction (Fig. 4.4A, MOD A).

To obtain the frameshift efficiencies of the ShortWT and its derivatives in cells with endogenous polyamine concentrations, we tested the cassettes from Fig.4.4A as well as the 3'STR DEL cassette from Fig.4.3 in mammalian HEK293T cells where the polyamine levels were not manipulated (Fig. 4.4C). ShortWT yielded a frameshift efficiency of 26% and its derivative with the 3'RNA structure deleted yielded a frameshift efficiency of 13% (Fig.4.4C, 3'STR DEL). NP+STR DEL and MOD A exhibited greatly reduced frameshift efficiencies, 0.8% and 0.5% respectively (Fig. 4.4C).

The frameshifting levels with ShortWT and its derivatives tested in cells treated with DFMO + 50 μM SPD closely matched the frameshifting levels obtained in HEK293T cells with endogenous polyamine levels (see Fig 4.4B,C). This observation suggested that the DFMO + 50 μM SPD treatment brings free intracellular polyamines close to their endogenous levels. In addition, DFMO + 50 μM SPD supported the greatest difference in frameshifting efficiency between ShortWT and its derivative constructs.

The mammalian HEK293T cell culture is a heterologous system for the analysis of regulatory frameshifting in the decoding of fungal antizyme mRNAs. The ShortWT and its derivative frameshift cassettes (Fig.4.4A) were also fused to the +1 frame of *lacZ* and transfected in *S.pombe* cells. When tested in the *S. pombe*,

β-galactosidase assays extrapolate to 16.5% frameshifting with the ShortWT (Fig.4.4D) (compared to 26% in HEK293T cells). Deletion of the 3'RNA structure in 3'STR DEL yielded 4.4% frameshifting efficiency, a 3.75 fold reduction compared to ShortWT. As with HEK293T cells, the two other constructs, NP+STR DEL and MOD A exhibited dramatically reduced frameshifting efficiencies: double deletion of the putative nascent peptide encoding signal and the 3'RNA structure produced a 22 fold reduction with frameshift levels of 0.74% (Fig.4.4D, NP+STR DEL). In the ShortWT derivative, changing the module A sequence to its complement yielded a 25 fold reduction with frameshift levels of 0.67% (Fig.4.4D, MOD A).

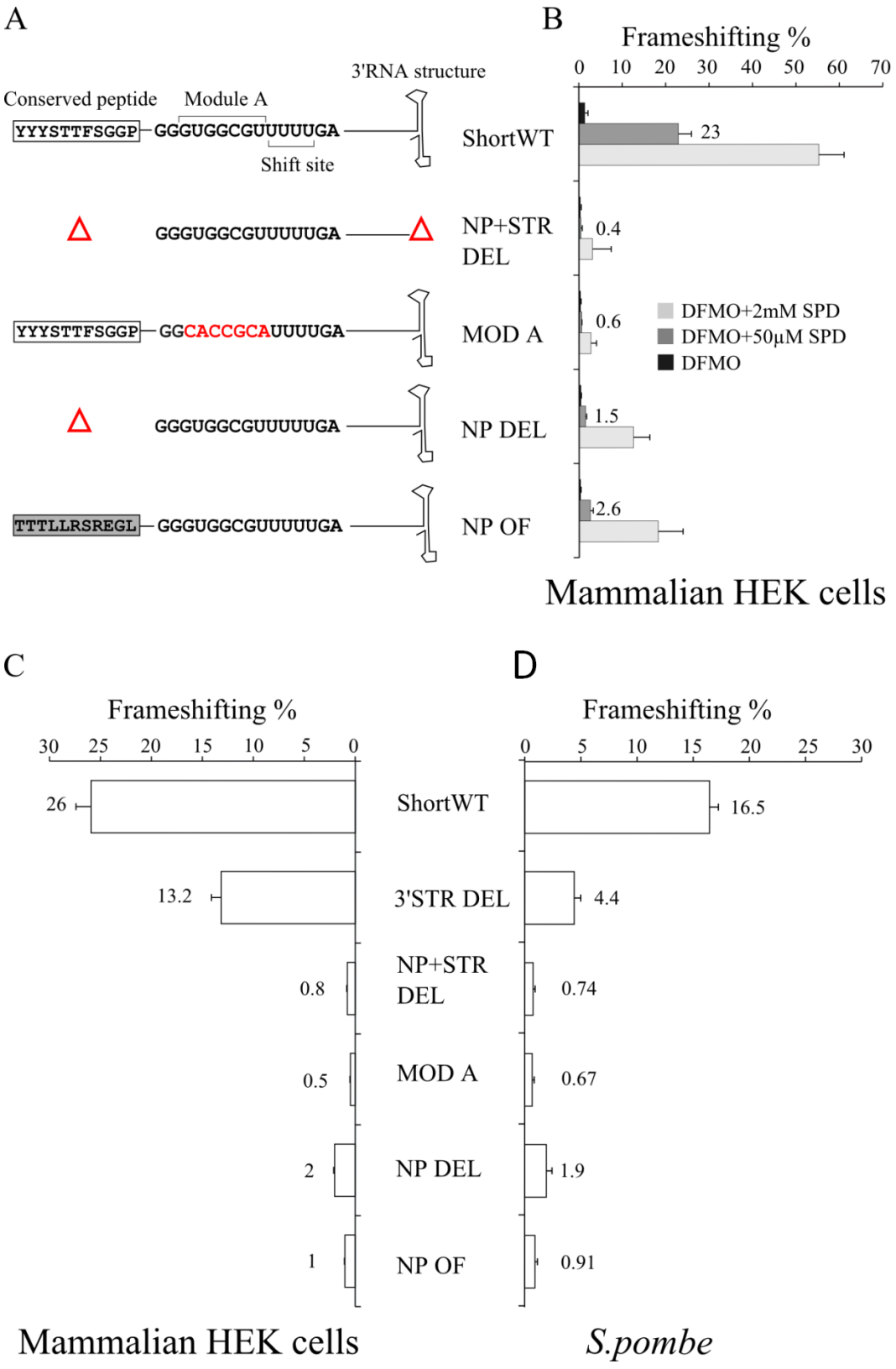

A
B
Frameshifting %
0 10 20 30 40 50 60 70
3'RNA structure
Conserved peptide
Module A
YYYSTTFSGGP
GGGUGGCGUUUUUGA
Shift site
ShortWT
23
NP+STR DEL
GGGUGGCGUUUUUGA
0.4
MOD A
YYYSTTFSGGP
GGCACCGCAUUUUGA
0.6
DFMO+2mM SPD
DFMO+50μM SPD
DFMO
NP DEL
GGGUGGCGUUUUUGA
1.5
NP OF
TTTLLRSREGL
GGGUGGCGUUUUUGA
2.6
Mammalian HEK cells
C
D
Frameshifting %
30 25 20 15 10 5 0
0 5 10 15 20 25 30
ShortWT
26
16.5
3'STR DEL
13.2
4.4
NP+STR DEL
0.8
0.74
MOD A
0.5
0.67
NP DEL
2
1.9
NP OF
1
0.91
Mammalian HEK cells
S.pombe

Figure 4.4 *Assessing the role of the conserved sequences on frameshifting from tests in HEK293T cells and S. pombe. A. Schematic representation of ShortWT and its derivative cassettes used in 4.4B, C and D. ShortWT, 3'STR DEL and NP DEL are as described in Fig.4.3. The nucleotide sequences of module A and the shift site are indicated. The amino acid sequence of the putative nascent peptide signal (boxed) is shown instead of the nucleotide sequence encoding it. The NP+STR DEL construct has both sequences encoding the putative nascent peptide signal and the 3'RNA structure deleted so that it contains only the 30 nucleotide sequence surrounding the frameshift site. The MOD A construct has the sequence of module A changed to its complement; the altered sequence is in red. The NP OF construct has the sequence coding for the nascent peptide put out-of-frame by deleting a nucleotide (U) at the 5' end of the sequence and inserting a nucleotide (G) at its 3' end. The altered nascent peptide sequence is boxed in grey. B. Frameshift efficiencies of ShortWT and its derivative constructs tested in HEK293T cells treated with DFMO and supplemented with Spermidine (SPD). The constructs were tested in three conditions: DFMO treatment only (black bars), DFMO treatment plus 50 μM SPD (grey bars) and DFMO treatment plus 2 mM SPD (light grey bars). C. Frameshift efficiencies of ShortWT and its derivative constructs tested in HEK293T cells where the polyamine content was not manipulated. D. Frameshift efficiencies of ShortWT and its derivative constructs tested in S.pombe.*

4.3.3 The putative nascent peptide signal

To probe the effect of the putative nascent peptide signal, the sequence encoding it was either deleted (NP DEL) or placed out-of-frame by deleting one nucleotide in the beginning of the sequence and inserting one nucleotide after it (NP OF) (Fig 4.4A). Both alterations were done in the context of ShortWT i.e. retaining the previously identified structure 3' of the shift site. They were tested in mammalian cells and *S. pombe*. Corresponding great reductions, 9 and 13 fold for NP DEL and 18 and 26 fold for NP OF, in frameshift levels were observed in the two systems respectively (Fig. 4.4C &D). A similar effect was observed in HEK293T cells with manipulated polyamine levels, with the greatest fold difference being obtained with DFMO + 50 μM SPD (Fig. 4.4B).

To further test if the phylogenetically conserved 5' stimulator within ShortWT functions at the amino acid or nucleotide levels, an additional cassette was

generated. In this construct, NP SYN, the sequence of the encoded peptide was preserved, but the nucleotide sequence encoding it was altered at the 3rd base of 11 codons by introducing synonymous substitutions. ShortWT, NP Del, NP OF and NP SYN were then tested in a SPD titration experiment. Synonymous substitutions had little effect on frameshifting efficiencies compared to ShortWT through most SPD supplementation concentrations tested (Fig. 4.5A). Even in the SPD concentration range where there was some difference – 10-500 μM – the frameshifting efficiencies of NP SYN were closer to ShortWT than to NP DEL or NP OF. Complete deletion of the sequence (NP DEL), and its out-of-frame variant (NP OF) resulted in similar greatly reduced frameshifting efficiencies throughout the concentration gradient. These results provide further evidence that high frameshifting efficiency depends on the peptide sequence (nascent peptide signal) rather than on its encoding nucleotide sequence. Additionally, the results show that with the NP DEL and NP OF cassettes, there is a lag in SPD stimulation of frameshifting in the first half of the titration gradient, compared to the pattern observed with ShortWT and NP SYN. Towards the later part of the gradient this pattern is reversed. This is illustrated by plotting the ratio of ShortWT to NP DEL frameshifting levels. This ratio was ca. 5 at the lowest concentrations of spermidine and peaks at ca. 19 with cells treated with DFMO and supplemented with 1 μM to 50 μM spermidine. It then declines at the highest concentrations of SPD (Fig. 4.5A). Interestingly, the position of the peak in the frameshifting lag coincided with the range of spermidine supplementation that corresponds to endogenous levels of free polyamines in HEK cells.

Titration experiments with another main cellular polyamine, spermine (SPM), and with putrescine (PUT), were also performed. The peak of the ratio of ShortWT to NP DEL frameshifting levels with spermine was at DFMO plus 1-2.5 μM SPM and with putrescine it was at DFMO plus 2.5 μM PUT (Fig. 4.5B, C).

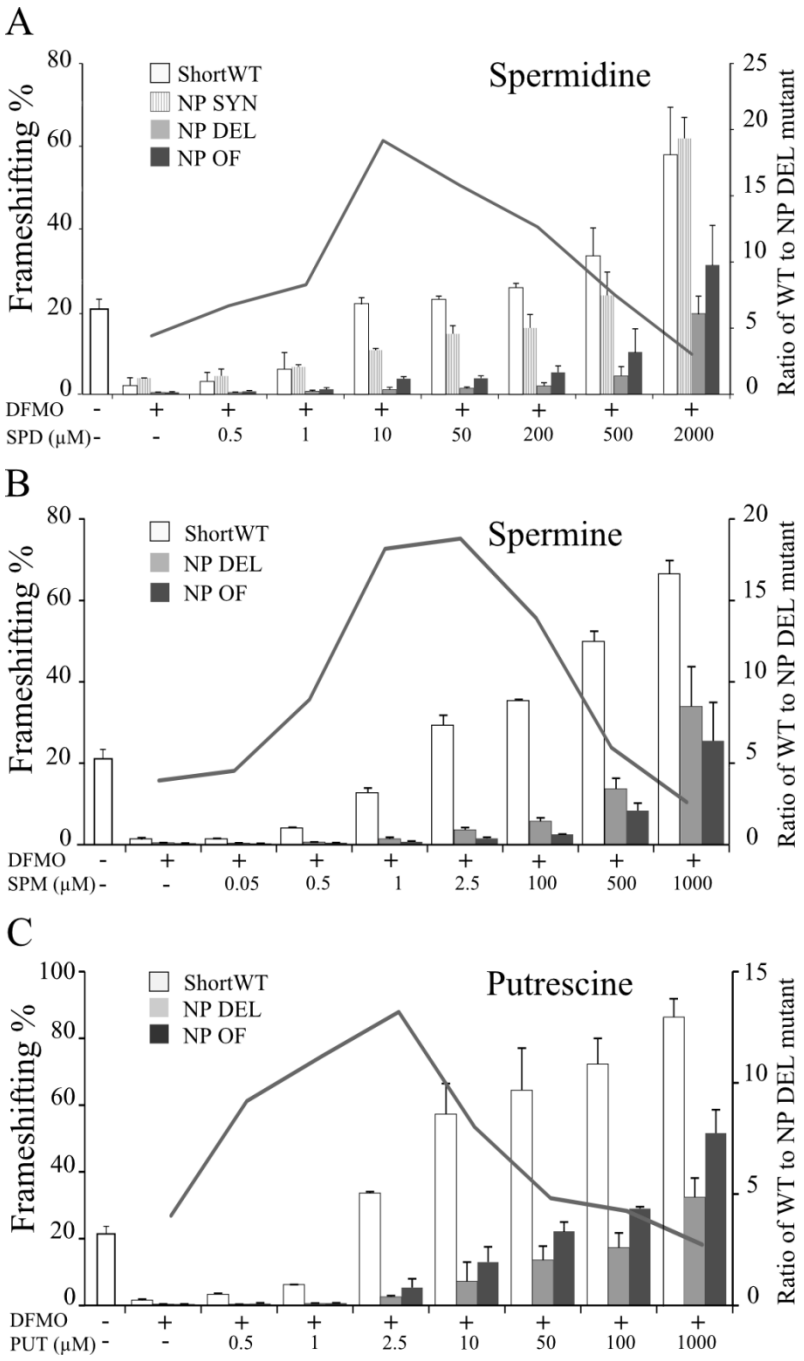


Figure 4.5: *Titration of polyamines with ShortWT and 5' mutant cassettes. ShortWT, NP DEL and NP OF constructs are as described in Figs.4.3 and 4.4. The NP SYN construct introduces 11 synonymous changes in the sequence encoding the putative nascent peptide signal. The leftmost white column in each graph represents the frameshift level with ShortWT cassette in untreated cells - i.e. cells in which free polyamines are in homeostasis. The curve represents the ratio of ShortWT to NP DEL frameshifting across the titration gradient. A. Titration of Spermidine (SPD)*

with ShortWT (white columns), NP DEL (grey columns), NP OF (black columns) and NP SYN (striped columns). B. Titration of Spermine (SPM) with ShortWT (white columns), NP DEL (grey columns) and NP OF (black columns). C. Titration of Putrescine (PUT) with ShortWT (white columns), NP DEL (grey columns) and NP OF (black columns).

The contribution of individual amino acids of the nascent peptide to the stimulatory effect on frameshifting was assessed by testing three series of constructs. In an alanine scan series, each codon of the conserved peptide sequence (from position -15 to -5) was sequentially replaced with an alanine codon (each had an in-frame control). None of the individual alanine substitutions produced a dramatic effect on frameshifting efficiency, suggesting that amino acid identity at any one position is not crucial (Fig.4.6A). The second, or deletion, series of constructs had a sequentially increasing number of codons from -15 to -2 deleted. In the third, or out-of-frame, series increasing one codon increments from -15 to -2 of the nascent peptide encoding sequence were put out-of-frame. This was accomplished by deleting one U nucleotide at the 5' end of the sequence and adding one U nucleotide at the 3' end of each increment. The results from the series of deletion and out-of-frame constructs are consistent with those from the alanine scan and indicate that the effect of residue deletion/alteration is cumulative – with progressive deletions yielding greater reductions of frameshifting efficiency (Fig.4.6B, C). Interestingly, the effect of the peptide alterations on the sensitivity of frameshifting to spermidine level is different with the different concentrations of SPD supplementation tested. At the highest concentration of SPD the frameshift site preserved a near maximum efficacy when the first eight amino acid residues were altered. By contrast, under conditions mimicking endogenous polyamine levels (DFMO + 50 μM SPD) and under low polyamine concentration (DFMO only), frameshifting was significantly reduced with as few as the first three amino acid residues being altered (Fig.4.6B, C). Deletion or out-of-frame mutations that encroach on module A result in dramatic

reduction of frameshifting efficiency, especially under condition of DFMO treatment plus 50 μM SPD supplementation.

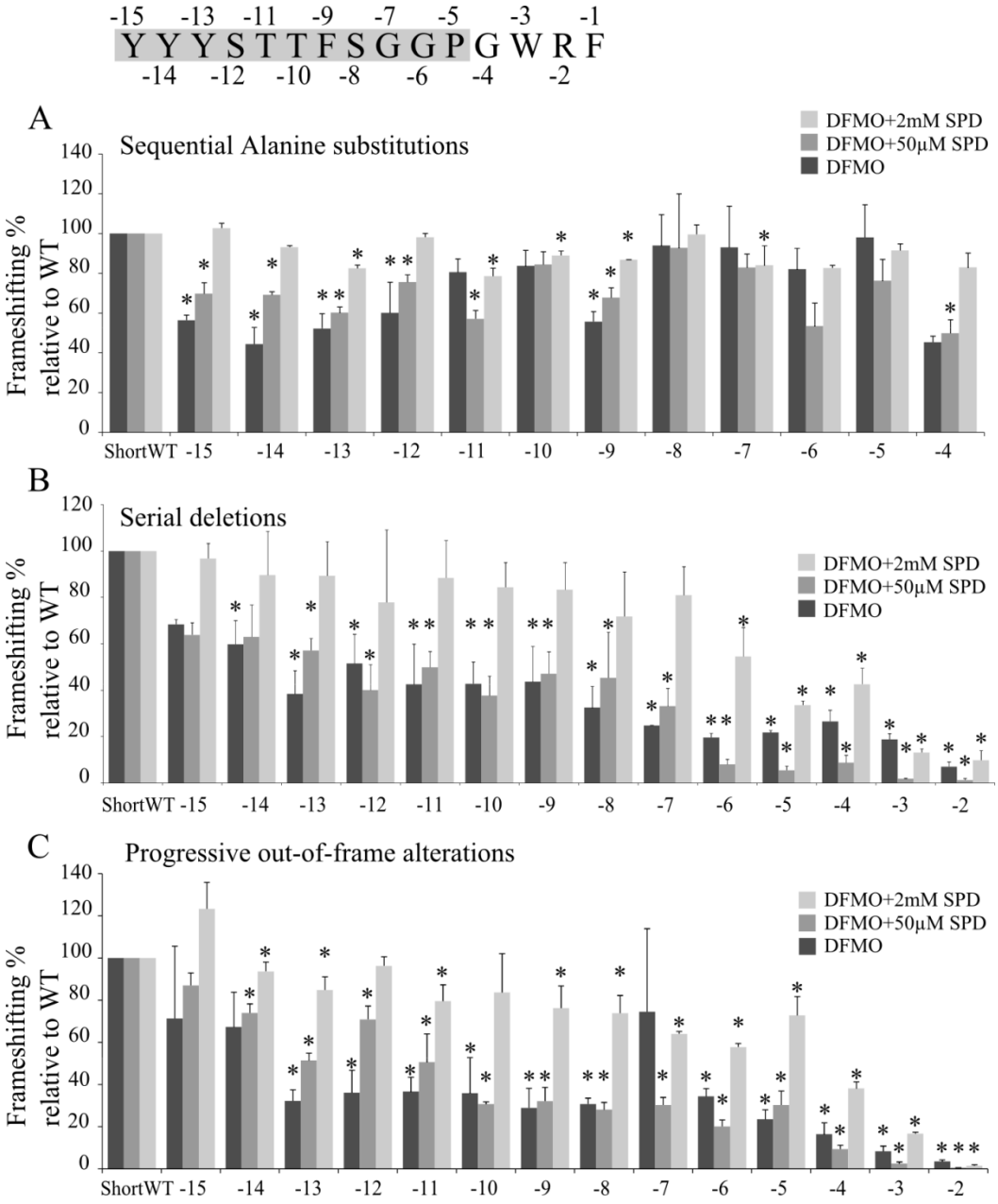


Figure 4.6: *Fine analysis of the 5'nascent peptide stimulator. The sequence of the 5' nascent peptide signal (residues -15 to -5) is shown above the figure highlighted in grey. The frameshifting efficiencies of three series of mutant*

constructs are presented as percentage of ShortWT efficiency. All constructs were tested in three conditions: DFMO treatment (black bars), DFMO treatment plus 50 µM SPD (grey bars) and DFMO treatment plus 2 mM SPD (light grey bars). A. Sequential alanine substitutions of individual codons at positions from -15 to -4; B. Serial deletions of codons from position -15 to -2. C. Progressive out-of-frame alterations of codons at positions -15 to -2.

4.3.4 Nascent peptide signal and its relationship to module A

To elucidate the effect of module A on frameshifting stimulation, two constructs with altered module A sequence were tested. The peptide signal encoding sequence is unaltered in MOD A in which module A sequence was substituted with its complement. In construct NP OF+MOD A, in addition to the module A substitution, the sequence of codons -15 to -5 that encode the nascent peptide signal, is placed out-of-frame. When tested under the three SPD supplementation conditions, frameshifting efficiencies with both, NP OF+MOD A and MOD A constructs, were dramatically lower compared to those with ShortWT or NP OF (Fig. 4.7A). This suggests that the peptide sequence alone is insufficient for high levels of frameshifting or conversely that module A is essential for efficient frameshifting.

To investigate if module A extends 7 nucleotides 5' of the frameshift site, as previously suggested (Ivanov and Atkins, 2007), we designed three constructs introducing nucleotide substitutions within the codon at position (-4) relative to the stop codon. The third nucleotide position in this codon, a G nucleotide, is the first nucleotide of module A, as proposed (Ivanov and Atkins, 2007). In the GGG(-4)GGC cassette the third nucleotide of this codon was changed from the wild-type G to C, a substitution which preserved the identity of the encoded amino acid (Gly). The other two constructs tested at the same time, have Glu at position (-4), however in the GGG(-4)GAG construct the Glu was encoded by a GAG codon, whereas in GGG(-4)GAA – it was encoded by a GAA codon. We chose to substitute the native (Gly) with (Glu) because in at least two mushroom antizyme mRNAs, the naturally occurring codon at position -4 is GAG Glu instead of GGG Gly. This amino acid

change does not change the identity of the nucleotide at position 3. With SPD supplementation mimicking endogenous levels of polyamines, the construct, GGG(-4)GAA, yielded a frameshifting efficiency closer to that of the GGG(-4)GGC construct, while the construct GGG(-4)GAG yielded a frameshifting efficiency similar to that of ShortWT (Fig.4.7B). The results suggest that the identity of the nucleotide at position 3 of codon -4 is more important than the identity of the amino acid encoded, consistent with it being part of the nucleotide specific module A. The nucleotides in the 5' adjacent codon, UGG (Trp), cannot be changed without changing the amino acid identity at this position and so precluding this type of analysis.

The codon at position (-2) was altered to test if it functions at the nucleotide or amino acid levels. Cassettes CGU(-2)CGA and CGU(-2)AGA introduced one and two nucleotide substitutions respectively, but both encode Arg which is the wild type amino acid at that position (-2). Both constructs yielded reduced frameshifting levels with the one having 2 nucleotide substitutions exhibiting a greater reduction (Fig.4.7B). The greatest effect was seen with DFMO + 50 μM SPD supplementation – frameshifting reduced to 60% (CGU(-2)CGA) and 36% (CGU(-2)AGA) of ShortWT. We also tested the construct CGU(-2)UGG which has two nucleotide substitutions that change the identity of the original Arg to Trp. This is a naturally occurring variation at codon (-2) in some Basidiomycotal antizyme mRNAs. Curiously, this alteration did not change the wild type FS levels.

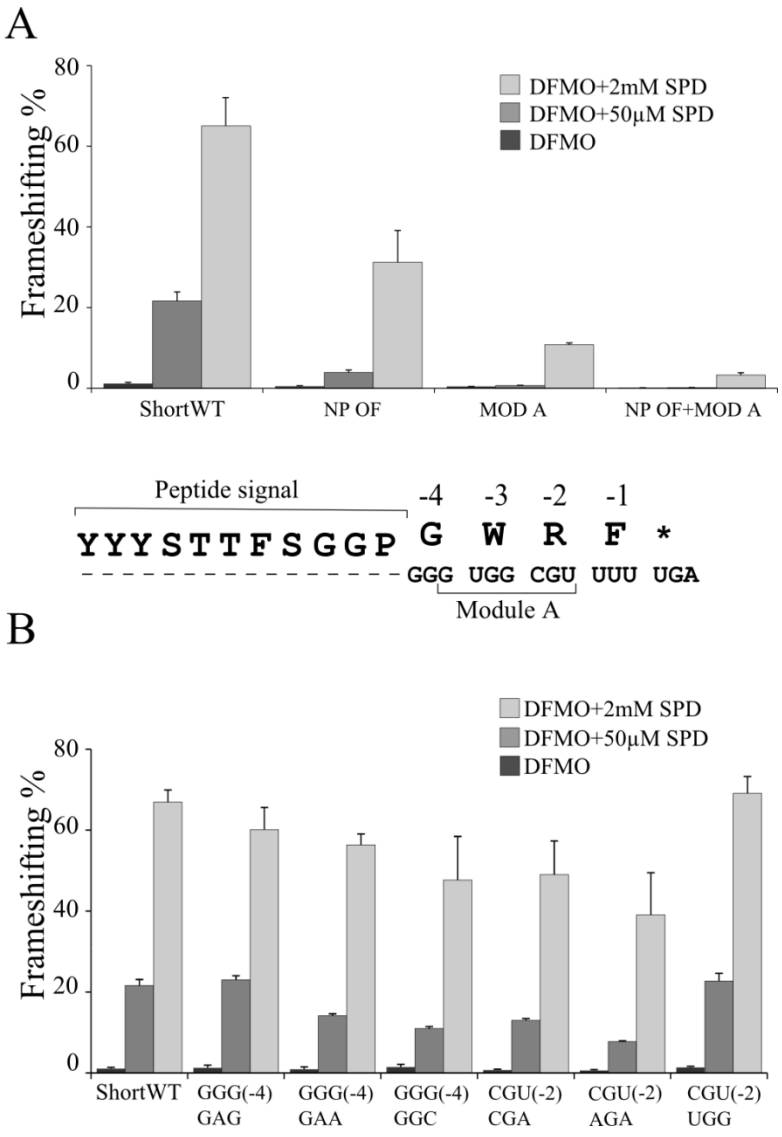


Figure 4.7: T*esting the effect of module A on antizyme frameshifting. ShortWT, NP OF and MOD A constructs are described in Fig.4.3 and 4.4A. The constructs were tested in three conditions: DFMO treatment (black bars), DFMO treatment plus 50 µM SPD (grey bars) and DFMO treatment plus 2 mM SPD (light grey bars). A). NP+MOD A construct combines an out-of-frame nascent peptide encoding sequence and a module A sequence that is the complement of its WT. B). Frameshifting efficiencies with ShortWT and its derivative cassettes with nucleotide substitutions in the codons at positions (-4) or (-2). The GGG(-4)GAG and GGG(-4)GAA constructs have the glycine codon (GGG) at position -4 changed to the glutamate (GAG) or (GAA) codon , either by one or two nucleotide substitutions respectively. The GGG(-4)GGC cassette has the third G nucleotide of that codon changed to C. This synonymous substitution preserves the identity of the encoded amino acid glycine. In CGU(-2)CGA construct the third nucleotide (U) in the CGU (Arg) codon at position*

-2 is changed to an (A). CGU(-2)AGA has two nucleotide changes which however do not alter the encoded amino acid (Arg). In CGU(-2)UGG construct the CGU (Arg) codon at position -2 is changed to UGG (Trp) codon.

Finally we tested the potential of the stimulatory nascent peptide and module A in ShortWT context to cause a pause that is detectable using a toe-printing assay in *Neurospora crassa* extracts (Wang and Sachs, 1997). The positive control was the Arginine Attenuator Peptide (AAP) encoding sequence in the presence or absence of externally added Arg (Wang and Sachs, 1997) (Fig.4.8). Stalling was not observed with the antizyme cassette under the conditions tested. Toe-printing with 3'STR DEL construct, and its derivative with the U of the stop codon deleted, in rabbit reticulocyte lysate (RRL) gave a similar result (Fig.4.9).

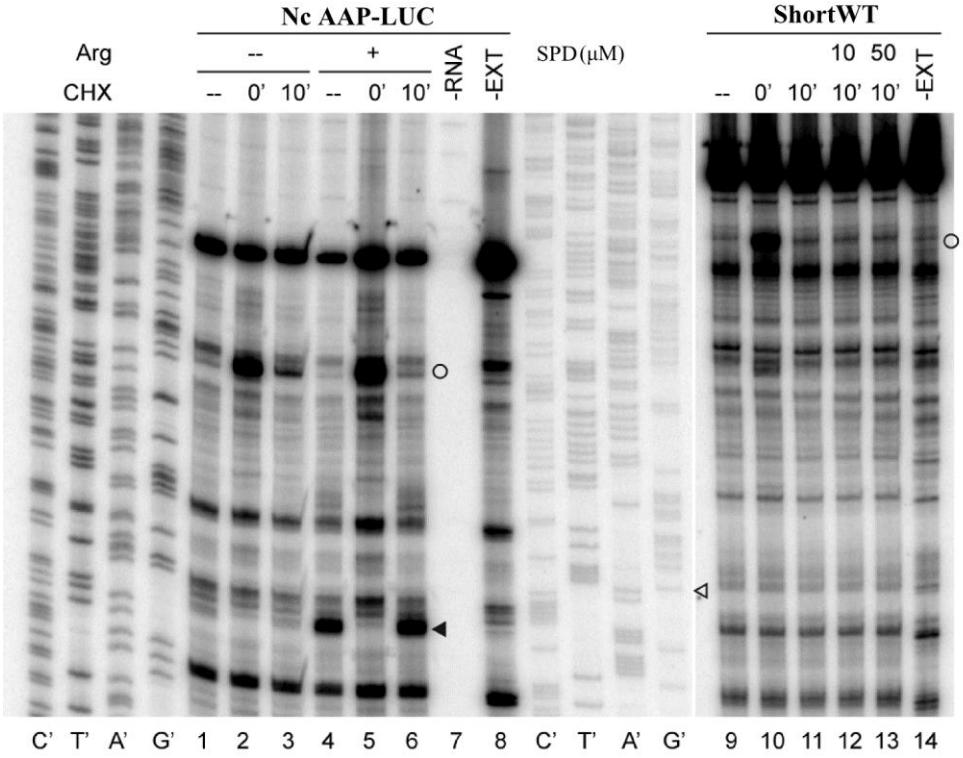


Figure 4.8. *Toe-printing of ribosomes translating C. cinerea antizyme mRNA in N. crassa extracts. The mRNAs used to program translation in N. crassa extracts are indicated on the top. On the left, reaction mixtures contained 10 μM (−) or 2 mM (+) Arg and 10 μM each of the other 19 amino acids. On the right, reaction mixtures contained 10 μM of the 20 amino acids and 0, 10, or 50 μM of spermidine (SPD). Cycloheximide was added to the reactions at indicated time points. Lanes indicated as no RNA or no EXT show toe-printing of extract without added RNA, and of RNA in the absence of extract, respectively. The same primer was also used for dideoxynucleotide sequencing of pRR301 (left sequencing markers) and pShortWT-IF (right sequencing markers). The open circles indicate the position of the toe-prints corresponding to ribosomes with the initiation AUG codon in the P-site. The black arrowhead indicates the position of the toe-prints corresponding to ribosomes with the AAP uORF termination codon in the A-site. The open arrowhead indicates the position of the G nucleotide in the ORF1 stop codon of ShortWT.*

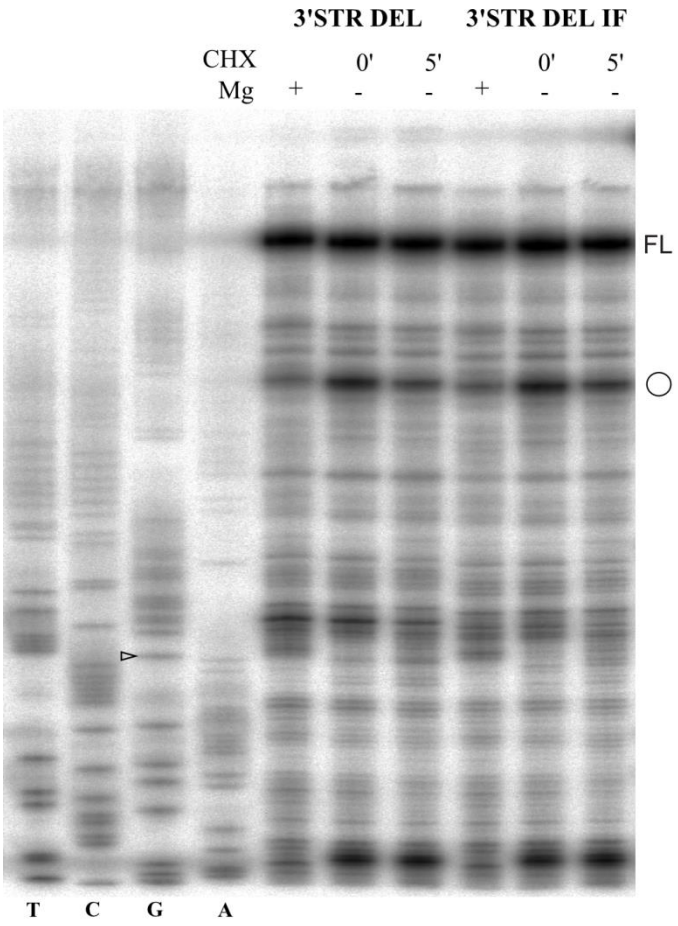


Figure 4.9*: Toe-printing of 3'STR DEL and 3'STR DEL IF mRNAs in rabbit reticulocyte lysate (RRL). Translation conditions are labelled on the top of the gel. The open circle indicates the main initiation signal at the AUG. The position of the G nucleotide in the shift site is indicated by an open arrowhead.*

4.4 Discussion

4.4.1 Nascent Peptide.

The results provide experimental and further phylogenetic evidence for a nascent peptide stimulator of Agaricomycetes antizyme mRNA +1 frameshifting. When the sequence encoding the 11 AA peptide (encoded -15 to -5 codons 5' of the shift site) is placed out-of-frame, frameshifting is reduced similarly to that of a complete deletion. In contrast, compared to wild type, introducing 11 synonymous nucleotide changes in the same region had a small effect on frameshifting. Serial 5' deletions,

out-of-frame mutations and an alanine scan (Fig. 4.6) suggest that the contribution of the amino acids to the effect is cooperative with no single amino acid being crucial for the stimulatory effect.

In distinction to the nascent peptide signal effects in *S. cerevisiae*, the peptide signal described here acts within the ribosome mediating the frameshift event. The 11 AA long peptide signal is expected to occupy approximately one third of the peptide exit tunnel. It is longer than MAGDIS, the hexapeptide product of the inhibitory uORF in mammalian AdoMetDC mRNA (Mize et al., 1998), but substantially shorter than the nascent peptide signal responsible for StopGo/Stop-Carry on (Donnelly et al., 2001a). Atomic level structure analysis of *S. cerevisiae* ribosomes reveal a constriction in the exit tunnel (Yusupova and Yusupov, 2014), that is the counterpart of that in bacteria at which SecM works – in bacteria this is approximately one third of the way into the tunnel from the PTC. Because of positioning of the Agaricomycete nascent peptide signal beginning 3AA distant from the PTC at the time of frameshifting, it may span the constriction likely also present in its ribosomes.

Small molecules, other than polyamines, can influence nascent peptide effects more generally within the peptide exit tunnel and an increasing number of examples are known where a specific nascent sequence in the exit tunnel causes a ribosome stall in eukaryotes (Luo et al., 1995, Wei et al., 2012) and in bacteria (Vazquez-Laslop et al., 2010, Gong and Yanofsky, 2002). In some cases (Cao and Geballe, 1996), the ribosome stalling occurs just prior to, or during, the decoding of a stop codon in the ribosomal A site, such that termination is inhibited. Since frameshifting and other recoding events are in competition with standard decoding, inhibition of the standard decoding step should push the equilibrium toward the recoding event (Atkins and Gesteland, 2010).

The results from the toe-printing assay could reflect instability of the particular stalled complex involved even though the counterpart paused complex in the positive control was stable. However it is more likely that at the sensitivity of the assay, no detectable pausing occurs. The peptide effect might not be due to ribosome stalling. If so, it would be conceptually similar to one case in bacteria recently

identified where ketolides promote -1 frameshifting when translating the leader peptide that results in activation of the translation of the downstream gene (Gupta et al., 2013). Recently highly sensitive single-molecule fluorescence has been applied to study the dynamics of a 5' stimulatory element on bacterial -1 frameshifting (Chen et al., 2014). As FRET approaches are developed for counterpart studies in eukaryotes much more refined step time data should be forthcoming. Knowledge of the likely non-canonical ribosome states in antizyme frameshifting are likely from such studies and other approaches especially cryo Electron Microscopy.

The results presented here, suggest that the role of the Agaricomycetes nascent peptide stimulators, presumably via interaction with exit tunnel components, is not simply to increase the efficiency of frameshifting but also to contribute to making the process especially sensitive to intracellular free polyamines at their endogenous levels. This is a novel feature for the function of *cis*-acting sequences in antizyme mRNA frameshifting.

4.4.2 Polyamine level.

Previous analyses of *cis*-acting stimulators of frameshifting in antizyme genes were usually performed in a way that only examined their effect in two extreme conditions with regard to polyamine concentrations – cells depleted for polyamines and supplemented with the highest dose of polyamines. This approach fails to investigate what function the *cis*-acting stimulators might have in intermediate concentrations of polyamines much closer to their endogenous levels. This approach fails to investigate what function the *cis*-acting stimulators have in intermediate concentrations of polyamines closer to their endogenous levels. When a spermidine titration experiment was performed with a wild type construct and three mutant constructs affecting the nascent peptide stimulator, a significant result was observed. The mutant constructs that substituted the amino acid sequence of this region exhibited a response lag to polyamine stimulation – i.e. intermediate concentrations of polyamines stimulate the wild type and the synonymous mutants far more than they do the two mutants that change the amino acid sequence of the nascent peptide

(Fig. 4.5A). The point on the titration experiment where the difference in stimulation is greatest coincides with the DFMO + polyamine supplementation that induces frameshifting levels in ShortWT close to 22%. This is very close to the frameshifting observed with the same sequence in untreated cells. In other words, the difference in response to polyamines between mutant cassettes that do, or do not, encode altered peptide sequence, is greatest under conditions that most closely mimic endogenous levels of free polyamines. This suggests that the sensor function of the nascent peptide stimulator is physiologically relevant. The potential for differential affinity sites for polyamine binding with contrasting effects on frameshifting makes it likely that discerning the mechanism will not be a simple task.

4.4.3 Module A

One of the 5' stimulators of antizyme frameshifting, apparently present in many if not most antizyme homologs, is a sequence of 6 to 7 nucleotides immediately abutting the shift site. At the time of the frameshifting the 5' adjacent codon to the shift site UUU_U would be in the E-site or in transit. Prior studies with different types of frameshifting have shown the importance of E-site codon interactions in some cases (Sanders and Curran, 2007, Baranov et al., 2002a, Leger et al., 2007). In Agaricomycete antizyme mRNA the E-site codon position with the greatest variability is definitely the third position (Fig. 1A). Experimentally changing the CGU (Arg) to CGA (Arg) or AGA (Arg) reduced frameshifting at all polyamine concentrations tested, with two changes leading to more severe reduction. In some Basidiomycotal antizyme mRNAs, outside of the Agaricomycetes lineage, there is a natural variation at the same codon, -2, that replaces the CGU Arg with UGG Trp. Substituting CGU with UGG, involving the same two codon positions did not reduce frameshifting at any of the polyamine concentrations tested. This is consistent with the overall strength of E-site codon:anticodon, irrespective of individual nt position, being relevant but is a weak inference.

Changing codon -4 from GGG (Gly) to GAG (Glu) had no effect on the efficiency, but changing it to GAA (Glu) resulted in a 40% reduction and to GCG

(Ala) had a 50% reduction. These data raise the possibility that the encoded amino acid may have an effect in the peptide exit tunnel and the nucleotide sequence may have an effect with the mRNA exit tunnel, perhaps even in interactions with rRNA.

Mutating module A to its complementary sequence had the same lag effect on polyamine stimulation across a titration gradient as seen with the nascent peptide mutants. Though the number of data points was limited (Fig. 4.7A), this suggests that module A too could be involved in sensitizing the shift site to polyamines in their endogenous levels range. As introduced above, module A is widely evolutionarily conserved and its original likely predates that of the nascent peptide signal in Agaricomycetes which has a restricted range. Though module A and the nascent peptide signal work at the same time, it may well be that future work will reveal instances of programmed frameshifting where the only 5'/5' encoded stimulator is a nascent peptide signal. The involvement of nascent peptide signals in stop codon readthrough, a different class of recoding, is also to be expected.

The experiments examining the mode of function of the conserved elements in Agaricomycetes antizyme mRNAs revealed that in addition to their stimulatory effect on the frameshift efficiency, these elements seem to sensitize the frameshift site for polyamines especially in the range of their endogenous concentrations. This model provides an example for how the *cis*-acting elements in antizyme mRNAs could exert their function.

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