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REVIEW

tRNA transfers to the limelight

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tRNA has a central role in biology as the adaptor between mRNA and protein, in which anticodon interactions with the mRNA codon at one end of the L-shaped tRNA allow an attached amino acid at the other end of the tRNA to condense with the peptide chain on a second tRNA through the action of the ribosome. This familiar role of tRNA requires mature tRNAs to be recognized by the aminoacyl tRNA synthetase to add the amino acid to its 3′ end, by EF1α to form a ternary complex with the aminoacylated tRNA and GTP, and by elements of the ribosome and associated components to allow binding, peptide synthesis, and translocation. Before fulfilling this role, all tRNA transcripts have to be extensively processed. Maturation of tRNAs requires five major steps: (1) removal of the 5′ leader by RNase P, which almost universally requires a ribonucleoprotein complex; (2) removal of the 3′ trailer sequence by some combination of endonucleases and exonucleases; (3) addition of CCA in eukaryotes, many eubacteria, and some archaea; (4) splicing of introns in some tRNAs of most (if not all) eukaryotes and some archaea, by an endonuclease that excises the intron and a ligase that joins the exons; and (5) numerous modifications of tRNA at multiple residues (see Fig. 1).

Recent results have led to several fascinating new insights into tRNA biology, which are described in this review. First, there has been dramatic progress in identification of the component enzymes and corresponding genes responsible for tRNA processing. In the yeast *Saccharomyces cerevisiae*, 38 gene products are now known to be directly responsible for tRNA processing, most of which have been identified in the last few years (Table 1). Because the genes corresponding to 16 more modification and processing steps remain to be identified, this means that ~1% of the yeast genome is directly involved in tRNA processing, not counting gene products involved in tRNA transcription and tRNA transport. Second, there has been significant progress in defining the broad outlines of specificity of several modification enzymes, and of the roles of the individual members of complexes in substrate recognition and catalysis, while

raising new questions about the precise nature of these functions. Third, there have been new insights into the cellular role of some tRNA modification enzymes whose role had previously been obscure because of the lack of mutant phenotypes, which was at odds with their obvious evolutionary conservation. Modifications are now seen as integral components of processing that interact with and influence translation at multiple levels, that affect nuclear transport, and that influence the stability of tRNA. Fourth, new evidence underscores the interplay of tRNA processing and tRNA-processing enzymes with other major pathways in the cell such as sterol biosynthesis, the unfolded protein response, and rRNA processing. Fifth, different organisms have evolved several different methods of maturing tRNA at almost every step, some of which involve novel mechanisms. This is reflected in multiple methods of building a 3′ end, a novel method of building a new 5′ end, two different pathways for splicing tRNA, new insight into the dynamics of CCA addition, and a new look at mechanisms that control tRNA quality. Sixth, the last few years have witnessed surprises and unexpected complexities from the study of the cell biology of tRNA and its processing enzymes. These include multiple nuclear locations of both tRNA and processing enzymes, the intersection of tRNA transport and tRNA aminoacylation in the nucleus, the existence of at least two pathways for tRNA transport from the nucleus to the cytoplasm, and the emergence of a whole new pathway of mitochondrial tRNA import. In short, tRNA is charging again.

Because progress has been most rapid and complete for the yeast *S. cerevisiae*, this review concentrates on developments in yeast. However, many exciting results that have emerged from study in other organisms are also discussed.

Genomic analysis and new technology have opened the field of tRNA-processing biochemistry

The importance of bioinformatics for identifying gene products involved in processing cannot be overstated. The analyses of Koonin, Santi, and coworkers (Gustafsson et al. 1996; Koonin 1996), using iterative approaches with BLAST with known genes encoding pseudouridylases and methyltransferases, yielded several predicted *Escherichia coli* pseudouridylases that ultimately

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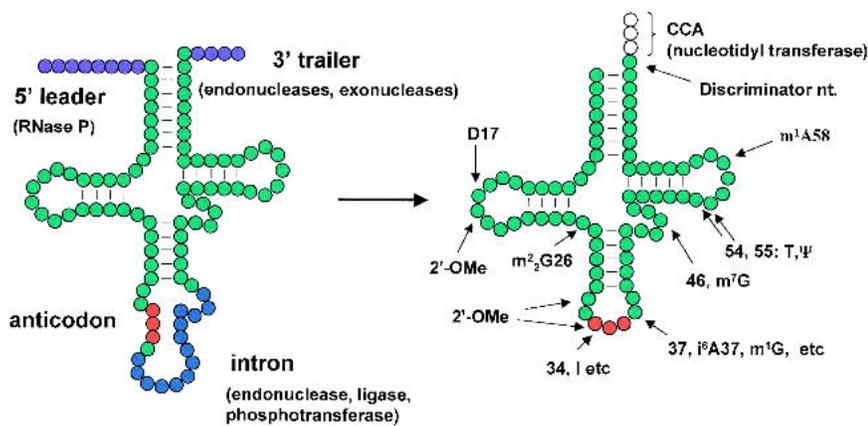
Figure 1. A schematic of a tRNA precursor and a mature tRNA. Each nucleotide is represented by a filled circle: part of the mature tRNA (green); leader and trailer sequences (purple); intron (blue); anticodon (red). The positions of several of the tRNA modifications discussed in the text are indicated on the canonical tRNA molecule shown at the *right*, using the usual tRNA numbering system. For reviews of the modifications, see Björk (1995) and Sprinzl et al. (1998).

proved correct (Del Campo et al. 2001, and references therein), as well as several candidate yeast enzymes. These analyses, coupled with similar analysis of yeast *PUS1* after its isolation in a genetic screen (Simos et al. 1996), and subsequent similar bioinformatics searches, ultimately led to the identification of genes encoding four different yeast tRNA pseudouridylases (Simos et al. 1996; Becker et al. 1997; Lecointe et al. 1998; Ansmant et al. 2001), four tRNA methyltransferases (Cavaillé et al. 1999; Motorin and Grosjean 1999; Nordlund et al. 2000; Pintard et al. 2002), and two adenosine deaminases (Gerber et al. 1998; Gerber and Keller 1999). Bioinformatics-based identification of Trm5p, the yeast m^1G methyltransferase responsible for modification at site 37, was slightly more complicated. To accomplish this, Björk and colleagues first identified an archaeal ortholog to the *E. coli* m^1G methyltransferase by a genetic selection, and then used a conserved region to identify the yeast gene (Björk et al. 2001).

Two very different genomic methods were used simultaneously to identify a family of dihydrouridine synthases in yeast and in *E. coli*. The yeast dihydrouridine synthase Dus1p was identified by a biochemical genomics approach involving parallel biochemical analysis of the yeast proteome (Xing et al. 2002). In this approach, a genomic set of 6144 yeast strains, each expressing a distinct GST-ORF fusion protein, was used to obtain 64 pools of purified yeast ORFs, each derived from 96 strains. Then pools were assayed for activity, and positive fractions were deconvoluted by preparation and analysis of subpools of the proteins (Martzen et al. 1999; Grayhack and Phizicky 2001). This biochemical genomics approach was also recently used to identify a complex of two yeast proteins that catalyze m^7G formation at position 46 of tRNAs (Alexandrov et al. 2002). The *E. coli* dihydrouridine synthase family was identified by an interesting bioinformatics analysis (Bishop et al. 2002). In this study, the COG (conserved orthologous genes) database was parsed to separate those COGs that did not have representatives in an organism lacking dihydrouridine, but did have representatives in several organisms that had dihydrouridine. Of the 86 COGs that were retrieved, six were candidates because they were poorly characterized, and one of these six coclustered physically

with tRNA modification activities, had some similarity to enzymes of similar biochemical activity, and proved to encode dihydrouridine synthase.

More conventional biochemical and genetic methods have also played a prominent role in identifying genes whose products are involved in tRNA processing. Brute force biochemical purification of yeast nuclear RNase P, followed by mass spectrometry analysis of polypeptides, yielded five new polypeptide subunits of the complex, together with four previously identified polypeptide subunits and an RNA subunit (Chamberlain et al. 1998). Similar brute force purification led to the identification of *TPT1*, encoding the 2'-phosphotransferase that removes the splice junction phosphate in the last step of yeast tRNA splicing (Culver et al. 1997). Identification of the four subunits of the yeast splicing endonuclease proteins was accomplished by coimmunoprecipitation with an epitope-tagged construct of the one known protein subunit, followed by peptide sequencing (Trotta et al. 1997). Finally, genetic analysis led to the identification of both subunits of the m^1A methyltransferase responsible for modification of A58 of tRNAs (Anderson et al. 1998, 2000), as well as of several other genes resonsible for various modifications (Table 1).

Gene discovery uncovers complexities in substrate specificity and enzyme composition

The identification of all these genes specifying tRNA-processing proteins has opened the door to two broad avenues of research: examination of the biochemical basis for substrate specificity and catalysis, and examination of the cellular role of the proteins. Biochemical analysis of modifications in vitro and analysis in vivo have shown that some modification enzymes, like Pus1p and Trm4p, catalyze modifications at a variety of different positions in different tRNAs (Simos et al. 1996; Motorin et al. 1998; Motorin and Grosjean 1999), whereas others, such as Pus3p and Trm7p, are region-specific (Lecointe et al. 1998; Pintard et al. 2002), and many other modification enzymes are completely position-specific. The nature of these differences in specificity is poorly understood, although it is known that the less specific enzymes Pus1p and Trm4p recognize only local struc-

Table 1. *Genes from* Saccharomyces cerevisiae *whose products catalyze steps in tRNA processing*

Yeast gene	Isolation method	Modification	Mutant phenotype	Location observed	References
PUS1	genetics	Ψ27, 28, 34 (35), 36 (26, 65, 67 likely); U2 snRNP 44	not essential; synthetic effects with pus4, los1, or altered minor tRNA^{Gln}	nucleoplasm	Simos et al. 1996; Motorin et al. 1998; Massenet et al. 1999; Grosshans et al. 2001
PUS3 (DEG1)	bioinformatics	Ψ38, 39 cyt., mito.	slow growth	primarily nucleus and cytosol	Lecointe et al. 1998
PUS4	bioinformatics	Ψ55 cyt., mito.	not essential; synthetic effects with pus1, or with altered tRNA$^{Ser}_{CGA}$	ND[a]	Becker et al. 1997; Grosshans et al. 2001; Johansson and Byström 2002
PUS6	bioinformatics	Ψ31 cyt., mito.	not essential	primarily cytosol	Ansmant et al. 2001
PUS8	ND	Ψ32	ND	ND	see Grosshan et al. 2001
TRM1	genetics; bioassay	m$^2_2$G26	not essential; synthetic effects with altered tRNA$^{Ser}_{CGA}$	nuclear rim; mitochondria	Ellis et al. 1986; Li et al. 1989; Johansson and Byström 2002
TRM2	genetics, bioinformatics	m^{5}U54 cyt., mito.	not essential; synthetic effects with altered tRNA$^{Ser}_{CGA}$	ND	Hopper et al. 1982; Nordlund et al. 2000; Johansson and Byström 2002
TRM3	bioinformatics	Gm18	not essential; synthetic with altered tRNA$^{Ser}_{CGA}$	ND	Cavaillé et al. 1999; Johansson and Byström 2002
TRM4	bioinformatics	m^{5}C 34, 40, 48, 49	not essential; paromomycin-sensitive	nuclear rim	Wu et al. 1998; Motorin and Grosjean 1999
TRM5	bioinformatics	m^{1}G37, m^{1}I,yW	very sick	ND	Björk et al. 2001
TRM7	bioinformatics	2′-O-Me 32 and 34	slow growth; paromomycin-sensitive; slowed translation	cytosol	Pintard et al. 2002
TRM8/TRM82	biochemical genomics	m^{7}G46	not essential	ND	Alexandrov et al. 2002
MOD5	genetics	i^{6}A37	loss of suppression	nucleoplasm; nucleolus; cytoplasm; mitochondria	Laten et al. 1978; Dihanich et al. 1987; Boguta et al. 1994
GCD10, GCD14	genetics	m^{1}A58	essential	nucleus	Anderson et al. 1998; Calvo et al. 1999; Anderson et al. 2000
TAD2, TAD3	bioinformatics	A34 to I34	essential	ND	Gerber and Keller 1999
DUS1,2	biochemical genomics; bioinformatics	D17 tRNA^{Phe} (Dus1p, in vitro)	not essential	ND	Bishop et al. 2002; Xing et al. 2002
TAD1	bioinformatics	A37 to I37 tRNA^{Ala}	not essential	ND	Gerber et al. 1998
RIT1	genetics	2′-O-ribosyl phosphate at 64 of tRNA$^{Met}_i$	not essential; synthetic effects with elF-2 mutations	ND	Åström and Byström 1994; Åström et al. 1999
SEN2, SEN15, SEN34, SEN54	genetics (SEN2); copurification	splicing endonuclease	essential	ND	Ho et al. 1990; Trotta et al. 1997
TRL1	purication	tRNA ligase	essential; tRNA splicing and HAC1 mRNA splicing	nuclear periphery; some cytosol activity	Clark and Abelson 1987; Phizicky et al. 1992
TPT1	purification	2′-phosphotransferase	essential	ND	Culver et al. 1997; Spinelli et al. 1997
POP1, POP3, POP4, POP5, POP6, POP7, POP8, RPP1, RPR2, RPR1	purification	RNase P	essential	nucleolus (some nucleoplasm)	Bertrand et al. 1998; Chamberlain et al. 1998

[a]Not determined.

tural features because they can catalyze reactions on minisubstrates (Motorin et al. 1998; Motorin and Grosjean 1999). Equally poorly understood for the majority of modifications is why only some tRNAs with the correct residue at the appropriate site are modified, whereas others are not. For several modifications, like i^{6}A in *E. coli*, the strict surrounding sequence requirements and features of the adjacent loop and helix can explain much of the specificity (Motorin et al. 1997); however, for many others there is little or no information.

Another active area concerns the precise role of individual subunits of multisubunit processing enzymes. For m^{1}A methyltransferase, which is catalyzed by Gcd10p/Gcd14p, it seems likely that Gcd10p directs binding of tRNA and Gcd14p binds the required cofactor *S*-adenosylmethionine (Anderson et al. 2000). However, for the adenosine deaminase Tad2p/Tad3p complex, both members of which share significant homology, there is no such clean split of functions. Mutational analysis indicates that Tad2p is the catalytic subunit, but both subunits appear necessary for binding (Gerber and Keller

1999). Still more complicated is the tetrameric splicing endonuclease of yeast, which like Tad2p/Tad3p, has two highly related subunits that are part of a widely conserved family. In this case each of the two related subunits, Sen2p and Sen34p, catalyzes one of the two endonucleolytic excision steps, thus taking the place of the homodimeric splicing enzyme in other organisms (Kleman-Leyer et al. 1997; Lykke-Andersen and Garrett 1997; Trotta et al. 1997; Li et al. 1998a). The roles of the other two subunits of the yeast enzyme, Sen15p and Sen54p, are unknown; in particular, there is no explanation for why the protein appears to be membrane-associated (Peebles et al. 1983; Trotta et al. 1997), and how tRNA recognition is accomplished. The largest complex in the yeast tRNA-processing pathway is RNase P, which is comprised of at least nine essential protein subunits and one essential RNA subunit (Chamberlain et al. 1998). Its composition belies the relatively simple nature of the reaction, cleavage of the 5′ leader from pre-tRNA molecules (Fig. 1); indeed, the corresponding bacterial enzyme accomplishes the same goal with just one catalytic RNA subunit and one protein subunit (Guerrier-Takada and Altman 1984). Although many of the details of the architecture of RNase P have been elucidated (Houser-Scott et al. 2002), the nature of the recognition domain and the catalytic domain is still unclear. Recently, it has been shown that the isolated Pop3p subunit binds pre-tRNAs tightly (Brusca et al. 2001). However, a precursor form of RNase P that lacks this subunit is active (Srisawat et al. 2002). Because this precursor form also lacks the only subunit that is unique between RNase P and the related nuclease RNase MRP, which is specific for rRNA and not tRNA, the suggestion has been made that the RNA subunit of RNase P is responsible for substrate specificity (Srisawat et al. 2002). Clearly, much remains to be done to elucidate precisely how this protein complex finds its substrates and catalyzes its activity, and the precise roles of the subunits in this function.

Unraveling the elusive functions of tRNA modification

Previous studies had indicated that although a few exceptional modifications were important for growth, the majority of tRNA modifications in yeast or other organisms had only modest effects on translation under defined circumstances, or had little or no measurable effects on growth. The failure to identify phenotypes of many modification mutants was distinctly unexpected, given their evolutionary conservation, the observation that almost all tRNAs have multiple modifications, and the fact that more than 80 modifications have been described in various organisms (Björk 1995; Sprinzl et al. 1998), and precluded further study of their function in meaningful ways. However, several examples of phenotypes of modification defects have now been described, which shed light on their roles.

For several modifications affecting the region around the anticodon, lack of the corresponding gene causes a distinct growth phenotype. Thus, slow growth is observed in strains lacking Pus3p, which modifies Ψ38 and

Ψ39 (Lecointe et al. 1998), Trm7p, which catalyzes 2′-O-methylation at positions 32 and 34 (Pintard et al. 2002), or Trm5p, which catalyzes m^{1}G and m^{1}I formation at position 37 and the likely first step of Y-base formation (Björk et al. 2001). A translation defect is associated with the *trm7* mutants, and almost certainly with *trm5* mutants, because the Trm5p homolog in *E. coli* is essential for reading frame maintenance (Urbonavicius et al. 2001). Similarly, each of the Tad2p and Tad3p subunits of the yeast tRNA A34 deaminase is essential, presumably to ensure that the wobble nucleotide can correctly pair with the mRNA codons during translation (Gerber and Keller 1999). In contrast, *mod5* mutants, which lack i^{6}A at position 37 in three cytoplasmic and two mitochondrial tRNAs, have no discernible phenotype other than altering efficiency of tRNA-mediated nonsense suppression (Laten et al. 1978). Surprisingly, the proteins for m^{1}A58 formation are also essential in yeast (Garcia-Barrio et al. 1995; Calvo et al. 1999), although this modification occurs well away from the critical anticodon loop.

For most other modifications, mutant phenotypes have been more difficult to find, until recent application of more sensitive assays. One such case concerns *E. coli truB* mutants, which are defective in Ψ55 formation. These mutants have almost no measurable phenotype in liquid culture, which is surprising because Ψ55 is almost universally found in the cytoplasmic tRNAs of all organisms as part of the highly conserved TΨC loop from nucleotides 54–56. Ofengand and coworkers (Gutgsell et al. 2000) have used competitive growth to demonstrate a distinct growth phenotype of *E. coli truB* mutants. When cocultured with wild-type strains through successive cycles of growth, these mutants are relatively rapidly lost, at a rate of a few percent per complete growth cycle (Gutgsell et al. 2000). Remarkably, this competitive growth defect of *truB* mutants could be complemented by a strain carrying a catalytically inactive truBp variant, demonstrating that there is some other function of TruB, possibly a chaperone function, that is responsible for the growth defect, rather than the Ψ55 modification itself. A similar competitive growth defect was previously reported for mutants lacking RluAp, which is responsible for formation of Ψ32 in tRNA as well as for two Ψ modifications in rRNA (Raychaudhuri et al. 1999).

A different approach using a synthetic (genetic interaction) screen recently demonstrated a phenotype for yeast *pus4* mutants, which lack Ψ55. In a synthetic lethal screen, a single mutant with no obvious phenotype is used to search for a second mutant that causes the cell to be sick or dead. In this way, a screen with *pus1* mutants, which lack many different Ψ residues in their tRNAs (Table 1), revealed that *pus1 pus4* double mutants are lethal, or slow growing and temperature-sensitive, depending on the exact background (Grosshans et al. 2001), as are *pus1* mutants that also have a defective minor tRNA species. Although it is not clear yet exactly how the *pus4* defect contributes to lack of viability of these double mutants, this result demonstrates the use of synthetic screens to expose otherwise subtle effects,

and provides a starting point for further genetics and cell biology experiments.

A similar synthetic screen was used by Johansson and Byström (2002) to explore the function of yeast Trm2p, which encodes the m^5U54 methyltransferase that forms T in the highly conserved TΨC loop of tRNAs. Their results demonstrated a synthetic interaction between *trm2* mutants and a *sup61* mutant specifying an altered $tRNA_{CGA}^{Ser}$, which was correlated with lower than normal levels of the mutant tRNA. A series of tests showed similar growth defects in *sup61 trm1*, *sup61 trm3*, and *sup61 pus4* double-mutant strains, all correlated with lower levels of $tRNA_{CGA}^{Ser}$, and no synthetic effects in *sup61 rit1* strains. Because all of the genes showing synthetic effects with *sup61* mutants are also involved in modification of this tRNA, and Rit1p is not involved in modification and has no synthetic effect, it seems likely that the growth defect is caused by direct interaction of these proteins with the tRNA. As described above with *truB* mutants, and observed earlier for the *E. coli* TrmA m^5U54 methyltransferase (Persson et al. 1992), the growth defect of *sup61 trm2* mutants could be partially complemented by expression of a catalytically inactive Trm2 protein, demonstrating a separate role of Trm2p distinct from its modification activity. It is not known if expression of catalytically inactive Trm1, Trm3, and Pus4 proteins will also complement their synthetic growth defects.

The essential function of m^1A58 modification that is catalyzed by Gcd10p/Gcd14p also appears to be caused by lowered tRNA levels, but remarkably, by only one of the 17 tRNA species that has this modification, $tRNA_i^{Met}$ (Anderson et al. 1998, 2000). Three lines of evidence support this claim: high copies of this tRNA (but not the elongator $tRNA^{Met}$, which also has this modification) rescued the lethal phenotype of a *gcd14-Δ* strain; a *gcd14* mutant had reduced levels of $tRNA_i^{Met}$; and a *gcd10* mutant coordinately exacerbates both the growth defect and the $tRNA_i^{Met}$ expression of a *gcd14* mutant (Anderson et al. 1998; Calvo et al. 1999).

In sum, the data indicate that many tRNA modifications at positions other than the anticodon and/or the corresponding modification proteins have a role in the stability of mature tRNAs.

New connections between tRNA processing, translation, and other metabolic pathways

Recent experiments have also shown a large number of new and intriguing connections between parts of the tRNA-processing pathway and the translation machinery as well as other pathways in the cell.

Translation

As discussed above, it is well known that a number of tRNA modification genes, particularly those affecting the region around the anticodon, exert their effects by altering translation at steps within the ribosome (see Ur-

bonavicius et al. 2001). However, recent experiments indicate other connections between tRNA processing and translation.

Recent evidence indicates a new link between the modification of $tRNA_i^{Met}$ by Rit1 protein and the translation initiation machinery. Rit1 protein adds a 2′-*O*-ribosyl phosphate to position 64 of $tRNA_i^{Met}$, which is the only tRNA bearing this modification (Åström and Byström 1994). *rit1* mutants have synthetic growth defects with mutations that either lower the number of initiator tRNA genes or perturb any of the subunits of eIF-2. Because eIF-2 is responsible for delivering $tRNA_i^{Met}$ to the 43S ribosome to initiate translation, this set of results implies that growth defects arise from any combination that lowers the amount of the eIF-2–$tRNA_i^{Met}$ complex, or the quality of $tRNA_i^{Met}$ in the complex. Consistent with this argument, overproduction of initiator tRNA genes can reverse the synthetic growth defect caused by *rit1* eIF-2 subunit double mutants, and overproduction of eEF1α can sequester $tRNA_i^{Met}$ that does not have the 2′-*O*-ribosyl phosphate and exacerbate formation of the complex (Åström et al. 1999).

An unexpected link has also been established between the status of tRNA processing in the nucleus and *GCN4* mRNA translation in the cytoplasm, because mutations that lead to 5′- or 3′-end-processing defects in the nucleus can induce *GCN4* mRNA translation (Qiu et al. 2000). Thus, *GCN4* mRNA translation can be induced by overexpression of either *NME1*, encoding the RNA subunit of RNase MRP (Tavernarakis et al. 1996), or of *PUS4*, encoding Ψ55 pseudouridylase (Qiu et al. 2000), and both phenotypes are correlated with increased accumulation of pre-tRNAs. Three lines of evidence link this pre-tRNA accumulation to a nuclear location: First, an altered $tRNA^{Val}$ that cannot be processed at its 3′ end, and which causes *GCN4* mRNA translation, is localized to the nucleus. Second, overproduction of the yeast exportin-t Los1 protein, which would be expected to move cargo to the cytoplasm, adversely affects *GCN4* translation, and *los1* mutants act to enhance *GCN4* translation. Third, overexpression of *PUS4* leads to nuclear accumulation of at least one tRNA, and this effect is abrogated by overexpression of *LOS1*. These results all support the argument for a surveillance mechanism in the nucleus that somehow conveys information to the translation machinery in the cytoplasm about the state of tRNA processing (Qiu et al. 2000).

Multiple effects of i^6A

One striking observation is the effect of i^6A formation, or of the protein that catalyzes the corresponding reaction, on multiple pathways. The protein Mod5, $Δ^2$-isopentenyl pyrophosphate:tRNA isopentenyl transferase, catalyzes transfer of the isopentenyl group from dimethylallyl pyrophosphate to A37 of certain tRNAs to form i^6A (Dihanich et al. 1987), and *mod5* mutants have lower translation efficiency in yeast strains with suppressor tRNAs such as *SUP7* (Laten et al. 1978). Recent experi-

ments indicate that Mod5p or its product i[6]A can affect several processes. First, Benko et al. (2000) have shown that the yeast enzyme Mod5p is in competition with Erg20p for their common substrate dimethylallyl pyrophosphate. Given that Erg20p catalyzes formation of geranyl pyrophosphate and farnesyl pyrophosphate, the precursors for sterols, prenylated proteins, uniquinone, dolichol, and HemeA, this result implies that a strict balance between the two proteins must be maintained to properly funnel dimethylallyl pyrpophosphate (Benko et al. 2000). Second, mutations in the *Caenorhabditis elegans GRO-1* gene, which encodes a highly related presumed MOD5 ortholog, grow slowly and have an increased life span (Lemieux et al. 2001). Like Mod5p in yeast (Boguta et al. 1994), GRO1 protein is found in the nucleus, cytoplasm, and mitochondria; only the mitochondrial form of the protein is required to complement the *gro1* defect (Lemieux et al. 2001). Third, there may also be a connection between i[6]A modification and selenocysteine tRNA. Thus, transient transfection of a mutant tRNA[Ser]Sec gene carrying a change at the 37 position, the site normally modified to i[6]A, causes the repression of translation of the cotransfected gene for the selenoprotein deiodinase (Warner et al. 2000). Addition of lovastatin, a drug that prevents formation of mevalonate (the precursor of the isopentenyl moiety), has the same effect, and also represses synthesis of endogenous selenoproteins (Warner et al. 2000).

RNase P and RNase MRP

The connection between RNase P and RNase MRP is underscored by the large number of shared components between the two proteins (Chamberlain et al. 1998). Each protein in yeast has nine polypeptide subunits and one RNA subunit, and eight of the nine subunits are identical. That RNase P and RNase MRP have an impact on each other in *S. cerevisiae* is evident by the apparent competition of their RNA components, NME1 and RPR1, for protein components, as measured by their competing effects for induction of *GCN4* mRNA translation (Qiu et al. 2000). Whereas the direct role of RNase P is to endonucleolytically remove the 5′ trailer from all tRNA transcripts, RNase MRPs have been implicated in processing 5.8S rRNA, as well as in exit from mitosis and various other roles (see Cai et al. 2002 and references therein). In sum, RNase P/RNase MRP balance can affect a number of processes in the cell.

tRNA splicing machinery in the unfolded protein response

The observation by the Walter lab that the unfolded protein response in yeast is mediated by a unique tRNA-like splicing event is another striking example of the sharing of an RNA processing pathway with an unanticipated, seemingly unrelated pathway. The unfolded protein response, which is activated by any of several stress treatments, leads to expression of the transcription factor Hac1p in yeast, which in turn activates expression of a number of downstream targets. Remarkably, expression of Hac1p in yeast is mediated by a tRNA-like splicing of *HAC1* mRNA, using an endonuclease activity of Ire1p, and ligation by the yeast tRNA splicing ligase (Sidrauski et al. 1996; Sidrauski and Walter 1997; Gonzalez et al. 1999) to remove the inhibitory function of the intron on translation of Hac1p (Ruegsegger et al. 2001). This unusual splicing reaction is also observed in mammals when yeast *HAC1* is expressed during the mammalian unfolded protein response (Niwa et al. 1999), and recently a true substrate-spliced mRNA has been found in both *C. elegans* and mice (Calfon et al. 2002). Therefore, at least some mRNA splicing occurs by a tRNA splicing mechanism in eukaryotes, sharing some of the tRNA splicing machinery, and factors that influence tRNA ligase may also regulate this class of splicing.

News about tRNA-processing mechanisms

Several recent experiments using different organisms have resulted in new insights into tRNA-processing biochemistry, and revealed unexpected diversity in tRNA-processing biochemistry. These are described below.

Elusive 3′-end-processing machinery

The mysterious mechanism by which 3′ ends of tRNAs are generated in eukaryotes and prokaryotes appears to be unfolding. It is known from earlier work in *E. coli* that several 3′ exonucleases can contribute to maturation of tRNAs, particularly RNase II and PNPase in trimming the longer 3′ trailers, and RNase PH and RNase T in trimming the last few nucleotides (Li and Deutscher 1994, 1996). However, the endonuclease has been elusive. Recent work by two groups has demonstrated that RNase E is likely the initial endonuclease that triggers subsequent tRNA processing, as demonstrated for a large number of different tRNA species in different types of operon contexts (Li and Deutscher 2002; Ow and Kushner 2002).

The 3′-end-processing machinery in eukaryotes is much more poorly understood, and is also complicated by multiple implicated endonucleases and exonucleases. In yeast two endonucleases and 3′ exonuclease activities have been partially purified (Papadimitriou and Gross 1996), and it is known that the yeast La protein, Lhp1p, is required for endonucleolytic processing of tRNAs, because strains without Lhp1p process tRNAs via exonucleases (Yoo and Wolin 1997). However, the identity of the endonuclease has been a mystery, because there is no indication that Lhp1p has endonuclease activity. Recently, Marchfelder and colleagues have identified a likely candidate from *Arabidopsis thaliana* and *Methanococcus jannaschii*, after analysis of the peptide sequences of a purified wheat protein (Schiffer et al. 2002). Expression of these proteins, called RNase Z, in *E. coli* results in extracts that can catalyze the same endonuclease reaction that was obtained from wheat germ,

namely, cleavage 3′ to the discriminator. This protein appears to be conserved in humans and in *S. cerevisiae*. It will be interesting to determine the function of this gene in vivo.

The puzzle of CCA addition

One longstanding conceptual problem is how CCA is added to the ends of tRNA in a precise fashion, without benefit of a template. Work in several labs has led to new views of how the CCA-adding enzyme accomplishes this feat. An original model depicted this enzyme as having three nucleotide-binding sites for progressive addition of the three nucleotides (Deutscher 1972). More recently, several different models have been proposed, based on a variety of experiments. Work from the Weiner lab has resulted in a new view of this protein as one with a single active-site pocket, whose active site appears to be recreated during the course of each addition reaction by refolding at the 3′ end of the tRNA. This model is based on the observation by Yue et al. (1998) that the *Sulfolobus shibatae* enzyme likely has a single active site, because mutation at either of two predicted critical Asp residues, identified by alignment, completely abolishes both C and A addition activities. Furthermore, there is strong evidence that the tRNA substrate is fixed to its protein for addition of the terminal C and A residues, because phosphate interference experiments demonstrate a requirement for the same phosphates for addition of C to tRNA-C as for addition of A to tRNA-CC, for both the *E. coli* enzyme and the *S. shibatae* enzyme, and because the *S. shibatae* enzyme is active for both addition reactions when cross-linked (Shi et al. 1998). It therefore seems likely that the active site reforms in collaboration with the growing 3′ end in the same pocket (Yue et al. 1998). A second model posits the existence of two nucleotide-binding sites, one catalytic CTP site and one ATP site that is both regulatory and catalytic (Hou 2000), to account for the observation that the *E. coli* enzyme can add more than the normal number of C residues in the absence of ATP, but not in its presence. A third model has been proposed by Steitz and coworkers (Li et al. 2000), based on the observation of tRNA-induced tetramer formation by the *S. shibatae* CCA-adding enzyme with only two tRNA molecules bound per tetramer. This half-of-the-sites occupancy by tRNA substrates led to the proposal of a scrunch-shuttling model according to which two proteins of each tetramer have adjacent active sites that are responsible for C addition and A addition, respectively. Addition of two C residues would require scrunching at one active site, after which the 3′ end would shuttle to the neighboring active site for addition of A (Li et al. 2000). Recently, it has been found that the eubacterium *Aquifex aeolicus* has separate enzymes for addition of CC and of A, as demonstrated by homology searches, activity assays, and function in *E. coli* strains lacking the CCA-adding enzyme (Tomita and Weiner 2001). This finding could be viewed as supporting an argument for separate sites for C addition and A addition in the *E. coli* and *S. shibatae* enzymes, or as a more primordial form of the enzyme. The true nature of the active site and the solution of this puzzle await the three-dimensional structure of the enzyme, and other chemical tests.

Splicing conserved at the outset but not at the end?

As described above, the groundbreaking work of the Abelson and Daniels labs led to the identification of related genes encoding part of the splicing endonuclease in yeast (Trotta et al. 1997) and the *Haloferax volcanii* protein (Kleman-Leyer et al. 1997), which then led directly to the rapid identification of orthologs in a number of archaeal, vertebrate, and plant species (Lykke-Andersen and Garrett 1997; Fabbri et al. 1998). Curiously, however, increasing evidence suggests that the subsequent ligation steps may not be conserved. The yeast ligation pathway is known to require two components: tRNA ligase (Trl1p), to join the excised exons forming a splice junction 2′-phosphate (Phizicky et al. 1992), and the 2′-phosphotransferase Tpt1p to transfer the phosphate to NAD to form ADP-ribose 1″–2″-cyclic phosphate (Culver et al. 1993; Spinelli et al. 1997). Similar ligase activities have been found in plants and humans (Konarska et al. 1982; Pick and Hurwitz 1986; Zillman et al. 1991), and Tpt1p activity or functional orthologs have been found widely in plants, vertebrates, and archaea (Zillman et al. 1992; Spinelli et al. 1998; Yukawa et al. 2001). However, the ligation pathway in some organisms may involve a completely different ligase, first discovered some time ago in vertebrates (Nishikura and De Robertis 1981; Filipowicz and Shatkin 1983; Laski et al. 1983), that does not produce a 2′-phosphate junction. Two papers describing a vertebrate-like ligase in *H. volcanii* have rekindled the argument for a division of this metabolic pathway into two branches of ligation (Gomes and Gupta 1997; Zofallova et al. 2000).

Equally curious, a functional Tpt1p ortholog is also found in bacteria such as *E. coli*. The *E. coli* 2′-phosphotransferase has the same activity as the yeast enzyme in vitro, can substitute for the yeast enzyme in vivo (Spinelli et al. 1998), and has almost the same substrate specificity requirements in vitro, and very similar kinetic parameters (Steiger et al. 2001). This is highly unexpected because there is no known ligase in *E. coli* that generates a junction 2′-phosphate, and no known RNA with this structure. However, the fact that the bacterial protein has been conserved through 3 billion years of evolution suggests that there is some related activity that it can catalyze or a related substrate upon which it acts. By extension, there may be a second function for the 2′-phosphotransferase in yeast in addition to its known role in removing the 2′-phosphate from ligated tRNA (Spinelli et al. 1997), and a corresponding function in other eukaryotes.

A multitude of tRNA editing/ modification mechanisms

Many recent experiments have uncovered extensive editing mechanisms in tRNAs, several of which were a

distinct surprise. One of the most unusual mechanisms of tRNA processing appears to require rebuilding the 5′ end of mitochondrial tRNAs in *Acanthamoeba castellanii*. All but three of the likely mitochondrial tRNAs in this organism have mismatches in one or more of the first three base pairs of the aminoacyl stem that would appear to require posttranscriptional alteration of the 5′ portion of the acceptor stem (Lonergan and Gray 1993; Burger et al. 1995). Experiments using crude extracts are consistent with the hypothesis that the 5′ ends of bulk tRNA and of model substrates are remodeled by removal of as many as three nucleotides at the 5′ end of the RNA, followed by the incorporation of nucleotides in the presence of ATP to reform a paired aminoacyl acceptor stem (Price and Gray 1999). This may be the first case of an authentic template-dependent polymerase that can add nucleotides in the 3′-to-5′ direction.

Other reports detail unusual editing mechanisms at the 3′ end. It has been known for several years that mitochondrial tRNAs can be edited at their 3′ ends in either of two template-independent ways, as judged by comparison of the sequences of cDNAs of circularized tRNA with that of the mitochondrial genome. Several animals such as land snail, chicken, and squid appear to have evolved a mechanism that formally corresponds to polyadenylation to fill in the end of the aminoacyl acceptor stem through to the discriminator position (see Fig. 1), presumably after processing of the tRNA precursor from its downstream overlapping gene (Yokobori and Paabo 1995a, 1997; Tomita et al. 1996). In contrast, platypus has evolved a mechanism in which the 3′ end is changed by introduction of two specific C residues in the acceptor stem and an A residue at the discriminator position, apparently in a template-independent manner, before the terminal CCA addition (Yokobori and Paabo 1995b). Recently, analysis of the mitochondrial tRNAs of the centipede *Lithobius forficatus* shows that for all but one of its 22 tRNA genes there is an apparent template-dependent editing requirement to fill in the aminoacyl acceptor stem and the discriminator base A; presumably this reaction requires an RNA-dependent RNA polymerase (Lavrov et al. 2000). Because all of these mechanisms are inferred from comparison of cDNA sequences with genomic sequence, each mechanism assumes that these cDNAs represent true tRNA-processing intermediates, rather than side products or dead-end products of this or another processing pathway. There is clearly more work necessary to prove this assumption is true and then to unravel these interesting tRNA-processing reactions.

Several previously described editing classes are also manifested in mitochondrial tRNA. The most prevalent of these editing events involves a C-to-U alteration corresponding to the likely deamination of cytidine. This type of editing was first observed in a marsupial tRNA[His] (Janke and Paabo 1993) and in plants (Marechal-Drouard et al. 1993; Binder et al. 1994), and appears in several cases to be required for processing of the corresponding tRNAs (Marchfelder et al. 1996; Marechal-Drouard et al. 1996a,b). C-to-U editing also occurs in *Leishmania*

tarentolae, where it appears to occur only when the tRNA is imported into the mitochondria (Alfonzo et al. 1999). Classical insertional editing of cytidine and uridine residues has also been observed in a number of mitochondrial tRNAs of *Physarum polycephalum* and *Didymium nigripes* (Antes et al. 1998).

One new twist of tRNA processing is the finding of a guide-RNA-mediated tRNA modification that is self-guided. A euryarchaeal tRNA[Trp] gene carries within its intron a box-C/D guide RNA motif specifying its own 2′-*O*-methylation at two positions (d'Orval et al. 2001). In eukaryotes and some archaea, the box-C/D motif specifies fibrillarin binding, and together with adjacent RNA sequences that pair to the target RNA, directs 2′-*O*-methylation at sites within the paired region (see Smith and Steitz 1997). This tRNA[Trp] and the corresponding intron-containing motif specifying 2′-*O*-methylation is found in several euryarchaeal species, and extracts from *H. volcanii* can faithfully reproduce the two ribose methylations in a manner that requires elements of the intron. This is the first example of a possible *cis*-acting methylation guide function, and the first such guide-modulated 2′-*O*-methylation reaction that has been reproduced in vitro.

tRNA quality control

Work in the Deutscher lab has uncovered a new dimension of tRNA biogenesis in *E. coli*, control of its quality by degradation of tRNAs that are incompletely 3′-end processed. Two lines of evidence support this claim. First is the demonstration that tRNA precursors (and other small stable RNAs) can be polyadenylated when tRNA maturation is deliberately slowed because of mutation of processing exonucleases (Li et al. 1998b). Because polyadenylation of mRNA (O'Hara et al. 1995) and a regulatory RNA (Xu et al. 1993) in *E. coli* has been linked to RNA degradation, polyadenylation of tRNAs may also trigger their degradation. Second is the demonstration that a tRNA[Trp] species with a mutation conferring a temperature-sensitive phenotype accumulates increased amounts of precursor tRNA in cells lacking poly(A) polymerase (Li et al. 2002). Because the precursor tRNAs did not accumulate in the corresponding strains with wild-type tRNA, and because even larger amounts of pre-tRNAs were observed in cells also lacking polynucleotide phosphorylase, which had previously been implicated as a degradative enzyme for mRNA (Xu et al. 1993; Carpousis et al. 1999), the implication from these studies is that misfolded tRNA precursors are subject to degradation, just as mRNAs are normally degraded in *E. coli*.

Surprising complexity for the nuclear organization of the tRNA-processing pathway(s)

Distinct preferred pathways for different organisms

Studies using pulse-labeled yeast cells and microinjected *Xenopus* oocytes that assessed appearance of tRNA-processing intermediates and presence of modified nucleo-

sides on these pre-tRNAs provided the first lines of evidence for the multistep reactions occurring in a preferred order (Etcheverry et al. 1979; Melton et al. 1980; Nishikura and De Robertis 1981). Surprisingly, the order of pre-tRNA-processing steps appears to differ among organisms; for example, in budding and fission yeast, end processing usually precedes splicing (O'Connor and Peebles 1991; for review, see Wolin and Matera 1999; Intine et al. 2002), whereas in *Xenopus* oocytes splicing precedes end processing (Lund and Dahlberg 1998).

Theoretically, ordered pathways could result from processing enzyme substrate specificities. In fact, there are clear examples of this. For instance, the enzymes that modify C to m^5C and U to Ψ of tRNA anticodon loops require intron-containing pre-tRNA, and these steps must occur prior to pre-tRNA splicing (Johnson and Abelson 1983; Szweykowska-Kulinska et al. 1994; for review, see Grosjean et al. 1997). Other modification activities use spliced tRNAs as substrates, and the resulting modifications occur only after splicing (Melton et al. 1980; Nishikura and De Robertis 1981; Grosjean et al. 1997; Spinelli et al. 1997).

Substrate specificity, however, does not provide an explanation for the ordering of the majority of tRNA-processing steps. Genetic and biochemical studies show that most tRNA biogenesis steps are not obligatorily ordered. For example, mutations of yeast genes encoding tRNA modification activities generally affect only the single nucleoside modification in question (Phillips and Kjellin-Stråby 1967; Hopper et al. 1982; Åström and Byström 1994; Anderson et al. 1998; Lecointe et al. 1998; Motorin et al. 1998; Cavaille et al. 1999; Motorin and Grosjean 1999; Nordlund et al. 2000; Pintard et al. 2002). Likewise, in vitro generated unmodified tRNAs generally serve as substrates for purified tRNA modification activities (Åström and Byström 1994; Becker et al. 1997; Anderson et al. 1998; Gerber et al. 1998; Cavaillé et al. 1999; Motorin and Grosjean 1999; Alexandrov et al. 2002; Pintard et al. 2002; Xing et al. 2002). In fact, we are unaware of a single example for which the addition of a given tRNA modification requires previous addition of a modification at a different location. Similarly, in wild-type yeast cells, RNase P generally removes tRNA 5' ends prior to endonucleolytic cleavage at 3' termini (O'Connor and Peebles 1991; Yoo and Wolin 1997). However, in cells lacking the tRNA-binding La protein, a 3' exonuclease usually acts prior to RNase P, changing the order of processing steps for many pre-tRNAs (Yoo and Wolin 1997). Moreover, in wild-type yeast, for at least tRNA^{Trp}, 3' processing precedes 5' processing (Kufel and Tollervey 2003). Therefore, the preferred order of 5' processing before 3' processing is not obligatory. Finally, the order of splicing versus end processing is not requisite because for both yeast and *Xenopus* oocytes the ordered paths can be reversed (Lund and Dahlberg 1998; Intine et al. 2002).

Location, location, location

The tRNAs that function in cytosolic protein synthesis appear to be processed at several locations—at multiple subnuclear sites as well as in the cytosol—and this partitioning may influence ordering of tRNA biogenesis steps. In the nucleus there is little evidence for tRNA processing occurring at transcription sites, unlike for pre-mRNA processing, which occurs cotranscriptionally via recruitment of processing activities to sites of mRNA transcription (for review, see Maniatis and Reed 2002). The La protein, involved in tRNA end maturation, is the only protein involved in tRNA processing that has been implicated in transcription, but its role in transcription remains controversial (for reviews, see Maraia and Intine 2002; Wolin and Cedervall 2002). Most other tRNA-processing proteins are concentrated at locations distinct from sites of transcription. Assuming that those sites of concentrations reflect sites of biochemical activity, it would appear that tRNAs are processed at several subnuclear locations.

The nucleolus, the location for rRNA transcription, processing, and ribosome assembly, also harbors some tRNA biosynthetic activities. For example, in budding yeast the RNA subunit of RNase P is primarily nucleolar (Bertrand et al. 1998). In contrast, the human RNA homolog appears to be distributed throughout the nucleoplasm (for review, see Jarrous 2002). As detailed above, most of the RNase P protein subunits are shared with RNase MRP functioning in pre-rRNA processing (for review, see Xiao et al. 2002). Therefore, the location of yeast RNase P in the nucleolus allows dual participation of proteins in rRNA and tRNA biogenesis. RNase P is not the only yeast tRNA-processing enzyme in the nucleolus, as a portion of the Mod5p-II pool has been reported to be located there (Tolerico et al. 1999). As rRNAs and snoRNAs do not appear to possess i^6A, it is unclear why Mod5p should be located in the nucleolus or even if it is active at this location. Consistent with the idea that part of the tRNA-processing pathway occurs in the nucleolus, some, but not all, intron-containing pre-tRNAs are located in the nucleolus (Bertrand et al. 1998; Sarkar and Hopper 1998; Grosshans et al. 2000).

Numerous nuclear tRNA biosynthetic enzymes are concentrated at subnuclear sites other than the nucleolus. For example, *S. cerevisiae* and *Schizosaccharomyces pombe* Pus1p, which catalyzes U to Ψ at several tRNA locations and at position 44 of U2 snRNA, is distributed throughout the nucleoplasm (Simos et al. 1996; Motorin et al. 1998; Massenet et al. 1999; Hellmuth et al. 2000). In contrast, yeast Trm1p-II, encoding m_2^2G methyltransferase, and Trm4p, encoding tRNA specific m^5C methyltransferase, are located at the inner nuclear membrane (INM; Li et al. 1989; Rose et al. 1995; Wu et al. 1998; Motorin and Grosjean 1999).

Yeast tRNA splicing endonuclease that catalyzes pre-tRNA intron removal purifies as an integral membrane heterotetrameric complex (Peebles et al. 1983; Trotta et al. 1997). It is widely believed that this membrane is the INM because pre-tRNA splicing is an intranuclear process in *Xenopus* oocytes (Melton et al. 1980) and because in budding yeast intron-containing pre-tRNAs appear to be located solely within the nucleus (Sarkar and Hopper 1998; Grosshans et al. 2000). However, the INM location

of tRNA splicing endonuclease has not yet been verified by microscopy. Even though, as assessed by indirect immunofluorescence and by electron microscopy, tRNA ligase that joins the cognate tRNA halves after splicing is located predominantly at the INM, there are nucleoplasmic and active cytosolic pools of this enzyme as well (Clark and Abelson 1987; Ruegsegger et al. 2001). The cytosolic pools appear to be involved in the unfolded protein response pathway described above. Despite the complexities, the most straightforward, but as yet unproven, conclusion from the yeast studies is that pre-tRNA intron removal occurs at the INM.

Modifications of the anticodon loop are among tRNA-processing steps that occur in the cytosol as these modifications appear only after splicing and, in *Xenopus* oocytes, are added only when substrates are introduced into the cytosol (Etcheverry et al. 1979; Nishikura and De Robertis 1981). Indeed, the majority of budding yeast Mod5p-II, responsible for modification of A_{37} to I^6A, is cytosolic (Boguta et al. 1994). Likewise, Trm7p, catalyzing methylation of ribose moieties at anticodon loop positions 32 and 34, and Pus6p, catalyzing formation of Ψ at position 31, have been reported to be largely cytosolic (Ansmant et al. 2001; Pintard et al. 2002).

In sum, substrate specificity can provide an explanation for the ordering of only a few of the steps of tRNA biogenesis. Rather, as tRNA biosynthetic activities appear to be spatially organized with several distinct subnuclear as well as cytosolic locations, it is likely that these cell biological constraints provide a major contribution to the preferred tRNA-processing order. However, there is insufficient information regarding whether the ordered events do, in fact, correlate with the subnuclear locations of the processing enzymes. Furthermore, at present there are no reports of the consequences of mislocating processing activities to alternative subnuclear locations on the order of processing steps. However, shifting *S. pombe* La protein from mostly nuclear to mostly cytosolic resulted in an altered processing order with pre-tRNA intron removal preceding 5'- and 3'-end maturation (Intine et al. 2002). If future studies to redistribute activities within the nucleus change tRNA-processing order, this would provide evidence that tRNAs travel to different subnuclear locations during maturation, a fundamentally different mechanism than for mRNA biogenesis.

Moving tRNAs around the cell

Although it has been long appreciated that eukaryotic cells possess mechanisms for delivering tRNAs from the nucleus to the cytosol, it has become obvious recently that the nuclear/cytoplasmic pathway is much more complicated than previously thought. In addition, it has become clear that cells also possess mechanisms for delivering tRNAs from the cytosol to mitochondria. Here we summarize present understanding of the mechanisms to accurately and efficiently move tRNAs around the cell.

Aminoacylation likely serves a proofreading role for tRNA nuclear export

Lund and Dahlberg (1998) provided paradigm-shifting data showing that tRNAs are aminoacylated while in the nucleus, and that nuclear tRNA aminoacylation facilitates, but is not essential for tRNA nuclear export in *Xenopus* oocytes (Arts et al. 1998b; Lund and Dahlberg 1998). Nuclear tRNA aminoacylation likely provides a proofreading step to ensure export of mature tRNAs to the cytosol because aminoacyl tRNA synthetases provide amino acids only to tRNAs possessing mature 3' termini (Lund and Dahlberg 1998). Nuclear tRNA aminoacylation also occurs in *S. cerevisiae*, where it also facilitates tRNA export (Sarkar et al. 1999; Grosshans et al. 2000), and nuclear pools of aminoacyl-tRNA synthetases have been verified both for vertebrate and yeast cells (Ko et al. 2000; Nathanson and Deutscher 2000; Azad et al. 2001; Galani et al. 2001). As aminoacylation of tRNAs was previously thought to occur solely in the cytosol, its occurrence also in the nucleus expands the known roles of the nucleus to include part of the translation process. Whether translation itself occurs in the nucleus remains controversial (Dahlberg et al. 2003). Despite the importance of nuclear aminoacylation of tRNA, it is unclear how nuclear aminoacylation fits into the tRNA export pathways described below.

Exportin-t/Los1p-dependent tRNA nuclear export

Movement of macromolecules between the nucleus and the cytoplasm is signal-mediated and often requires a small GTPase, Ran, its regulators, and members of the Ran-binding importin-β family (for review, see Görlich and Kutay 1999). Importin-β family members, in addition to binding to Ran, interact with NPC components and cargo and shuttle between the nucleus and cytosol. According to a simple model, a given importin-β family member would provide unidirectional nucleus/cytosol movement for a specific subset of cargoes (Görlich and Kutay 1999). Some of the components of the tRNA nuclear export machinery, such as Los1p and Rna1p, were identified in yeast more than two decades ago by the effects of mutations in the corresponding genes on tRNA biogenesis (Hopper et al. 1978, 1980). However, their roles in the tRNA export process were first deciphered for the homologs in vertebrate cells.

A large body of literature now demonstrates that the vertebrate importin-β family member exportin-t and its yeast homolog Los1p serve to export tRNA from the nucleus to the cytosol (Fig. 2). Exportin-t directly binds tRNA in a Ran-GTP-dependent mechanism (Arts et al. 1998a; Kutay et al. 1998). Exportin-t also interacts with distinct nuclear pore proteins on the nucleoplasmic and the cytosolic nuclear surfaces, facilitating tRNA movement from the nuclear interior to the cytosol (Kuersten et al. 2002). In vitro studies showed that exportin-t interacts with ~10-fold higher apparent affinity with tRNAs possessing mature 5' and 3' termini than with tRNAs with terminal extensions or with tRNAs lacking
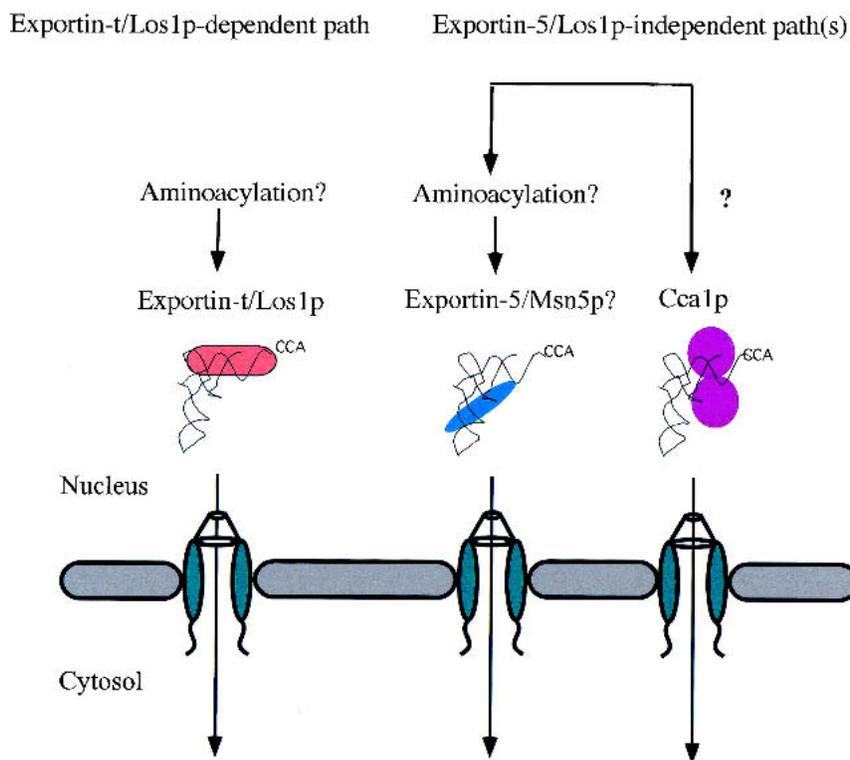
Figure 2. Multiple pathways to export tRNA from the nucleus to the cytosol. On the *left*, the classical exportin-t/Los1p tRNA nuclear export pathway shared between vertebrates and yeast is depicted. It is unclear whether tRNA aminoacylation is important for this pathway, and it is therefore indicated by a question mark. On the *right*, the Los1p-independent tRNA nuclear export possible mechanisms are depicted. Exportin-5 likely provides a minor tRNA nuclear egress route for vertebrates, but it is not clear whether its homolog, Msn5p, provides the same function for yeast. A major tRNA nuclear egress route for yeast is known to exist, and aminoacylation and Cca1p participate in this mechanism, but the details of their actions are not known and are indicated by question marks.

the 3′ CCA nucleotides. Exportin-t interacts with ~5-fold higher apparent affinity with modified tRNAs than with in vitro generated unmodified tRNAs (Arts et al. 1998b; Lipowsky et al. 1999). Despite the fact that the relative in vitro binding affinities differences are not large, tRNAs with abnormal 5′ and/or 3′ termini or with mutations affecting the overall three-dimensional structure are exported inefficiently to the cytosol in *Xenopus* oocytes, providing supporting in vivo data that exportin-t substrate specificity provides a proofreading role to ensure nuclear export of end-matured tRNAs (Arts et al. 1998b; Lund and Dahlberg 1998; Lipowsky et al. 1999). However, exportin-t does not distinguish between intron-containing and spliced tRNAs (Arts et al. 1998b; Lipowsky et al. 1999). Export of only spliced tRNA to the cytosol in *Xenopus* oocytes results from the preferred processing order of splicing prior to end processing (Lund and Dahlberg 1998). It is unknown whether exportin-t binds preferentially aminoacylated, rather than uncharged tRNAs, thus the role of aminoacylation in this pathway requires further study.

Despite its well-described substrate specificity for mature 3′ tRNA termini, there is at least one indication that exportin-t may sometimes interact with tRNAs possessing 3′ extensions. Chimeric tRNAs with 3′-attached ribozymes (tRNA-Rzs) have been reported to be efficiently exported to the cytosol in somatic cells, but not in *Xenopus* oocytes (summarized in Kuwabara et al. 2001). Based on exportin-t's substrate specificity (Arts et al. 1998b; Lipowsky et al. 1999), tRNA-Rz nuclear export would have been anticipated to be exportin-t-independent. Surprisingly, these chimeric molecules appear to

interact with exportin-t in vitro, and tRNA-Rz export in somatic cells appears to be exportin-t-dependent, inconsistent with the prediction. One possible explanation is that weak interactions between exportin-t and tRNA-RZs might be sufficient for export.

Two lines of evidence show that *S. cerevisiae* Los1p is the yeast exportin-t homolog even though it has limited sequence similarity to exportin-t. First, *los1* mutant cells accumulate nuclear pools of tRNA; and second, Los1p binds tRNA in an Ran-GTP-dependent manner (Hellmuth et al. 1998; Sarkar and Hopper 1998). Although the tRNA-binding activity of Los1p has not been examined in detail, it has been assumed that Los1p, like exportin-t, also preferentially interacts with tRNAs with mature 5′ and 3′ termini and does not distinguish between intron-containing and spliced tRNAs. If this indeed proves true, then there must be an additional mechanism (see discussions below) in yeast to prevent nuclear export of unspliced pre-tRNAs. This is because, unlike for *Xenopus* oocytes, tRNA end processing usually precedes splicing in *S. cerevisiae*, preventing efficient use of the vertebrate kinetic pathway favoring interaction of exportin-t with already spliced pre-tRNAs.

Redundant exportin-t/Los1p-independent tRNA nuclear export pathway(s)

Exportin-t/Los1p does not provide the sole means by which tRNAs exit the nucleus. Studies showing that the *LOS1* genes of both *S. cerevisiae* and *S. pombe* are nonessential (Hurt et al. 1987; S. Sazer, pers. comm.) provide strong genetic evidence for the existence of an alterna-

tive tRNA nuclear export pathway(s). Recent biochemical studies indicate the same is likely also the case for vertebrate cells.

In vertebrate cells inhibition of exportin-t via introduction of antibodies specific for exportin-t inhibited tRNA nuclear export >80%, but did not completely prohibit export (Arts et al. 1998b; Lipowsky et al. 1999). Therefore, exportin-t likely provides the major route for tRNA nuclear export in vertebrates. Nevertheless, two recent studies demonstrate that vertebrate cells also possess an exportin-t-independent tRNA nuclear export pathway. The importin-β family member exportin-5 directly binds to either aminoacylated or nonaminoacylated tRNAs in an Ran-GTP-dependent mechanism, providing a secondary tRNA nuclear export pathway for vertebrates. Exportin-5 bound to aminoacylated tRNA also actively depletes the translation elongation factor eEF1α from the nucleoplasm (Bohnsack et al. 2002; Calado et al. 2002).

Exportin-5 likely also functions to export other RNAs from the nucleus. Studies of nuclear export of adenoviral VA1 RNA (also transcribed by RNA polymerase III) in monkey COS1 cells and *Xenopus* oocytes identified a minihelix sufficient for nuclear export via Ran-dependent reaction (Gwizdek et al. 2001). Interestingly, excess tRNA could block export of VA1 RNA, but excess minihelix RNAs did not block tRNA export. The data are consistent with the existence of an export pathway responsive to minihelix-containing RNAs that tRNAs are able to access (Gwizdek et al. 2001). Not surprisingly, an unpublished report indicates that the exportin for VA1 RNA is exportin-5 (Gwizdek and Dargemont, pers. comm., cited in Calado et al. 2002).

It is not clear if the yeast Los1p-independent pathway uses the exportin-5 homolog Msn5p. Overexpression of eEF1α compensates for *los1* null mutations, and mutations of the genes encoding eEF1α cause a defect in tRNA nuclear export, implicating eEF1α in an alternative tRNA nuclear export pathway in *S. cerevisiae* (Grosshans et al. 2001). However, as there is no evidence that yeast eEF1α shuttles between the nucleus and the cytosol, eEF1α may somehow be involved in tRNA nuclear export via an indirect mechanism (Grosshans et al. 2001). Moreover, although the phenotype of *los1 msn5* (encoding the yeast exportin-5 homolog) double mutants has not been reported, deletion of Msn5p does not cause an obvious defect in tRNA nuclear export (Feng and Hopper 2002), indicating that Msn5p (exportin-5) could only provide a minor tRNA nuclear egress pathway for *S. cerevisiae*. It is therefore likely that yeast possess nuclear export pathways that are independent of both Los1p and Msn5p.

Although no yeast importin-β family member, other than Los1p, has been identified that is important for tRNA nuclear export, other proteins that function in the Los1p-independent nuclear export pathway have been identified. Mutation of *PUS1* causes defects in nuclear tRNA export, and there are synthetic growth defects in *los1 pus1* double mutants, perhaps implicating Pus1p in an alternative tRNA export pathway (Simos et al. 1996;

Grosshans et al. 2001). Cca1p, which catalyzes addition of the 3'-terminal C, C, and A nucleotides to tRNA, and Mes1p, methionyl-tRNA synthetase, are also implicated in an Los1p-independent tRNA nuclear export pathway because Cca1p and Mes1p each are multicopy suppressors of the tRNA nuclear export defect caused by *los1* null mutations, and mutations of the genes encoding these proteins cause nuclear tRNA accumulation (Feng and Hopper 2002). How these proteins function in an Los1p-independent export pathway is unknown. However, *S. shibatae* CCA-adding enzyme forms stable complexes with CCA-containing tRNAs (Shi et al. 1998), and yeast Cca1p shuttles between the nucleus and cytosol, providing the potential for Cca1p to function directly as an exporter or indirectly as an adapter in an Los1p-independent pathway. Alternatively, pseudouridylation, CCA addition, and aminoacylation could prepare tRNA for interaction with an unidentified exportin (Fig. 2; Feng and Hopper 2002).

In sum, it appears that there are at least two, and perhaps more than two, parallel tRNA nuclear export pathways for both vertebrates and lower eukaryotes. Whether the same exportin-t/Los1p-independent alternative tRNA nuclear export pathways are used in fungi and vertebrates requires further studies.

Coupling of tRNA nuclear export and splicing in S. cerevisiae

S. cerevisiae mutations in the Ran pathway, *LOS1*, or any of several genes encoding nucleoporins, which should solely affect tRNA nuclear export, instead also cause defects in pre-tRNA intron removal (Hopper et al. 1978, 1980; Sharma et al. 1996; Simos et al. 1996). The results are in contrast to studies of *Xenopus* oocytes for which there appears to be no effect of the Ran pathway on pre-tRNA splicing (Lund and Dahlberg 1998). It has been concluded that tRNA nuclear export and splicing are coupled in yeast, but not in vertebrates. However, the effect of the yeast mutations on pre-tRNA splicing can not be absolute because cytosolic pools of spliced tRNAs are essential and some of the genes in question are nonessential. Moreover, mutations of *CCA1* or overexpression of Cca1p affect tRNA nuclear export but do not affect pre-tRNA splicing (Sarkar et al. 1999; Grosshans et al. 2000; Feng and Hopper 2002).

What accounts for the partial coupling of pre-tRNA splicing and nuclear export in budding yeast but not in vertebrates? Perhaps yeast Los1p functions directly to couple tRNA splicing to export. In HeLa cells exportin-t location is dynamic, with nucleoplasmic, nuclear rim, and cytosolic locations, accumulating at the nuclear rim when the RanGTPase cycle is disrupted (Kuersten et al. 2002). Although yeast Los1p also has multiple locations, primarily at nuclear pores, but also throughout the nucleoplasm and in the cytosol, it is not known if its location changes in response to RanGTPase, like its exportin-t counterpart (Shen et al. 1993; Simos et al. 1996). If Los1p also has dynamic subnuclear distribution, it could have a role in delivering intron-containing tRNAs from

the nucleoplasm to the NPC-located splicing machinery, providing an explanation as to why mutations of *LOS1* or the RanGTPase cycle affect splicing. However, this would not explain why mutant nucleopore proteins affect splicing. Although it is formally possible that increased nuclear tRNA pools resulting from tRNA nuclear export defects would saturate the tRNA splicing machinery, in vitro studies provided no evidence for product inhibition of the splicing endonuclease (Peebles et al. 1979). Another possibility, reminiscent of models for coupling mRNA processing and export (Ishigaki et al. 2001 and references therein), is that tRNA introns could be marked by a tRNA nuclear retention protein that prohibits splicing in the absence of appropriate export machinery, providing a proofreading role for tRNAs whose termini are processed before splicing (Feng and Hopper 2002). Alternatively, as the yeast tRNA splicing enzyme complex has not been verified to be located at the INM (discussed above), an alternative membrane association could result in pre-tRNA splicing only after export.

Import of tRNAs into mitochondria

Numerous fungi, protists, plants, and animals import tRNA from the cytosol into mitochondria. Mitochondrial genomes vary in the number of encoded tRNAs, ranging from zero, as in some trypanosomatids, to the full complement used in mitochondria protein synthesis in organisms like *S. cerevisiae* and humans. Thus, for some organisms, mitochondria tRNA import from the cytosol is essential. Mitochondrial genomes use a nonstandard genetic code with, for example, mitochondria tRNAs inserting amino acids in response to stop codons. The corresponding imported tRNAs must be prevented from participating in protein synthesis while in the cytosol. Indeed, both tRNA editing and tRNA modification events occur inside the mitochondria that activate some of the imported tRNAs (for review, see Schneider and Marechal-Drouard 2000; also, see discussions above).

Import of tRNAs into mitochondria has been studied in the most detail for *S. cerevisiae*, and for several different trypanosomatids. *S. cerevisiae* imports 3%–5% of the total cytosolic pool of a single tRNA, $tRNA_{CUU}^{Lys}$, into mitochondria. This tRNA is first aminoacylated by cytosolic lysyl-tRNA synthetase. In general, mutant tRNAs that cannot be aminoacylated also are not imported into mitochondria, showing that aminoacylation is required for import. However, it appears that import does not require the presence of an amino acid on tRNA per se because a few particular mutant tRNAs that are unable to be aminoacylated are still imported. Rather, the data support the idea that amino acid addition in the cytosol alters tRNA conformation, allowing subsequent mitochondria import steps (Entelis et al. 1998). Aminoacylated $tRNA_{CUU}^{Lys}$ interacts in the cytosol with the precursor to mitochondria lysyl-tRNA synthetase (pre-MSK), followed by mitochondria import. The import requirements are similar to those for mitochondria-imported proteins (summarized in Entelis et al. 1998). As imported proteins are thought to be unfolded on the cy-

tosolic face of mitochondria prior to import, it is unclear whether $tRNA_{CUU}^{Lys}$–pre-MSK is imported as a complex or, instead, whether interaction with pre-MSK alters $tRNA_{CUU}^{Lys}$, making it import-competent (Entelis et al. 1998; for review, see Entelis et al. 2001).

Although import of $tRNA_{CUU}^{Lys}$ into yeast mitochondria is not essential for mitochondrial protein synthesis, and, in fact, $tRNA_{CUU}^{Lys}$ appears not to be aminoacylated by mitochondrial lysyl-tRNA synthetase (Entelis et al. 2001), this imported tRNA can function in translation. Recent studies changing the identity elements of $tRNA_{CUU}^{Lys}$ to $tRNA^{Met}$ or to a $tRNA^{Ala}$, able to base-pair with nonsense codons, resulted in aminoacylation of the mutant tRNAs by methionyl-tRNA or alanyl-tRNA synthetase, respectively. In vitro import studies and in vivo nonsense suppression assays showed the imported tRNAs are active in mitochondria protein synthesis (Kolesnikova et al. 2000). Moreover, human mitochondria, not known to import tRNA, were able to import yeast $tRNA_{CUU}^{Lys}$ in vitro, provided that human cytosolic extracts contained the yeast pre-MSK (Kolesnikova et al. 2000). The combined studies demonstrate that for *S. cerevisiae*: (1) mitochondrial tRNA import requires *trans*-acting proteins like the cytosolic and precursor mitochondrial tRNA synthetases; (2) identity of the amino acid on the tRNA is unimportant for mitochondria import; (3) imported tRNAs can participate in protein synthesis; and (4) the import machinery is similar from yeast to humans.

The mechanism of mitochondrial tRNA import for trypanosomatids likely differs from that of budding yeast. Trypanosomatid mitochondrial tRNA import appears not to require cytosolic proteins, but, rather, only proteinaceous receptors on the mitochondrial surface; however, it is difficult to completely rule out cytosolic contamination in the in vitro import systems (Schneider and Marechal-Drouard 2000). The nature of imported tRNA is controversial and may differ between species. Some studies conclude that only pre-tRNAs with 5′ extensions are imported, whereas other studies show the sequence of the 5′ extension to be unimportant, supporting the model that only mature tRNAs are imported (Yermovsky-Kammerer and Hajduk 1999; Tan et al. 2002). If, indeed, pre-tRNAs are imported in vivo, these tRNAs would have to somehow escape the processing and proofreading activities located in the nucleus and be exported to the cytosol with intact 5′ and 3′ extensions.

For both *S. cerevisiae* and trypanosomatids there have been numerous studies to map tRNA determinants important to their mitochondrial import. Studies of the yeast $tRNA_{CUU}^{Lys}$ are complicated because tRNA determinants affect interaction with the cytosolic and pre-MSK. Recently, the SELEX procedure has been used to characterize *Leishmania* tRNA motifs important for mitochondrial import. The studies uncovered two types of tRNA-like motifs. The former were efficiently imported, but the latter, remarkably, were imported efficiently only in the presence of the former. The investigators propose that tRNA–tRNA interactions regulate the pool of tRNAs imported into mitochondria (Bhattacharyya et al. 2002).

Conclusions

The excitement in tRNA biology resulting from the explosion of newly identified tRNA-processing genes, the investigation of tRNA processing in a wide array of organisms, and from increasingly sophisticated use of powerful cell biological and genetic tools to track tRNAs movement, is just beginning. It is clear that the next few years will bring valuable insights into the function of many genes involved in tRNA biogenesis, new revelations about the intricacies of tRNA-processing biochemistry, more connections between tRNA-processing machinery and other cellular functions, and greater understanding of the movement of tRNA in the cell, and factors influencing its movement. Understanding tRNA biology has become cool again, and it seems likely that it will remain so during the next decade.

Acknowledgments

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