Chapter 5
tRNA Biogenesis and Processing

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Abstract tRNAs are essential in all domains of life; this becomes especially important in trypanosomatids, where for all purposes the same set of tRNAs are utilized for cytoplasmic and mitochondrial protein synthesis. What makes the system special is that although tRNA biogenesis starts in the nucleus, the resulting products will satisfy translational requirements in two very different compartments. The balance between intracellular tRNA transport and post-transcriptional modifications may modulate tRNA function in gene expression. This chapter will summarize what is currently known about various processes that a tRNA must undergo in a trypanosomatid cell to become fully functional. Whenever possible, we will highlight both commonalities and differences with other systems, while emphasizing open questions that may lead to new and surprising discoveries in this group of evolutionarily divergent organisms.

Keywords Editing • Mitochondria • Modification • Processing • tRNA transport • Trypanosomes

5.1 Introduction

Due to intricate membrane systems eukaryotic cells exhibit a great deal of intracellular compartmentalization, which provides an organizational hierarchy for various cellular and metabolic pathways. As a consequence, eukaryotic cells have evolved

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efficient transport systems that ensure that a given molecule reaches its final destination, which often differs from its original site of synthesis. This intracellular organization is highlighted especially in the maturation pathways of tRNAs in trypanosomatid parasites. In most eukaryotes, tRNAs are encoded in either of two DNA-containing compartments: the nucleus or the genome-containing organelles (chloroplast and mitochondria). However, in trypanosomatids, mitochondrial genomes do not appear to contain any tRNA genes. Therefore, the complete set of tRNAs used in both cytoplasmic and mitochondrial protein synthesis is encoded solely in the nuclear genome. In these organisms tRNAs are thus transcribed in the nucleus, exported to the cytoplasm, and later a subset of cytoplasmic tRNAs is actively imported into the mitochondria. However, before tRNAs can be rendered functional in any cellular compartment, they face many enzymatic reactions including end trimming, intron splicing, tRNA editing, and other modifications. Some of these processes, for example those involved in trimming of extraneous sequences at the tRNA ends, occur in the nucleus, usually preceding cytoplasmic export. Others, like editing and modification, may occur at any point in the tRNA maturation pathway and in any of the tRNA-containing compartments. The following sections will cover what is known about these maturation processes in trypanosomatids, focusing primarily on the genera *Trypanosoma* and *Leishmania* where most of the research has been performed. As tRNA transcription will be extensively covered elsewhere in this book, we will focus on steps immediately following transcription by polymerase III in the nucleus and steps that precede tRNA degradation in any compartment. Therefore, tRNA synthesis and stability will be covered only when needed to explain certain aspects of tRNA maturation. Special emphasis will be placed throughout this chapter on how transport dynamics may affect tRNA maturation, which in turn may have direct bearing on tRNA function.

5.2 tRNA Biogenesis and Processing

5.2.1 Nuclear Trimming of 5′ and 3′-Sequences to Generate a Full-Length tRNA

In trypanosomatids, like in most organisms, tRNAs are transcribed as precursor molecules, containing extra sequences at their 5′- and 3′-ends (5′-leader and 3′-trailer, respectively) that have to be removed as part of a tRNA's normal maturation process. The exact order of end-trimming events depends on the tRNA species, but most commonly in eukaryotic organisms, processing begins in the nucleus with 5′-leader removal followed by 3′-trailer cleavage (Fig. 5.1).

Maturation of the 3′-end of eukaryotic pre-tRNA involves two sequential events: removal of the 3′-extension followed by CCA addition. Cleavage of the 3′-trailer of pre-tRNAs is accomplished by the highly conserved tRNase Z, often found as two homologous proteins in eukaryotes. The smaller of the two enzymes, tRNase ZS,

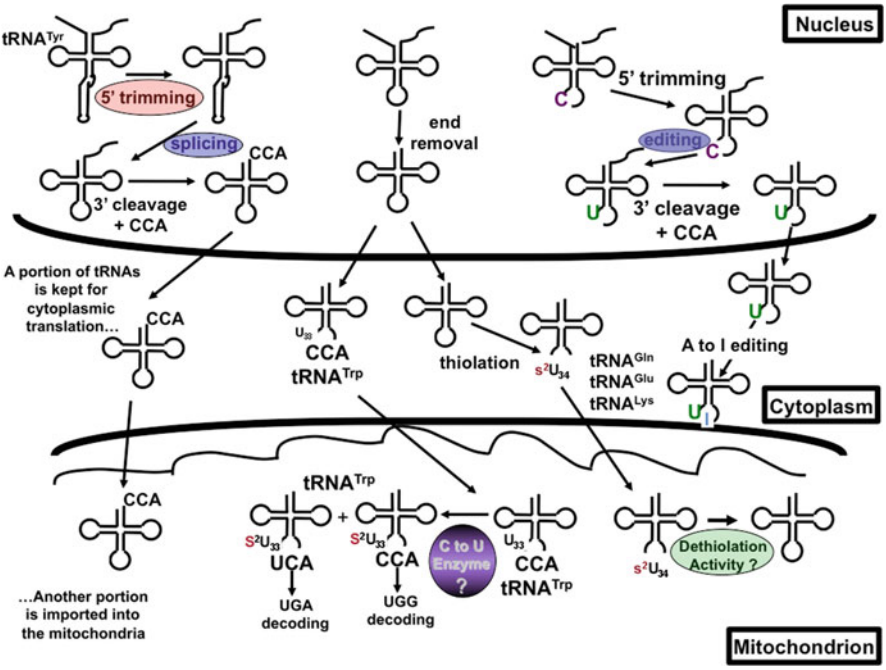

Fig. 5.1 tRNA maturation processes in trypanosomatids. Following transcription tRNAs undergo numerous processing events, those depicted here are the few pathways that have been studied in trypanosomatids. Shown on the *left* is the fate of a newly transcribed tRNA, specifically highlighting $tRNA^{Tyr}$, the only intron containing tRNA in trypanosomatids. The 5′-leader sequence is removed followed by intron splicing and 3′-trailer cleavage. CCA is added to the 3′-end of the tRNA, which is then exported into the cytoplasm where a portion of the tRNAs is kept for cytoplasmic translation and another portion is imported into the mitochondrion for mitochondrial translation. Equally important are tRNA editing and modification events that occur throughout the maturation process. Editing as exemplified by cytidine to uridine (C to U) and adenosine to inosine (A to I) can occur in all three compartments. *Highlighted* in the pathway in the *middle* is mitochondrial C to U editing that permits $tRNA^{Trp}$ to decode UGA as tryptophan. Highlighted in the maturation pathway on the *right* is the C to U editing of $tRNA^{Thr}$ that occurs in the nucleus before 3′-trailer cleavage and addition of the CCA end. This tRNA is further edited (A to I) after being exported to the cytoplasm. tRNA modifications such as thiolation (s^2U) have been observed in the cytoplasm (i.e., $tRNA^{Gln}$, $tRNA^{Glu}$, $tRNA^{Lys}$) as well as in the mitochondrion (i.e., $tRNA^{Trp}$) (*middle* pathway). Activities depicted as *colored* circles represent reactions known to occur in trypanosomatids; however, the enzymes responsible for those reactions remain unknown

appears in all domains of life (bacteria, eukarya, and archaea), while the larger, tRNase ZL, is only found in eukarya (formerly referred to as Elac1 and Elac2, respectively). It is not yet entirely clear why the two forms are present in some eukaryotes, but differences in substrate specificity (Takaku et al. 2004) and cellular localization of the two enzymes may explain it. tRNase ZL usually contains a predicted mitochondrial (Levinger et al. 2004) or chloroplastic (Vogel et al. 2005) targeting sequence, while tRNase ZS always localizes to the nucleus. In trypanosomatids, not much is known about the nature of the 3′-end maturation

enzyme(s). However, bioinformatic analysis in *T. brucei* reveals the presence of a potential homolog of the larger eukaryotic form of the enzyme, tRNase ZL (unpublished results). The other homolog is either absent or not easily detectable by database searches. Furthermore, attempts to identify a version of tRNA Z that contains a mitochondrial targeting sequence have been unsuccessful, suggesting that only tRNase ZL exists in trypanosomatids, but unexpectedly localizes to and functions only in the nucleus. However, one must admit this is just a computer-based prediction. If this protein is truly only nuclear, this should not be at all surprising due to growing evidence that mitochondrial tRNAs are imported from the cytoplasm as mature molecules (as described in the sections below). Thus tRNA import obviates the need for 3′-processing inside the mitochondria. It is possible that some version of tRNA Z may be involved in processing other RNAs in various organisms, including trypanosomatids, judging by the enzyme′s substrate specificity. It has been noted that the minimal substrate for in vitro cleavage by tRNase Z is an RNA molecule consisting of the T-stem loop, acceptor stem, and a minimum of an 11-nucleotide 3′-overhang (Mayer et al. 2000; Nashimoto et al. 1999; Schiffer et al. 2001), raising the possibility of alternative substrates.

Besides tRNA Z other cellular components, including the multisubstrate RNA-binding La protein, have been shown to play a less direct but important role in the removal of the 3′-trailer in eukaryotes (Yoo and Wolin 1997). In *T. brucei*, RNA polymerase III transcripts such as tRNAs end in a string of uridines that serves as the binding substrate for the La protein, which has been shown to prevent nuclease degradation of bound RNA in other systems (Wolin and Cedervall 2002). The *T. brucei* La protein homolog has been identified and recent attempts to characterize its function have produced interesting results. In yeast, downregulation of La is nonlethal; this is likely due to redundant mechanisms of 3′-trimming by exonucleases (Yoo and Wolin 1997). Conversely, RNAi of the *T. brucei* La protein arrested growth shortly after induction, indicating that it is essential for cell viability (Arhin et al. 2005; Foldynova-Trantirkova et al. 2005).

It has also been observed that the downregulation of La caused a 50% decrease in elongator $tRNA^{Met}$ and a fourfold increase in intron-containing $tRNA^{Tyr}$. Despite the various proposed interactions of La with different RNA species (for example other tRNAs), these were the only two to be noticeably affected. It should also be noted that immunoprecipitation experiments with La-specific antibodies and *T. brucei* extracts only pulled down the intron-containing $tRNA^{Tyr}$, pointing to its definite involvement in pre-$tRNA^{Tyr}$ processing. How it affects elongator $tRNA^{Met}$ and whether it interacts with other pre-tRNAs is still unknown (Arhin et al. 2005). It has been proposed that La plays a role in either recruiting the tRNA splicing machinery to the tRNA substrate and/or the recruitment of a tRNA Z to cleave the 3′-end trailer sequence. Still, the precise role the La protein plays in tRNA maturation remains a mystery, and subsequent experiments are required to elucidate its function in *T. brucei*. In a different study of *T. brucei* initiator-$tRNA^{Met}$, nuclear-localized polypeptides with similarity to an *S. cerevisiae* protein, involved in formation of SAM-dependent 1-methyladenosine at position 58 of the TΨC loop of tRNAs (Anderson et al. 1998), were shown to affect tRNA processing when

down-regulated. RNAi of the predicted adenosine methyltransferase, *Tb*MT40, produced an accumulation of pre-tRNAs with 5′-leaders but a trimmed 3′-end. This provides another example in trypanosomatids in which a 3′-to-5′ order of processing is observed. Curiously, downregulation of *Tb*MT40 and its partner protein *Tb*MT53, which is predicted to bind $tRNA^{iMet}$, produced an abundance of elongator $tRNA^{Met}$. The reason for this is unknown. The authors also show via immunoprecipitation that *Tb*MT40 and *Tb*MT53 are part of a 300 kDa multimeric complex with unknown components and function (Arhin et al. 2004).

Similarly, the mechanism for removal of the 5′-leader from tRNAs in trypanosomatids is not exactly clear. It was expected that, like in most eukaryotic tRNAs, 5′-removal is mediated by the highly conserved ribonuclease P (Xiao et al. 2002). RNase P was one of the first examples of a ribonucleoprotein using RNA as its catalytic moiety (Guerrier-Takada et al. 1983), cleaving its RNA substrate to produce 5′-monophosphate and 3′-hydroxyl end products. This metalloenzyme requires divalent ions such as Mg^{2+} for catalysis (Frank and Pace 1998) and correct folding of its RNA component (Baird et al. 2007; Hsieh and Fierke 2009; Kazantsev et al. 2009). It is believed that eukaryotic RNase P recognizes the pre-tRNA via the acceptor stem, the TΨC loop (Baird et al. 2007; Carrara et al. 1995; Levinger et al. 1995; Yuan and Altman 1995), and is aided by its interaction with pre-tRNA bound to the La protein (Esakova and Krasilnikov 2010; Yoo and Wolin 1997).

The exact nature of the enzyme(s) responsible for the removal of the 5′-leader sequence of trypanosomatid pre-tRNAs is not yet clear. However, *a T. brucei* mitochondrial RNase P-like activity has been partially characterized and shown to effectively cleave the 5′-leader of pre-tRNAs. Curiously, there is evidence suggesting that the RNA component of this mitochondrial RNase P is not needed for cleavage activity (Salavati et al. 2001). This observation is consistent with a recent report that the RNase P activity of human mitochondria is a protein-only enzyme (Holzmann et al. 2008). Interestingly, humans still encode an RNA-containing RNase P used for the processing of nucleus-encoded tRNAs. Since the presence of RNase P RNA has been difficult to discern by bioinformatic means, it is possible that in these organisms the requirement for the RNA component has been lost during evolution and that, in fact, even the nuclear RNase P is a protein-only enzyme. Paradoxically, it is not clear why an RNase P-like activity would be required in trypanosomatid mitochondria, since as mentioned above, tRNAs are imported as mature molecules. Likely, trypanosomatid mitochondrial RNase P has an alternative function, perhaps in the maturation of mitochondrial polycistronic transcripts, including mitochondrial rRNA.

Before the end-matured tRNA can participate in aminoacylation, a highly conserved three-nucleotide sequence, CCA, must be added posttranscriptionally by the 3′-specific CCA nucleotidyltransferase, the CCA-adding enzyme. In general, there are two classes of CCA-adding enzymes: the archaeal class I and the eukaryotic and bacterial class II (Xiong and Steitz 2006). Both facilitate the addition of CCA using CTP and ATP in a template-independent polymerization reaction. Currently, no published information is available for tRNA nucleotidyltransferases in *T. brucei*, but expectedly, a BLAST search of the *T. brucei* sequence database revealed the

presence of a putative class II CCA-adding enzyme and no matches for class I. However, this enzyme has not been genetically or biochemically characterized.

5.2.2 Intron Removal and Nuclear Export

In *T. brucei*, the only intron-containing tRNA is tyrosine tRNA ($tRNA^{Tyr}$), which has an 11 nucleotide intervening sequence. Intron-containing tRNAs can be found in all of the phylogenic kingdoms and usually consist of a short sequence of nucleotides that occur immediately 3′ of the anticodon loop in eukaryotes. The function of introns within tRNAs is largely unknown; however, in yeast $tRNA^{Tyr}$, the intron is required for conversion of the central uridine of the anticodon to pseudouridine (Johnson and Abelson 1983). The mechanism of tRNA intron splicing proceeds in two steps. The first involves the removal of the intron by a specific endonuclease that recognizes conserved elements of the anticodon stem. The two halves generated by the endonuclease are then joined by a ligase to complete the process (Greer et al. 1987; Fig. 5.1).

What is eye-catching about the *T. brucei* $tRNA^{Tyr}$ intron is its length; at 11 nucleotides it is one of the shortest tRNA introns known in nature. Assuming that tRNA splicing is as conserved in *T. brucei* as it is in other organisms, the *T. brucei* system may inherently provide insight into a very simplified minimal substrate for the *T. brucei* endonuclease. Although neither the endonuclease nor the ligase have been identified in trypanosomatids, earlier work showed that unlike the yeast endonuclease, site-specific mutations in the anticodon loop of *T. brucei* $tRNA^{Tyr}$ disrupted splicing, implying the strict necessity for a specific structure for cleavage. This is reminiscent of tRNA splicing in plants (Stange et al. 1988). Notably, $tRNA^{Tyr}$ mutants deficient in tRNA splicing were observed to accumulate with 3′-extensions, while 5′-extensions were not detected with probes specific for the 5′-leader sequence, suggesting that the 5′-mature and unspliced intermediate serves as the substrate for 3′-end processing. This order of events is different from that observed in other organisms where 5′- and 3′-maturation occur before splicing (De Robertis and Olson 1979; Melton et al. 1980).

Once the pre-tRNA ends have been cleaved off and in the case of $tRNA^{Tyr}$, once the intron has been removed, the mature tRNA must be exported from the nucleus to the cytoplasm. Again, little is known about this process in *T. brucei*, but in other eukaryotic systems a Ran-GTP dependent protein, exportin T, carries tRNA across the nuclear membrane and releases it after Ran-GTP hydrolysis to form Ran-GDP (Hopper et al. 2010). It has been proposed that exportin T contributes to tRNA proofreading before export by assessing the condition of the 5′- and 3′-ends as well as the structure provided by the acceptor stem and T-stem loop (Arts et al. 1998; Cook et al. 2009; Lipowsky et al. 1999). The maturation status of tRNAs destined for mitochondrial import is currently a point of contention, so it will be interesting to see how exportin T discriminates substrates for different localization or if other factors are also involved in tRNA export, as has been seen in fungi (Hopper et al. 2010).

In summary, although the various activities that mature a tRNA via end trimming and CCA addition are predictably present in trypanosomatids, little is known about the biochemical nature and the mechanisms of any of the enzymes or processing events.

5.2.3 tRNA Editing and Modifications

Maturation of tRNAs involves more than the typical splicing of introns and the removal of the 5′-leader and 3′-trailer. Posttranscriptional editing and modification of many nucleotides throughout the tRNA sequence represents in all domains of life another necessary step before a tRNA becomes fully functional. Over 100 different modified nucleotides have been characterized with a median of 8 modifications per tRNA (Phizicky and Alfonzo 2009). While individually many of the modifications are dispensable, with functions ranging from tRNA stability, to tRNA structure and folding to translational fidelity, editing and modification events can be integral parts of normal cell function, some even being essential for viability (Phizicky and Alfonzo 2009). In a few cases, trypanosomatid tRNAs provide the only example for a particular modification or editing event. There are, of course, the modifications that are nearly universally conserved and after which tRNA regions are named, including dihydrouridine (D) in the D arm and pseudouridine (Ψ) in the TΨC arm, but perhaps of greater interest are those modifications that may be unique to trypanosomatid physiology. Alternatively, equally interesting are those modifications that despite being in common with other systems, in trypanosomatids, still provide nuances in their mechanisms of synthesis. These may promise to become potential chemotherapeutic targets. In general, little is known on tRNA editing and modification in trypanosomatids. The following sections will explore various themes that are slowly developing in the study of modifications in these organisms, but unfortunately the discussion will be limited to the few examples of modifications that have been studied. These include cytidine-to-uridine (C to U) and adenosine-to-inosine editing (A to I), inside and outside of organelles (Alfonzo et al. 1999; Crain et al. 2002; Rubio et al. 2006, 2007), and tRNA thiolation (Bruske et al. 2009; Crain et al. 2002; Wohlgamuth-Benedum et al. 2009).

5.2.3.1 tRNA Editing

As originally stated, “RNA editing” is broadly defined as a programmed posttranscriptional alteration of sequence information in mRNA beyond what is encoded in the DNA genome (Benne et al. 1986; Gray 2003). This definition was originally used to describe only canonical nucleotide changes, but in the following subsections we will use an even more generalized definition of editing that includes the replacement of canonical nucleotides for noncanonical ones (e.g., adenosine for inosine) that have a direct effect on either tRNA structure or function.

C to U Editing

The first studies of tRNA editing in trypanosomatids came from the fact that in these organisms, all the tRNAs used in mitochondrial translation are nucleus encoded and subsequently imported into the mitochondrion. A problem is encountered when considering the translation of tryptophan codons because, like with many eukaryotic organisms, in the trypanosomatid mitochondrial genome the canonical UGG tryptophan codon is often replaced by UGA, a stop codon in cytoplasmic translation (Alfonzo et al. 1999). This led to the question of how organisms with a single $tRNA^{Trp}$ with anticodon CCA could decode UGG as tryptophan and UGA as stop in the cytoplasm, while decoding UGA as tryptophan once imported into the mitochondrion. As first shown in *Leishmania tarentolae* (Alfonzo et al. 1999) more than a decade ago and later corroborated in *Trypanosoma brucei* (Charriere et al. 2006), these organisms have solved this decoding conundrum in a simple yet rather elegant way. The tRNA undergoes cytidine (C) to uridine (U) editing at the first position of the anticodon, thus changing the CCA anticodon to UCA which can now decode UGA by canonical base pairing and even UGG by wobbling (Alfonzo et al. 1999). Clearly, mitochondrial compartmentalization of such an activity avoids the potential generation of a high-copy suppressor tRNA (with anticodon UCA) in the cytoplasm (Fig. 5.1).

While the reason for this type of editing is clear, its mechanism remains uncertain. The simplest activity accounting for the observed C to U editing would involve a tRNA-specific cytidine deaminase. A precedent for a polynucleotide-specific cytidine deaminase already exists in the editing of mRNA in mammals (Navaratnam et al. 1993). The possibility of such an enzyme in trypanosomatid mitochondria has been most recently reinforced with the discovery of an analogous activity in archaea, where the first *c*ytidine *d*eaminase *a*cting on *t*RNAs (CDAT8) has been described (Randau et al. 2009). This enzyme, a member of the larger cytidine deaminase family, catalyzes C to U via a conserved hydrolytic deamination reaction in a zinc-dependent manner. Unfortunately, no protein similar to CDAT8 in *Trypanosoma* or *Leishmania* could be identified by bioinformatic analysis. Therefore, the enzyme responsible for this mitochondrial editing activity remains elusive. However, an in vivo approach still allowed defining certain features of the tRNA substrate that are important for $tRNA^{Trp}$ editing. We took advantage of the fact that tRNA variants expressed from a plasmid in *L. tarentolae*, when transcribed could still be imported into the mitochondria. We used this approach to establish that a single base pair reversal, at the last position of the $tRNA^{Trp}$ anticodon stem, abolished C to U editing in vivo (Crain et al. 2002). Beyond this position, it is not known what other determinants of editing exist in the natural $tRNA^{Trp}$ substrate in *Leishmania,* but in *T. brucei* (as discussed below) modifications may serve as an antideterminant (Wohlgamuth-Benedum et al. 2009). Clearly, C to U editing has direct bearing on translation and because an estimated 88% of the conserved mitochondrial tryptophan codons are UGA, it has been concluded that this editing is essential for mitochondrial translation and cell survival.

The only other example of C to U editing in trypanosomatids occurs outside the mitochondrion and is, in fact, the first example of C to U editing outside organelles in eukarya (Gaston et al. 2007; Rubio et al. 2006). Occurring just 5′ of the tRNA anticodon (position 32) in all three isoacceptors of $tRNA^{Thr}$, this editing event takes place while the tRNA is still in the nucleus and before the removal of the 5′-leader from the tRNA (Fig. 5.1) (Gaston et al. 2007). Unlike the mitochondrial editing of $tRNA^{Trp}$, C_{32} to U_{32} editing has no direct bearing on decoding; however, in vitro, C_{32} to U_{32} stimulates, although is not required for, the essential adenosine-to-inosine formation at the wobble position of $tRNA_{AGU}^{Thr}$. As A to I has a direct effect on translation and is essential for cell viability, it may stand to reason that C_{32} to U_{32} indirectly affects translational efficiency of some, if not all, threonine codons.

A to I Editing

By far, the most common form of editing occurring in eukaryotic tRNAs is the adenosine to inosine substitution at the first position of the anticodon. Mechanistically, this type of editing proceeds via a conserved hydrolytic deamination reaction, much the same as that described for the C to U activity of archaeal CDAT8 (Randau et al. 2009). Unlike the case of C to U editing of $tRNA^{Trp}$, the enzyme responsible for A to I editing has been identified in *T. brucei* in our laboratory (*Tb*ADAT2/3) (Rubio et al. 2007). This enzyme functions as a heterodimer, comprised of two subunits, ADAT2 and ADAT3, which harbor the Zn-coordinating motifs, H(C)xE and PCxxC, characteristic of all members of the cytidine deaminase superfamily. In these enzymes, the conserved histidine and two cysteines coordinate a zinc ion, while the fourth ligand is an activated water molecule. A conserved glutamate in ADAT2 then acts as a proton shuttle between the activated water and the exocyclic nitrogen at C6 of the purine ring. This set of ligands and cofactors thus act in concert to hydrolyze the amino group at C6 of the adenosine ring releasing ammonia as the leaving group. Although inosine deamination has been studied for several years in various systems, the enzyme from *T. brucei* still produced a few interesting surprises. For instance, the core sequences of these deaminases, including the residues involved in catalysis, resemble those found in cytidine deaminases despite the fact that the enzyme catalyzes an adenosine deamination reaction (Gerber and Keller 1999). This observation in itself may be of no consequence, but given our finding that tRNAs may undergo both A to I and C to U editing (as discussed briefly in the preceding section), we decided to explore the possibility that this enzyme could perform both reactions. To our surprise, downregulation of TbADAT2 (the presumed catalytic subunit) in *T. brucei* led to a reduction in both A to I and C to U editing of $tRNA^{Thr}$, suggesting that the enzyme is involved in both reactions in vivo. However, in vitro we found that TbADAT2/3 could robustly catalyze A to I, but not C to U, editing in tRNA. This raises the question of what factor may be missing in the in vitro reaction with recombinant proteins. We could, however, demonstrate that TbADAT2/3 efficiently catalyzes C to U deamination of DNA (Rubio et al. 2007). Although the biological significance of the DNA reaction

is not clear, the observed activity demonstrated that the enzyme had the inherent capacity to catalyze both reactions as previously hypothesized. The question still remains, why it cannot perform the reaction on tRNA? One explanation may be that there are posttranscriptional modifications in the natural substrate that may be important specificity determinants and are missing in our in vitro substrate. Alternatively, the enzyme itself may be missing some additional cofactor found in vivo. Here it is worth noting that the A to I reaction at the wobble position occurs after tRNA export from the nucleus, but interestingly, both subunits (*Tb*ADAT2 and 3) localize to both the nucleus and the cytoplasm (Gaston and Alfonzo, unpublished results). This raises the possibility that within a cellular compartment, the substrate specificity can change by virtue of other proteins associating with ADAT2 within a given compartment. In this realm, compartmentalization will determine enzyme specificity in a manner reminiscent of the previously described C to U editing of $tRNA^{Trp}$ in mitochondria of trypanosomatids. Currently, the answers to many of these questions are still far from clear.

5.2.4 Modifications

Beyond C to U and A to I editing, trypanosomatid tRNAs also undergo a number of posttranscriptional modifications. Initially, a number of modifications were identified by a combination of liquid chromatography and mass spectrometry analysis of cytoplasmic and mitochondrial $tRNA^{Trp}$ (Crain et al. 2002). This of course was prompted by the discovery of the C to U editing event described above. From these studies, 12 different modifications were identified in *Leishmania* $tRNA^{Trp}$ including dihydrouridine (D), pseudouridine (Ψ), 2-thiouridine (s^2U), N^6-isopentenyladenosine, and a number of sugar and base methylations (i.e., 2′-O-methylcytidine, 2′-O-methyluridine, 2′-O-methylguanosine, 5-methyluridine, 7-methylcytidine, and N^2-methylguanosine). These are by no mean unusual and occur in tRNAs from other organisms (Juhling et al. 2009; Sprinzl and Vassilenko 2005). What generated interest was the finding that many modifications were added to the tRNA following mitochondrial import.

Most surprising was the discovery that this tRNA undergoes thiolation at position 33 of the anticodon (adjacent to the edited nucleotide). Although previous work had raised the possibility of this tRNA position being modified, this finding, however, constituted the first demonstration of modification at this position in any organism. U_{33} plays an important function in shaping the anticodon loop structure allowing a tRNA to splay the anticodon nucleotides, priming them for translation. This unusual thiolation of $tRNA^{Trp}$ eventually led to further exploration of the thiolation pathways of trypanosomatids. In *T. brucei*, like in most eukaryotes, there are two places where tRNAs can be thiolated: the cytoplasm and the mitochondria (Fig. 5.1). Cytoplasmic thiolation seems to require the same components as in yeast, but the specific contributions of factors like Urm1, Uba, Ncs 1, and Ncs 6 have not been formally tested (Leidel et al. 2009). These are identifiable by genomic

searches and are expected to provide similar functions as in the yeast system. Trypanosomatids and other eukaryotes differ in the nature of the tRNAs used for mitochondrial thiolation. In the *T. brucei* cytoplasm, $tRNA_{UUG}^{Gln}$, $tRNA_{UUC}^{Glu}$, and $tRNA_{UUU}^{Lys}$ are the only known targets for thiolation, but because of tRNA import, these tRNAs enter the mitochondria already containing the thiol group added by the cytoplasmic thiolation system. So far, the only tRNA known to undergo mitochondrial thiolation in these organisms is $tRNA^{Trp}$ (Alfonzo et al. 1999; Charriere et al. 2006), which as described above also undergoes C to U editing. Surprisingly, in our studies of thiolation, we found that RNAi downregulation of any of the mitochondrial thiolation factors (including Nfs, the key desulfurase essential for thiolation) led to upregulation of tRNA editing to almost 100% (Wohlgamuth-Benedum et al. 2009). This observation implies that s^2U_{33} acts as a negative determinant for tRNA editing and helps maintain the levels of the two isoacceptors as required for UGG and UGA decoding. Notably, $tRNA^{Trp}$ is not thiolated in the cytoplasm during transit. This raised the question of how this tRNA avoids cytoplasmic thiolation. We showed that editing is not required for thiolation at U_{33} in *L. tarentolae*, a close relative of *T. brucei* (Crain et al. 2002). Therefore, the only viable explanation is that the cytoplasmic and mitochondrial tRNA thiolation systems differ in their substrate recognition and that, in fact, there are features common to $tRNA^{Gln}$, $tRNA^{Glu}$, and $tRNA^{Lys}$ required for thiolation that are not present in $tRNA^{Trp}$. Recently, it was shown that following import, $tRNA^{Gln}$, $tRNA^{Glu}$, and $tRNA^{Lys}$ become dethiolated by an unidentified activity, raising the possibility that the mitochondrial thiolation may play a "repair" role for this tRNA set; again, this has not been formally tested (Bruske et al. 2009).

An additionally surprising discovery with the *T. brucei* system involves the fate of the cytoplasmic tRNAs in the absence of thiolation. We showed that $tRNA^{Gln}$, $tRNA^{Glu}$, and $tRNA^{Lys}$ become unstable and are quickly degraded if thiolation is impaired (Wohlgamuth-Benedum et al. 2009). This instability was specific only to the thiolated tRNA set and differs in this respect from a more general rapid tRNA degradation pathway described (Alexandrov et al. 2006; Engelke and Hopper 2006). The nature of the enzymes or factors mediating this degradation is currently unknown. Overall, the thiolation system of *T. brucei* shows not only how intracellular compartmentalization affects tRNA modification, but it even exemplifies how location may affect modification enzyme substrate specificity. A curious corollary of the thiolation story is the remarkable finding that the same desulfurase required for iron–sulfur (FeS) assembly is also required for tRNA thiolation in both the cytoplasm and the mitochondria. In the mitochondria, subunits of respiratory complex III require an FeS cluster, therefore downregulation of Nfs could lead to downregulation of respiratory rates. We suggest a model by which the divergence of the two pathways (FeS assembly and tRNA editing/thiolation) from a common key enzyme may be exploited by these cells to carefully match respiratory rates to mitochondrial translation perhaps by offsetting the 50/50 ratio of edited and unedited $tRNA^{Trp}$. It is also worth mentioning that a number of cytoplasmic modification enzymes require FeS clusters for activity, thus this hypothesis may even include cytoplasmic modification systems in connection with FeS-cluster

assembly for global metabolic regulation (Lill and Muhlenhoff 2006). These proposals are of course largely speculative, but their exploration may reveal important global aspects in the coordination of these facets of cellular metabolism.

5.3 tRNA Import into the Mitochondria

The mitochondrion is the powerhouse of the cell in that it provides the bulk of the energy required for various cellular transactions in the form of ATP. The mitochondrial genome of kinetoplastids encodes only a small subset of genes required for the assembly of complete respiratory complexes and generation of ATP. Glaringly missing from the mitochondrial genomes of trypanosomatids are any recognizable tRNA genes. This is not unique to the trypanosomatids and indeed most, if not all, mitochondria-containing organisms have lost tRNA genes from their mitochondrial genomes during evolution. Historically, the case of missing tRNA genes in mitochondria dates back to the 1960s when Suyama and coworkers while working with the single-cell protist *Tetrahymena pyriformis* first introduced the concept of mitochondrial import of tRNAs from the cytosol (Suyama 1967). This was needed in order to supplement the protein synthesis machinery with the missing tRNAs, which in conjunction with those tRNAs still encoded in the organelle, could account for the decoding of all the codons used in mitochondrial translation (Suyama 1967).

To date, multiple occurrences of tRNA import have been described in diverse organisms: the ciliate *Tetrahymena* (Rusconi and Cech 1996; Suyama 1967), kinetoplastid flagellates *Trypanosoma* and *Leishmania* (Hancock and Hajduk 1990; Mottram et al. 1991; Simpson et al. 1989), yeast (Martin et al. 1979; Rinehart et al. 2005), various marsupials (Dorner et al. 2001), the apicomplexan *Toxoplasma* (Esseiva et al. 2004), plants (Marechal-Drouard et al. 1988), and, most recently, mammals (Rubio et al. 2008). The number of imported tRNAs varies greatly from as few as one tRNA in mammals to a full set of tRNAs in kinetoplastids and apicomplexans (Salinas et al. 2008). The kinetoplastids *Leishmania* and *Trypanosoma* represent the most extreme case of missing tRNA genes, in that all cytoplasmic tRNAs, with the exception of initiator $tRNA^{Met}$, have to be imported from the cytoplasm. In this section our discussion will focus on the studies of tRNA import into the mitochondria of *Leishmania* and *Trypanosoma*, two systems that cover the lion's share of what is known about tRNA import in this group of evolutionary diverse organisms.

The disappearance of a varying number of tRNA genes from mitochondrial genomes among different organisms has perhaps driven the independent evolution of systems that permit the import of tRNAs from the cytoplasm (Lithgow and Schneider 2010; Salinas et al. 2008); still, the factors that control tRNA import, specifically the nature of the import machinery, remain to be fully understood. In the absence of clear knowledge of the import machinery, here we will refrain from the relaxed use of the term "mechanism," and instead we will divide import

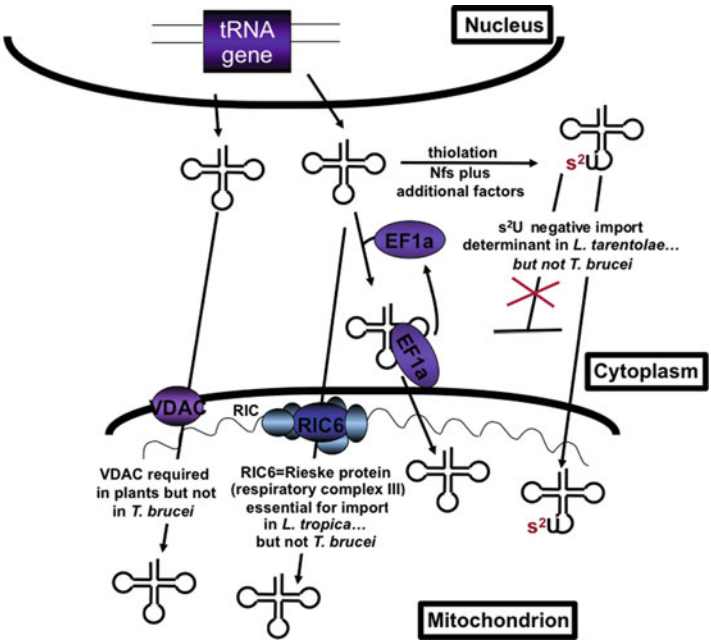

Fig. 5.2 Pathway for tRNA import into mitochondria independent of the protein import pathway. The pathways shown highlight important features of tRNA import into the mitochondria that occurs independently of the protein import pathway. Specific factors and complexes affect import differently in different organisms. For example, thiolation (s^2U) is a negative import determinant for tRNA import in *L. tarentolae*, but is not a determinant in *T. brucei*. Likewise, RIC6 (Rieske protein) of the RNA import complex (RIC) is necessary for import in *L. tropica*, but not in *T. brucei*. Elongation factor EF1a is also involved in import in *T. brucei*. Other factors, like VDAC, play a role in other organisms, but not in *T. brucei* (as shown)

systems into two broadly defined types (a) those utilizing the known protein import pathway, and (b) those in which tRNA import is shown to occur independent of the protein import machinery (Alfonzo and Soll 2009). For clarity, type I import, is limited to the single known example from yeast where direct participation of the canonical and well-characterized protein import machinery itself helps drive the tRNA across the mitochondrial double membrane. Since this type of import has not been described in trypanosomatids, for all practical purposes in these organisms tRNA import occurs independently of the protein import machinery (Fig. 5.2). Clearly tRNA import components themselves have to be transported and inserted in the membranes; careful teasing of the secondary effects of protein import from that of the downstream import of the tRNA remains a delicate nuance in the identification of proteins involved in the transport of tRNAs.

Common to all organisms that import tRNAs into the mitochondrion, including trypanosomatids, is the need for tRNAs to traverse both the outer and inner mitochondrial membranes to reach their final destination in the mitochondrial matrix, where translation takes place. There are various hypotheses that explain

how a given tRNA is recognized in the cytosol and delivered to the mitochondrion, but currently no single proposal for tRNA delivery explains all the data published so far. While the tRNA import process can be efficiently reproduced in vitro in the absence of cytoplasmic factors, the contribution of cytoplasmic factor(s) to the process also has to be addressed. Given that tRNAs in the cell are integrally associated with components of the translation machinery, they need to be recycled during protein synthesis. The missing link in the series of events is how the tRNA frees itself from the translation machinery and the mode by which the tRNA reaches the mitochondrion. One hypothesis proposes that a fraction of the nuclear-encoded tRNAs escapes the cytoplasmic translation machinery by interacting with protein factors that will consequently direct it to the mitochondrion. In *Trypanosoma brucei*, the best example is provided by the cytoplasmic translation elongation factor 1α (eEF1α), which plays a role as a specificity determinant for a small subset of imported tRNAs (Bouzaidi-Tiali et al. 2007), but by extrapolation may play a similar role with all cytoplasmic tRNAs prior to transport (Fig. 5.2). In vivo experiments suggested that in *T. brucei*, $tRNA^{Met}$, $tRNA^{Ile}$, and $tRNA^{Lys}$ contain a major localization determinant within the TΨC-stem at nucleotides 51 and 63, which form a base pair in the canonical tRNA structure. This finding was a logical derivation of an earlier observation that in the initiator $tRNA^{Met}$, one of two tRNAs that is not imported into the *T. brucei* mitochondrion, this base pair is identical to the main antideterminant preventing interaction with elongation factor (eEF1α). This is different from all the imported tRNAs, which contain the base pair needed for eEF1α binding, implicating this factor in tRNA delivery to the mitochondrial surface. Indeed, knockdown of cytoplasmic eEF1α, but not of translation initiation factor 2, was found to inhibit mitochondrial import of newly synthesized tRNAs, long before cytoplasmic translation or cell growth is affected. Additionally, $tRNA^{Sec}$, the only other cytosol-specific tRNA in *T. brucei*, which has its own elongation factor and does not bind eEF1α, could be redirected to the mitochondrion by simple introduction in its sequence the same base pair required for eEF1α binding. Therefore, for tRNAs that are imported, binding to eEF1α provides an additional level of import specificity beyond what is already provided by the import machinery itself (Lye et al. 1993; Shi et al. 1994; Bouzaidi-Tiali et al. 2007).

In terms of actual mitochondrial import factors intrinsically associated with the membranes, a system has been described in *Leishmania tropica*. This system is independent of the mitochondrial protein import pathway and involves the so-called *R*NA *i*mport *c*omplex (RIC) (Mukherjee et al. 2007). The RIC was derived from a detergent-solubilized extract of *L. tropica* inner mitochondrial membranes that was subjected to chromatography. This affinity column harbored an immobilized RNA oligonucleotide corresponding to what has been identified previously as a legitimate import signal by the same authors. This affinity substrate corresponded to the D arm of $tRNA^{Tyr}$ and when used for affinity chromatography yielded a massive multiprotein aggregate (Bhattacharyya et al. 2000). Sequences resulting from mass spectrometry analysis of the resulting complex produced a total of 122 nuclear-encoded ORFs. These were then used to mine the *Leishmania* genome sequence database, and the role of specific proteins in tRNA import was further analyzed by

Western blotting and gene knockdown using RNAi (Mukherjee et al. 2007). The RIC is comprised of an 11-protein core complex assembled at the mitochondrial inner membrane with a stoichiometry adding up to a total mass of ~580 kDa. The import complex requires ATP and membrane potential to import tRNAs. Within the complex, there are three mitochondrial- and eight nuclear-encoded subunits. Analyses by knockdown and in vitro reconstitution experiments indicated that six of the eight nuclear-encoded subunits, RIC 1, 4A, 6, 8A, 8B, and 9, are essential for import. The RIC has been affinity-purified and resolved from other mitochondrial complexes by native gel electrophoresis (Goswami et al. 2006). Functional complexes could be reconstituted with recombinant subunits expressed in *Escherichia coli*. Several essential RIC subunits are identical to specific subunits of respiratory complexes. The two nonessential subunits were identified as RIC3, an M16 metalloproteinases, and RIC5, a trypanosomatid-specific protein. It is proposed that RIC1 and RIC8A are the two receptors involved in initial tRNA binding. Then, trimeric RIC6 and RIC9 form the translocation pore, while RIC4A and RIC8B anchor the complex to the membrane. Membrane-embedded mitochondrial-encoded subunits 2 (dimeric), 4B (substoichiometric), and 7 interact with RIC4A (Fig. 5.2). The dispensable subunits RIC3 and RIC5 are assembled peripherally (Goswami et al. 2006).

Despite many reports, the relevance of the *Leishmania* import complex is still controversial. For instance, one of its essential components is the Rieske protein, but downregulation of its expression by RNA interference in *T. brucei* had no effect on tRNA import (Paris et al. 2009), despite the predicted effects on membrane potential and mitochondrial function traditionally ascribed to Rieske function. Thus in the kinetoplastid system, the true nature of the tRNA import machinery is not yet clear. In plants, inhibition of the VDAC (voltage-dependent anion channel) by the addition of reuthenium red impaired tRNA import into mitochondria (Salinas et al. 2006), suggesting that the VDAC plays a critical role for tRNA transport across the outer membrane. However, the same is not true in *T. brucei*, where a knockout of the gene encoding the VDAC protein had no effect on tRNA transport (Pusnik et al. 2009) (Fig. 5.2). In conclusion, to date no single protein has been identified and characterize that directly contributes to tRNA translocation across the mitochondrial membranes in trypanosomatids. Thus far, what all protein-import independent mechanisms, including those of trypanosomatids, have in common is the requirement for ATP (although the exact role of ATP is still not clear) and lack of requirement for membrane potential (Alfonzo and Soll 2009). However, since the actual transporters have not been identified, it is difficult to conclude more on the actual transport mechanism.

5.3.1 Analyses of tRNA Molecules as Import Substrates

Earlier observations suggested that in *Trypanosoma brucei*, the 5′-leader-containing precursor tRNA is the substrate for in vivo import. The leader sequence

was, in fact, supposed to contain sequence information needed for import. Specifically, the 5′-flanking sequence of the precursor $tRNA^{Leu}$ was shown to be important for its in vivo localization in *T. brucei* mitochondria (Sherrer et al. 2003). The proposed model was analogous to the protein import mechanism, where the preprotein N terminus serves as a "zip code" for organellar targeting. However, conflicting experiments showed that at least in the case of $tRNA^{Leu}$ (CAA) isoacceptor, import occurs regardless of the sequence context of the imported gene, implying that the tRNA presequence could not be an import determinant for every tRNA in *T. brucei* (Tan et al. 2002).

To address the contribution of sequence determinants within the mature tRNA molecule, the in vivo import of tRNAs into the mitochondrion *of L. tarentolae* was studied using two tRNAs that differentially localize within the cell (Lima and Simpson 1996). In *L. tarentolae*, $tRNA^{Ile}$(UAU) is mostly localized within the mitochondrion while $tRNA^{Gln}$(CUG) is primarily in the cytosol (Lye et al. 1993; Shi et al. 1994). To permit discrimination of the exogenously transfected tRNA from that of the endogenous tRNAs in these in vivo import experiments, plasmids encoding sequence-tagged variants at the D loop of either $tRNA^{Ile}$ or $tRNA^{Gln}$ were transfected into *L. tarentolae* cells. The import of RNAs, assessed by primer extension analysis, revealed that the plasmid-encoded genes were expressed and that the tagged tRNAs showed a similar intracellular localization as the endogenous tRNAs. The in vivo import experiments in *L. tarentolae* further demonstrated that the exchange or deletion of the 5′-flanking genomic sequences had no effect on the expression or mitochondrial localization of the tagged $tRNA^{Ile}$ or on the expression or cytoplasmic localization of the tagged $tRNA^{Gln}$, suggesting that the signals for importation are localized within the tRNA itself. Swapping the D stem and loop from the mainly cytoplasmic $tRNA^{Gln}$ with that from the $tRNA^{Ile}$ produced an increased mitochondrial localization of the plasmid-expressed mutated $tRNA^{Gln}$. Given that the D loop exchange between the two differentially localized tRNAs did not eliminate the mitochondrial localization of the plasmid-expressed tagged $tRNA^{Ile}$, the role of tertiary tRNA structure or additional sequence elements were proposed to contribute an essential role in signaling the mitochondrion to import the tRNA (Lima and Simpson 1996).

Similar experiments were performed in vitro with the same tRNAs used for the in vivo studies, namely $tRNA^{Ile}$ and $tRNA^{Gln}$. Worth highlighting with these studies in the *L. tarentolae* in vitro import system is the observation that the system is saturable. This strongly implies that the in vitro assay exhibits dynamics akin to receptor-mediated systems. Moreover, the amount of tRNA protected from nuclease digestion in the presence of isolated mitochondria reaches a plateau at different concentrations, 25 μM for $tRNA^{Ile}$ and 3.8 μM for $tRNA^{Gln}$ (Rubio et al. 2000). This difference seen in the in vitro saturation levels of imported tRNA is consistent with the levels of in vivo localization of these tRNAs, where $tRNA^{Ile}$ is primarily mitochondrial and $tRNA^{Gln}$ is primarily cytoplasmic (Kapushoc et al. 2002; Lye et al. 1993). In vitro, swapping the D-loops of full-length $tRNA^{Ile}$ and $tRNA^{Gln}$ led to the reversal of their import efficiency. This was also consistent with the previous in vivo import studies, which strongly supports the fact that at least for these two

tRNAs the in vitro import system was a valid mimic of the in vivo situation. This observation not only argues for the specificity of this process, but also implicates the importance of structural interaction between the D-arm and the TΨC-arm in the tRNA in providing discrimination for mitochondrial import. Again, in the absence of any additional cytoplasmic factors, the mitochondrion can itself mediate imported levels of tRNA reminiscent of the in vivo situation. This does not, of course, discount the possibility that cytoplasmic factors play a role in vivo.

In *Leishmania tropica,* the import of tRNAs into mitochondria has been proposed to involve two signature consensus sequences dividing tRNAs into either class I tRNAs or class II tRNAs. Type I tRNAs are proposed to contain a conserved sequence motif in the D arm needed for efficient import across the inner mitochondrial membrane, and positively stimulates import of type II molecules into the mitochondrial matrix. In contrast, type II tRNAs exhibit a conserved sequence motif within the variable region and the TΨC domain and are poorly imported into the mitochondrion. Additionally, type II tRNAs inhibit import of type I RNAs into the mitochondrial matrix. It is further speculated that a limited number of receptors regulate the rate of import of individual tRNAs and that regulatory interactions exist between the two types of tRNA molecules (Mahapatra et al. 1998). Furthermore, Mahapatra and Adhya suggest that in vitro, an import protein receptor at the mitochondrial surface specifically recognizes a sequence motif only at the D-arm for $tRNA^{Tyr}$ (Mahapatra et al. 1994, 1998). Specifically, the D-arm sequence motif AUGGCAGAG is proposed to interact with *L. tropica* RIC. This D-loop has been proposed as the receptor recognition motif and a key import determinant. The import probe was quite unique, in that it consisted of antisense RNA transcripts between −53 and +25 nucleotides of the *Leishmania* β-tubulin gene. Experimental evidence showed that mitochondrial uptake in vitro of this transcript could be competitively displaced by *Leishmania* tRNA. Accordingly, they suggested that the nucleotide sequence AUGGCAGAG or its motif within the antisense RNA might account for this competitive displacement, since this sequence motif exhibited homology to the sequence AU(U)GGC/UA within the D-loop region of two imported tRNA species, $tRNA^{Thr}$ and $tRNA^{Tyr}$ (Bhattacharyya et al. 2000).

In order to further test the proposal that an import signal resides within the D loop, a different study explored the possible presence of the proposed sequence determinant in all tRNA sequences available at the time. These sequences were extensively surveyed and examined for the existence of this D-loop motif and whether the motif could determine the import fate of a given tRNA (Suyama et al. 1998). No obvious consensus sequence at the D-loop was obtained from the analysis that could correlate tRNA localization within the cell to the presence of the D-loop motif, calling into question its in vivo importance. Therefore, the nucleotide sequence at the D-loop alone does not explain the observation of variable import phenotypes between tRNAs and in these arguments one must include the contribution of tertiary contacts between different regions of the highly conserved L-shaped tRNA structure (Suyama et al. 1998).

In *L. tarentolae*, further studies on the import of short RNAs, containing either the class I or II D-loop consensus demonstrated that both were imported in vitro into *L. tarentolae* mitochondria (Rubio et al. 2000). Strikingly, the in vitro import of the short RNA substrates led to the loss of discrimination for mitochondrial localization, which is in contrast to the import behavior observed with the full-length tRNA. This indicated that even though shorter RNA molecules may be imported in vitro, the ability of the mitochondria to discriminate between the substrates to be imported becomes compromised. To ascertain the limits of import specificity, smaller RNAs including five different 16- to 17-nucleotide mini-helix RNAs and one unstructured 17-nucleotide substrate (nonhelix forming) were also tested; all could be efficiently imported in vitro. However, unstructured RNAs of greater sizes (19, 24, and 33 nucleotides) failed to support import. The fact that in vitro import loses its specificity when the RNA substrate becomes less than ~17 nucleotides in length suggests that although a feature contributed by the D-loop may be important, it is within the context of a full-length tRNA that import discrimination is achieved.

One common feature between the in vitro tRNA import systems in *L. tarentolae* and *L. tropica* mitochondria is the observation of in vitro import in the absence of added cytoplasmic factors. These two systems, however, still yield conflicting data; as discussed above, a membrane potential is not required for import in *L. tarentolae*, but it is absolutely required in *L. tropica*. However, in both systems there is a need for ATP hydrolysis. Significantly, the lack of requirement for membrane potential suggests that tRNAs are actively imported via a route other than the protein import pathway, which has a strict requirement for a membrane potential. Coincidentally, the lack of requirement for membrane potential in *Leishmania* is similar to that of the import of $tRNA^{Gln}$ in yeast and human mitochondria (Rubio et al. 2008). These systems radically differ from that of yeast $tRNA^{Lys}$, which has a strict dependence on membrane potential due to the involvement of the protein import pathway. Despite these differences, it is clear from experiments where mitochondria were pretreated with proteinase K prior to import, that the machinery that imports tRNAs is proteinaceous in nature.

Aside from studies focused on determining positive elements contributing to tRNA import, possible negative import determinants have also been described. Given that posttranscriptional modifications are known to affect tRNA structure, the possibility that nucleotide modifications affect the tRNA in a positive or negative manner is likely. One report suggested that tRNA thiolation of uridines to form 2-thiouridine (s^2U) is a negative determinant for import in *L. tarentolae* (Fig. 5.2; Kaneko et al. 2003; Paris et al. 2009). Using RNA interference (RNAi), we knocked down a key component of the tRNA thiolation pathway in *T. brucei*, Nfs. We showed that although the inhibition of this protein led to a concomitant decrease of $tRNA^{Glu}$ thiolation, it had no effect on the distribution of this tRNA species in vivo or in vitro, suggesting that s^2U is not a negative determinant for tRNA import in *T. brucei* (Paris et al. 2009).

5.4 Concluding Remarks

If history has taught us something it is that with trypanosomatids the only thing that can be expected is often the unexpected. This highlights a more complex truth that reflects the evolutionary position of these medically important single-cell protists, highly divergent from the more commonly studied eukaryotic organisms. In this chapter, we tried our best to highlight recent developments in the field of tRNA biogenesis in trypanosomatids. What should become evident to the reader is that in fact, little is known about most of the events leading to formation of mature tRNAs in these cells, despite much progress made in other systems. One should, however, never discard the importance of digging further into biological processes in organisms that have been a constant source of awesome surprises.

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