Chapter 10
Mitochondrial Translation in Trypanosomatids

Dmitri A. Maslov and Rajendra K. Agrawal

Abstract For a long period of time, the functional properties and the very existence of mitochondrial translation in trypanosomatids remained controversial. The unusual resistance of the system to the known inhibitors of translation, such as chloramphenicol, in conjunction with the extreme hydrophobicity of the translation products appeared to be the main factors that made detection and characterization of this system so difficult. As of today, only two mitochondrial translation products have been reliably identified in *Leishmania tarentolae* and *Trypanosoma brucei*: cytochrome *c* oxidase subunit I (COI) and apocytochrome *b* (Cyb) that are encoded by nonedited and 5′-edited mRNAs, respectively. A large body of circumstantial evidence suggests that the F_1F_0 ATPase subunit A6 and ribosomal protein S12 are also expressed in trypanosomatid mitochondria. The issue of existence of kinetoplast-mitochondrial ribosomes has been addressed lately by the isolation of the 50S monosome particles and reconstruction of their 3D-structure using single-particle cryo-electron microscopy. The overall architecture of these particles strikingly resembles that of eubacterial ribosomes, despite profound differences in the size of ribosomal RNAs and the protein content of these two classes of ribosomes. Evidence begins to accumulate that in order to selectively achieve translation of the fully edited mRNA templates the mitoribosomes are involved in higher-order interactions with mRNA editing and polyadenylation machineries. The pentatricopeptide repeat (PPR) proteins emerge as important participants in these interactions.

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A. Bindereif (ed.), *RNA Metabolism in Trypanosomes*, Nucleic Acids and Molecular Biology 28, DOI 10.1007/978-3-642-28687-2_10,

Keywords Cryo-electron microscopy • Leishmania tarentolae • Mitochondrial translation • Mitoribosome • Pentatricopeptide repeat (PPR) proteins • RNA editing • Trypanosoma brucei

10.1 Introduction

The initial discovery of mitochondrial protein synthesis (or translation) in human beings (McLean et al. 1958) was rapidly followed by several findings of this process in other organisms. The distinct feature of mitochondrial translation that allowed for its clear separation from the translation in the cell cytoplasm was its sensitivity to chloramphenicol and other inhibitors of bacterial protein synthesis, although cytoplasmic translation was insensitive to these inhibitors (Lamb et al. 1968). Conversely, mitochondrial translation was found to be resistant to cycloheximide, an efficient inhibitor of cytoplasmic translation. These observations were consistent with the bacterial origins of mitochondria and were generally regarded as universal. The finding of the separate mitochondrial protein synthesis was rapidly followed by the identification of mitochondrial ribosomes (henceforth referred to as mitoribosomes), first indirectly as membrane-associated ribonucleoprotein granules observed by electron microscopy and later by cosedimentation of ribosomal RNA (rRNA)-containing complexes with nascent polypeptides (reviewed by Curgy 1985). As expected, mitoribosomes proved to be quite different from cytoplasmic ribosomes (O'Brien 2002; O'Brien and Denslow 1996; O'Brien and Kalf 1967a). However, in spite of their likely common origin and the shared sensitivity to certain inhibitors, mitoribosomes apparently diverged from their bacterial counterparts, with changes affecting both their functional properties and the architecture (Borst and Grivell 1971; O'Brien 2003; Pel and Grivell 1994; Sharma et al. 2003; Spremulli et al. 2004). Mitoribosomes are specialized in translation of a limited number of templates (usually under 20) that, with few exceptions, encode integral membrane proteins—subunits of the respiratory chain complexes. The extreme hydrophobicity of these products requires a tight association of mitoribosomes with membranes (Gruschke and Ott 2010; Ott and Herrmann 2010). Apparently in order to accommodate these requirements, the mitochondrial rRNAs, which are invariably encoded by mitochondrial DNA, have undergone a significant reduction in their sizes (Mears et al. 2002, 2006). This loss in rRNA, to some degree, is compensated by a large and diverse set of ribosomal proteins that are encoded in the nucleus, synthesized in the cytoplasm and then imported into mitochondria (O'Brien 2002; Pietromonaco et al. 1991; Smits et al. 2007). Although a large amount of data describing structural and functional aspects of mitochondrial ribosomes from various sources have been accumulated, their detailed structural characterization has been achieved only in a few cases using three-dimensional (3D) cryo-electron microscopy (cryo-EM) (Sharma et al. 2003, 2009). In this chapter, we first present a brief historical account followed by recent developments in the studies of trypanosomatid mitochondrial translation and the machinery involved in the process.

10.2 Protein Synthesis in Kinetoplast-Mitochondria

The early attempts to detect mitochondrial translation in trypanosomatids using the differential sensitivity approach yielded somewhat inconclusive, and often controversial, results. In an insect trypanosomatid, *Crithidia luciliae*, a large amount (up to 50%) of the total protein synthesis was found to be chloramphenicol-sensitive (Laub-Kupersztejn and Thirion 1974). In line with the findings in other systems, this effect was attributed to the mitochondrial protein synthesis. The authors, however, used unusually high (500–800 μg/ml) concentrations of the antibiotic, as in other systems a nearly complete inhibition is achieved usually at much lower concentrations (100 μg/ml). Moreover, the work of Laub-Kupersztejn and Thirion was almost immediately challenged by other group (Kleisen and Borst 1975), which found that only a 2% inhibition of the total protein synthesis in this organism is caused by a similarly high (400 μg/ml) concentration of the drug.

Likewise, in *Trypanosoma brucei*, a strong inhibitory effect of chloramphenicol was observed only at the concentrations exceeding 300 μg/ml; however, this effect was attributed to the secondary effects related to a concomitant inhibition of respiration (Spithill et al. 1981). The lack of a direct inhibitory effect at the lower concentrations was attributed to the membrane permeability barriers; however, even with isolated mitochondria, any significant (~50%) effect on the total protein synthesis was observed only at high concentrations of D-chloramphenicol. Similarly, other bacterial protein-synthesis inhibitors were effective only at high concentrations. The mitochondrial protein synthesis in *T. brucei* was revisited using digitonin-permeabilized cells (Shu and Göringer 1998), and a small (~9%) fraction of the total synthesis was found to be resistant to the effect of cycloheximide. It was further reduced to ~2% by treatment with chloramphenicol at 142 μg/ml, leading to the conclusion that this fraction represented the mitochondrial protein synthesis that was intrinsically sensitive to this antibiotic. This conclusion was reiterated using isolated mitochondria from *T. brucei* that were treated with a low concentration (50 μg/ml) of the inhibitor without additional permeabilization (Nabholz et al. 1999).

The drug permeability issue was also addressed by using digitonin-treated mitochondria from an insect trypanosomatid, *Crithidia fasciculata* (Hill et al. 1975). As is typical for trypanosomatids, this organism was refractory to the presence of chloramphenicol in the medium, although its growth was severely inhibited even at a low (10 μg/ml) concentration of cycloheximide. Accordingly, the incorporation of radiolabeled amino acids in isolated mitochondria was not affected by chloramphenicol, while that in the cytosolic fraction was sensitive to cycloheximide. Remarkably, although the treatment of isolated mitochondria with digitonin stimulated the incorporation of labeled amino acids, this did not cause the organelles to become sensitive to chloramphenicol even at a 250 μg/ml concentration. As a control, the synthesis was shown to be sensitive to puromycin, a structural mimic of the CCA end of an aminoacylated tRNA and the universal inhibitor of translation. The resistance of mitochondrial translation to chloramphenicol in

isolated organelles from *C. fasciculata* was also confirmed later (Tittawella 1998). These results strongly suggested that the lack of sensitivity to chloramphenicol at concentrations effective in other systems was not an artifact, but represented an intrinsic property of the mitoribosomes. This view was further strengthened by the sequence analysis of *T. brucei* and *L. tarentolae* mitochondrial rRNAs (further discussed below) which showed that the peptidyl transferase center in these mitoribosomes was altered in a way that would confer resistance to chloramphenicol (de la Cruz et al. 1985b; Eperon et al. 1983).

Although the contradictory results described above still remain unexplained, the issue of sensitivity or insensitivity of the kinetoplast mitochondrial translation to inhibitors is secondary to the main question about the very existence and uniqueness of the process itself. Because of the failure of the previous approaches to unequivocally address this question, the emphasis over the time has shifted from analysis of the bulk protein synthesis toward the detection and identification of individual proteins encoded by the kinetoplastid mitochondrial genome. Besides the two rRNAs, one each corresponding to two ribosomal subunits, the maxicircles of kDNA in *T. brucei*, *L. tarentolae*, and *C. fasciculata* encode 18 polypeptides. The mRNAs corresponding to 12 of these polypeptides must undergo posttranscriptional editing to various extents ("edited" mRNA) in order to become translation-competent (Arts et al. 1993; Benne et al. 1986; Feagin 2000; Simpson et al. 1998, 2000; Stuart and Feagin 1992). The remaining polypeptides are encoded by mRNAs that do not require editing ("never-edited" or "nonedited" mRNA). A significant body of circumstantial evidence, mainly the fact that edited mRNAs in trypanosomes encode polypeptides homologous to functional mitochondrial proteins in other organisms, indicated that these edited mRNAs are indeed translated (Blum et al. 1990; Feagin et al. 1988; Shaw et al. 1988). Additional evidence included the finding of an immunoreactive polypeptide of the size expected for COII (Breek et al. 1997; Shaw et al. 1989), detection of a subunit with the mass matching that of COII by mass spectrometry analysis of the purified cytochrome *c* oxidase complex (Breek et al. 1997), and induction of an antimycin A resistance mutation in the maxicircle gene encoding apocytochrome *b* (Cyb) mRNA (Schnaufer et al. 2000). However, the direct proof in the form of amino acid sequence was still missing. Furthermore, the anticipated mitochondrially encoded polypeptides could not be detected among subunits of the biochemically purified respiratory complexes (Priest and Hajduk 1992; Speijer et al. 1996, 1997). That was puzzling given the enzymatic activity of the isolated complexes and the crucial role played by their mitochondrially encoded subunits (Berry et al. 2000; Ludwig et al. 2001; Mulkidjanian et al. 2007; Remacle et al. 2008). Therefore, it was hypothesized that an extreme hydrophobicity of the mitochondrial subunits had led to preferential loss of these polypeptides due to aggregation and precipitation, upon disassembly of the respiratory enzyme complexes with SDS (Breek et al. 1997).

The effect of hydrophobicity was employed to identify mitochondrially encoded subunits of cytochrome *c* oxidase and cytochrome bc_1 isolated from mitochondria of *Leishmania tarentolae*. The method is based on the abnormal electrophoretic

mobility of hydrophobic polypeptides in electrophoretic gels; apparently due to a higher SDS content, these polypeptides migrate faster in low-concentration gels as compared to most proteins (Marres and Slater 1977). As a result, in a two-dimensional 9% versus 14% polyacrylamide Tris–glycine–SDS gel, such polypeptides are found as spots located off the main diagonal formed by proteins with normal migration properties. Therefore, even the low-intensity fuzzy spots formed by mitochondrially encoded polypeptides would no longer be obscured by the prominent bands of nuclear-encoded polypeptides of the respiratory enzyme complexes. This approach led to the identification of a series of off-diagonal spots in cytochrome *c* oxidase (Horváth et al. 2000b). The subunit I (COI) polypeptide is encoded by the nonedited mRNA (Simpson et al. 1998). Antibodies raised against an internal peptide inferred from the COI mRNA sequence reacted with some of these spots and this identification was confirmed by partial N-terminal amino acid sequencing. Apparently, a partial oligomerization of the hydrophobic COI polypeptide had occurred upon dissociation of the complex. Another off-diagonal spot was putatively identified by partial sequencing as cytochrome *c* oxidase subunit II (COII). The *L. tarentolae* COII mRNA is minimally edited by insertion of four U-residues in the internal pre-edited site (Shaw et al. 1989) and, therefore, the N-terminal sequence determined is translated from the nonedited segment. A few off-diagonal spots were also detected with cytochrome bc_1 (Horváth et al. 2000a). This complex contained a single, mitochondrially encoded subunit—cytochrome *b* (Cyb), which in *L. tarentolae* is encoded by an mRNA that undergoes 5′-editing by insertion of 39 U-residues at 15 editing sites (Feagin et al. 1988). The anticipated N-terminal amino acid sequence is therefore derived from the edited sequence. The match of the determined Cyb sequence from two of these spots, with the sequence predicted by edited mRNA, was the first direct confirmation of the functionality of RNA editing in trypanosome mitochondria. Again, a partial oligomerization was responsible for multiplicity of spots observed in the gel. Interestingly, in each of the cases described above, the deblocking of the N-terminal methionine was necessary, suggesting that the initiator tRNA was formylated.

The predicted A6 subunit of F_1F_0 ATPase (Bhat et al. 1990) and ribosomal protein S12 (Maslov et al. 1992; Maslov and Simpson 1992), as well as putative subunits of NADH dehydrogenase, still remain to be identified as mitochondrially encoded gene products in trypanosomatids. The latter set represents a more challenging task because the respective complex seems to be relatively low in quantity and high in complexity, as compared to the other respiratory enzymes in trypanosomatids (Čermáková et al. 2007; Fang et al. 2001; Opperdoes and Michels 2008).

The de novo synthesis of COI and Cyb polypeptides was subsequently investigated via labeling of *L. tarentolae* cells with radioactive amino acids in the presence of cycloheximide followed by the 2D gel analysis (Horváth et al. 2002). Several off-diagonal radiolabeled spots were observed, with some of them matching the positions of monomeric COI and Cyb and identified by direct sequencing. The similar set of products was also observed using translation in isolated kinetoplast-mitochondrial fraction. After pulse-chase labeling, radioactive

COI and Cyb polypeptides were incorporated in the respective respiratory complexes, as evidenced by native gel electrophoresis of the detergent-solubilized mitochondrial membranes. Analysis of mitochondrial translation in cycloheximide-inhibited *T. brucei* cells has also revealed several off-diagonal spots (Aphasizheva et al. 2011; Neboháčová et al. 2004). Among the radioactive products observed, the COI product was identified by matching its gel migration with *L. tarentolae* COI and tracing its incorporation in the cytochrome *c* oxidase complex (D. A. Maslov, unpublished observations). The comigration of radiolabeled putative Cyb product with a discrete protein spot, which was identified by mass spectrometry, provided another marker for mitochondrially encoded proteins in *T. brucei* (Neboháčová et al. 2004). As expected, the synthesis of COI and Cyb polypeptides in *L. tarentolae* and *T. brucei* was insensitive to chloramphenicol and other inhibitors at a concentration of 100 μg/ml, but was sensitive to puromycin (Horváth et al. 2002; Neboháčová et al. 2004).

It was noticed that some fully edited mRNA sequences, e.g., pan-edited RPS12 (G6 or CR6) and G4 (CR4) in *T. brucei* (Corell et al. 1994; Read et al. 1992), and G3 in *Phytomonas serpens* (Maslov et al. 1999), contain alternative reading frames encompassing almost the entire mRNA-coding regions but have no sequence homology in the databases. Whether just one or both polypeptides are expressed in each of these cases remains an open question. Nevertheless, it is possible that RNA editing expands the coding capabilities of the trypanosomatid mitochondrial genome, by defining variations in amino acid sequence through alternative editing patterns. Thus, there seem to exist two mature editing patterns for the C terminus of ND3 (CR5) mRNA in *T. brucei* (Read et al. 1994b) and ND9 (G2) in *Leishmania mexicana amazonensis* (Maslov 2010). In *T. brucei*, a partially edited COIII mRNA seems to be translated to produce a protein that is involved in kinetoplast DNA maintenance (Ochsenreiter et al. 2008; Ochsenreiter and Hajduk 2006). However, experimental determination of amino acid sequences of the predicted polypeptides in each of these cases is still missing.

A panel of radiolabeled products and immunoreactive polypeptides with apparently normal gel-migration properties was identified in *Leishmania tropica* to represent several mitochondrial proteins including the components of cytochrome *c* oxidase and cytochrome bc_1 complexes (Goswami et al. 2006; Mukherjee et al. 2007). Given the unusual properties of the *bona-fide* COI and Cyb proteins in the closely related *L. tarentolae*, this identification needs further verification.

10.3 Kinetoplast-Mitochondrial Ribosomes, tRNA, and Translation Factors

Historically, the task of detecting mitoribosomes in other systems was achieved by using their cosedimentation with nascent peptides produced in the presence of cycloheximide (Attardi and Ojala 1971; Brega and Vesco 1971; Greco et al. 1973;

O'Brien and Kalf 1967a; b). The mitochondrial origin of these particles was ascertained by the sensitivity of this labeling to chloramphenicol. In trypanosomatids, however, this approach proved to be unsuccessful, and the nature of mitoribosomes of trypanosomatids remained an open question for a long time. In their pioneer work, Hanas and coworkers (1975) were able to detect 72S particles in a total cell lysate of *T. brucei* that were distinct from the cytoplasmic ~80S ribosomes. The mitochondrial origin of the 72S particles was tentatively deduced from the sensitivity of association of the nascent peptides to chloramphenicol, although a high concentration of the antibiotic was used. In a manner characteristic for mitoribosomes, these particles did not dissociate at low Mg^{2+} concentrations, but their dissociation could be achieved by increasing the K^+ concentration. However, in a later study, at odds with the previous findings, a rather heterogeneous population of the ribonucleoprotein particles, containing the ribosomal small subunit (SSU) 9S and large subunit (LSU) 12S RNAs, was observed in a mitochondrial lysate of *T. brucei* (Shu and Göringer 1998). Among those, the 80S particles were found that apparently contained both rRNAs, and, therefore, they were also the candidate monosomes. These particles dissociated into smaller subcomplexes at a lower Mg^{2+} concentration. Yet in *C. fasciculata*, the ribosomal ribonucleoprotein (RNP) complexes that could be detected by the association with nascent polypeptides were much smaller, about 35S (Tittawella et al. 2003), and, also at odds with the previous report for the same species (Tittawella 1998), this association appeared to be sensitive to chloramphenicol. The reasons for these irreconcilable findings remain unclear.

Besides these results, the earlier reports described the chloramphenicol-sensitive 68S ribosomes in *Crithidia oncopelti* (Zaitseva et al. 1977) and 60S ribosomes in *C. luciliae* (Laub-Kuperszteijn and Thirion 1974). More recently, 75S mitoribosomes were reported from *L. tropica* and *L. donovani* (Maarouf et al. 1995). From their sensitivity to bacterial inhibitors of protein synthesis and the dissociation of their subunits at lower Mg^{2+} concentrations, these ribosomes showed similarity to their bacterial counterparts. In none of these cases, however, the characterization of the isolated particles was sufficiently detailed, while the case of *C. oncopelti* was additionally complicated by the presence of intracellular bacterial endosymbionts (Chang 1974).

In *L. tarentolae*, particles with morphologies reminiscent of the typical monosomes and their small and large subunits were detected by electron microscopic analysis of a mitochondrial lysate (Scheinman et al. 1993). Subsequently, analysis of the sedimentation profiles of the SSU 9S and LSU 12S rRNAs in the gradients was used to detect and isolate ribosomal particles in a detergent lysate of the mitochondria (Maslov et al. 2006). The most abundant of these, termed 45S SSU*, could be purified to near homogeneity. They were shown to contain only the 9S rRNA and nearly 50 proteins, including several universal homologs of small subunit ribosomal proteins, such as S5, S6, S9, S11, S15–S18 and MRPS29 (Maslov et al. 2007). Besides these, there were several pentatricopeptide repeat (PPR)-containing proteins that in other systems are known to participate in various aspects of mRNA maturation (Delannoy et al. 2007; Schmitz-Linneweber and

Small 2008). As determined by electron microscopy analysis, the 45S SSU* particles displayed an unusual bilobed morphology. The current view is that they represent a mixture of homodimers of the 30S SSU and heterodimers of the 30S SSU and an unknown protein complex of about the same size as the 30S SSU. The function of these usual complexes remains unclear. The presence of the PPR proteins among its components suggests that these complexes may participate in sequence-specific mRNA recognition for translation, or in the biogenesis of the ribosomes.

The pool of the free 30S SSU particles was relatively small (Maslov et al. 2006, 2007). On the contrary, the pool of the free LSU was large with the majority of the 12S rRNA found within these 40S particles. The typical LSU morphology of these complexes was easily recognizable by transmission electron microscopy. Both 45S SSU* and 40S LSU complexes tend to dimerize, adding to the multiplicity of the ribosomal RNP complexes present in the mitochondrial lysate.

There was no prominent peak of the monosomes that could be expected to contain both rRNAs with an equimolar ratio. The only candidate for this role was the 50S complex that was obscured in the gradients by the peaks of more abundant 40S LSU, 45S SSU* complexes and their dimers. However, electron microscopy analysis of the 50S material clearly revealed the presence of the characteristic monosome-like particles in this fraction.

The subsequent detailed structural analysis of these particles by single-particle 3D cryo-EM (Sharma et al. 2009) revealed that the overall morphology and dimensions of the *Leishmania* mitoribosome (~245 Å in diameter; henceforth referred to as Lmr) are more similar to those of the 70S eubacterial ribosome (~260 Å) rather than to its mammalian mitochondrial counterpart (~320 Å). The common morphological features, such as the body, the head, and the platform of the small subunit, and the central protuberance and the side-stalks of the large subunit are obvious in Lmr (Fig. 10.1; for a detailed comparison with eubacterial and mammalian mitochondrial ribosomes, see Agrawal et al. 2011). Such a conventional morphology of Lmr was unanticipated given the large differences in the composition of Lmr and bacterial ribosomes. For example, the L1-like stock is obvious in the structure, although there is no protein homologous to eubacterial L1. Moreover, the sizes of the small (9S) and large (12S) rRNAs are greatly (by more than 60%) reduced compared with *E. coli* (de la Cruz et al. 1985a, b; Eperon et al. 1983). In *L. tarentolae* these RNAs are only of 610 and 1,173 nt, respectively, and these are even smaller than the rRNAs in mammalian mitochondria, which are among the smallest (12S for the SSU and 16S for the LSU rRNA). A superimposition of the secondary structures of the *Leishmania* mitochondrial and bacterial rRNA molecules (Figs. 10.2a, b) shows that this reduction in sizes was due to the absence of several stem–loop segments in the former. Thus, the 9S SSU rRNA does not contain 24 out of 45 stem–loops present in the *E. coli* structure. In the 12S LSU rRNA only 42 helical regions are preserved out of 101 such regions of *E. coli*. The entire Domain I is absent, while Domain III is greatly reduced. Domains II, IV, and VI (in particular, the linker to its α-sarcin/ricin stem–loop) are also reduced in size. Only Domain V, which forms the peptidyl-transferase

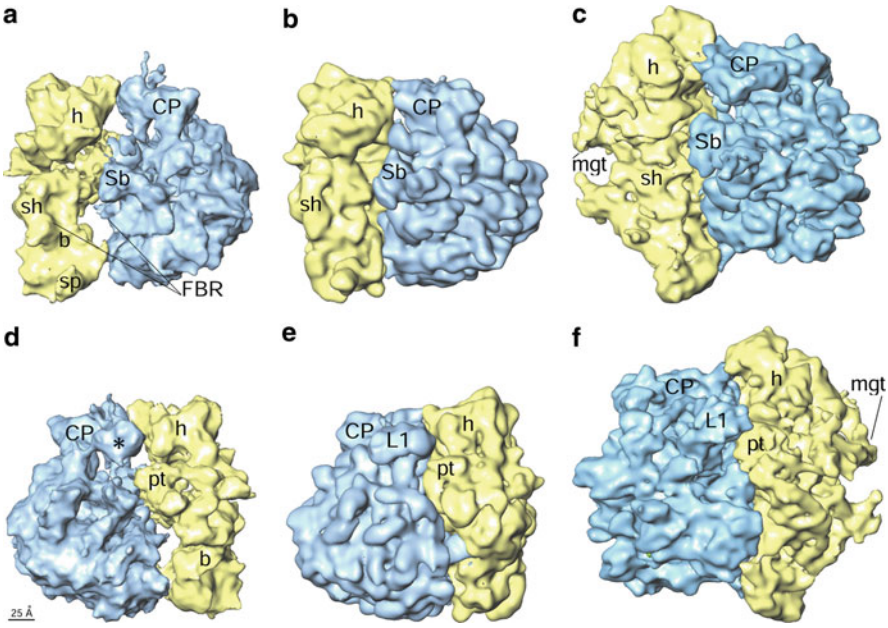

Fig. 10.1 Three-dimensional cryo-electron microscopic map of the *Leishmania* 50S mitoribosome (Lmr) (**a**, **d**) (Sharma et al. 2009) and its comparison with the *E. coli* 70S ribosome (**b**, **e**) (Gabashvili et al. 2000) and the bovine 55S mitoribosome (**c**, **f**) (Sharma et al. 2003). **a**, **b**, **c**—view from the L7/L12 stalk side; **d**, **e**, **f**—view from the L1 side. The small and large ribosomal subunits are depicted in *yellow* and *blue* colors, respectively. Landmarks: *b* body, *CP* central protuberance, *FBR* factor binding region, *h* head, *mgt* mRNA gate (in the bovine mitoribosome), *pt* platform, *Sb* L7/L12 stalk base, *sp* spur, *asterisks* the density in Lmr that substitutes the missing L1 protein

center, is relatively well preserved in the 12S rRNA. With respect to ribosomal proteins, the 50S Lmr also contains 10 proteins that are homologous to eubacterial proteins in the SSU (out of 20 eubacterial proteins) and 21 homologs in the LSU (out of 34 eubacterial proteins). The missing RNA segments (Fig. 10.2) and proteins are only partially (by 50–60%) replaced by the *Leishmania*-specific ribosomal proteins. The result of such an incomplete compensation is a highly porous ribosome structure with several noticeable tunnel-like features transversing the body, the platform, and the head of the SSU, as well as body and the base of central protuberance of the LSU.

Although the recent proteomics analyses of mitoribosomal fractions in *L. tarentolae* (Maslov et al. 2006, 2007) and *T. brucei* (Zíková et al. 2008) failed to detect the predicted ribosomal protein S12 (Maslov et al. 1992; Read et al. 1992), the cryo-EM structure clearly shows the presence of a corresponding protein mass within the SSU of Lmr. A likely reason for the absence of the S12 peptides among the proteolytic digestion products might be a tight association of this protein with the 9S rRNA.

The intersubunit space in Lmr contains a large gap between the SSU and LSU bodies. This gap is mainly due to the absence of a large part of the SSU rRNA helix

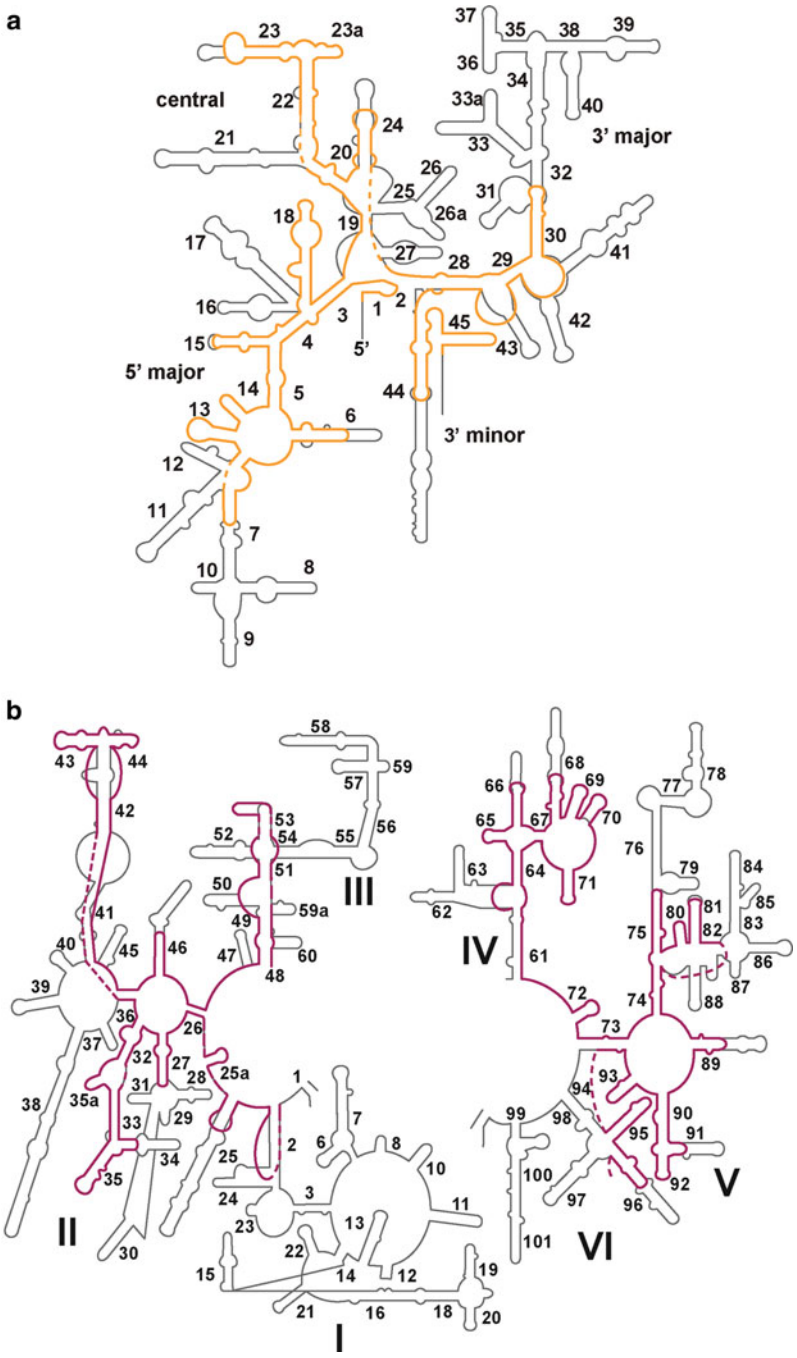

Fig. 10.2 Secondary structure of the trypanosomatid ribosomal RNAs (Sharma et al. 2009). (**a**) Small subunit ribosomal RNA: the structure of *L. tarentolae* 9S rRNA (*orange*) superimposed on the structure of a bacterial 16S rRNA (*gray*). The numbers identify the 16S rRNA helices.

44 (h44) which in eubacterial ribosomes forms much of the SSU:LSU interface in this region. Also, the remaining part of h44 is slightly shifted in Lmr. There are fewer intersubunit bridges in Lmr (nine) compared to eubacterial ribosomes (thirteen) (Yusupov et al. 2001) or mammalian mitochondrial ribosomes (fifteen) (Sharma et al. 2003), and those which are present include only three RNA–RNA contacts, with others being either an RNA–protein or a protein–protein contact.

Several features of the Lmr structure clearly suggest that its *modus operandi* is different from that of eubacterial counterparts. For example, the mRNA channel along the SSU head appears to be lined with Lmr-specific proteins, and the mRNA entrance is rather wide. Although the identities and exact roles of the participating putative proteins remain unknown, it has been suggested that these features reflect the novel modes of recognition of the mRNA by the ribosome, and interactions with specific translation factors (Sharma et al. 2009). It should be noted that the mRNA path in mammalian mitoribosomes is also protein-rich (Sharma et al. 2003).

Furthermore, there appears to be greater contributions of Lmr-specific proteins in the formation of tRNA-binding sites, since several of the eubacterial rRNA segments that form the aminoacyl (A) and peptidyl (P) sites in eubacterial ribosomes are absent in Lmr. The E site seems to be entirely absent in the Lmr. There is also a protein mass that extends from the central protuberance of LSU into the intersubunit space adjacent to A and P sites (referred to as A- and P-site finger or APSF, Fig. 10.3). A similar structure is observed in the mammalian mitoribosome, but there it is smaller, extending only up to the P-site (PSF) (Sharma et al. 2003). It should be noted that, unlike many other mitochondrial systems in which tRNAs are encoded by mitochondrial DNA, all tRNAs in trypanosomatid mitochondria are imported from the cytoplasm and the same tRNAs are also involved in protein synthesis by the cytoplasmic ribosomes (Hancock and Hajduk 1990; Schneider et al. 1994; Shi et al. 1994; Simpson et al. 1989). This includes the mitochondrial initiator tRNA which represents the cytoplasmic elongation Met-tRNA$_m$. When formylated, this tRNA is used for initiation (fMet-tRNA$_i$), and when left unformylated, the same tRNA is used for elongation (Tan et al. 2002). It is conceivable that a set of Lmr-specific proteins has evolved to allow for accommodation of the typical set of eukaryotic tRNAs and the proper discrimination between the two types of Met-tRNA. The homolog of initiation factor IF-2, which brings fMet-tRNA$_i$ to the ribosome, is identifiable in the trypanosomatid genomes (tritrypdb.org/), although it is rather diverged. Homologs of IF-1 and IF-3 are not detectable, further testifying to the peculiarity of the initiation of translation in this system.

Fig. 10.2 (continued) (**b**) Large subunit ribosomal RNA: the *L. tarentolae* 12S rRNA (*purple*) superimposed on a bacterial 23S rRNA (*gray*). The numbers identify the 23S rRNA helices, while the secondary structure domains are identified with *roman numerals*. *Dashed lines* represent the regions with unassigned secondary structure

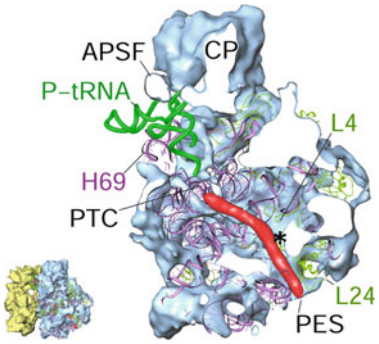

Fig. 10.3 The cut-away view of the 3D cryo-EM structure of the large subunit of the *Leishmania* mitoribosome (Sharma et al. 2009). *White* regions represent the cutting plane. The view is from the L7/L17-stalk side, with the small ribosomal subunit interface to the *left*. Position of the polypeptide-exit tunnel is depicted by docking a low-resolution model of a polypeptide chain (*red*). P-site is *highlighted* by docking a tRNA model (P-tRNA). *APSF* A- and P-site finger, *PES* polypeptide-exit site, *PTC* peptidyltransferase center. The *asterisk* denotes the location of polypeptide accessible site (PAS), which is situated slightly behind the cutting plane. L4 and L24—ribosomal proteins

Besides the canonical AUG initiation codon, which is observed in the majority of cases, trypanosomatids appear to utilize several noncanonical codons for translation initiation. These were predicted initiation codons found in homologous positions for the start codons in multiple alignments. For example, the ORF present in the fully edited RPS12 mRNA sequence from *L. m. amazonensis* contains isoleucine codon Auu (with low case u's added by editing) instead of AUG (Maslov 2010). The ORFs in fully edited sequences of G3 mRNA from *L. amazonensis* and *P. serpens* begin with another isoleucine codon AuA, although there is also an in-frame AuG downstream in the *L. amazonensis* sequence (Maslov et al. 1999; Maslov 2010). There are other cases of predicted noncanonical codons, including the leucine codon UUG (Corell et al. 1994; Read et al. 1994b). In none of such cases, however, was the utilization of the noncanonical codons for initiation verified experimentally. Another codon reassignment is utilization of UGA (along with UGG) for tryptophan, as in many other mitochondrial systems. This is achieved by a C-to-U editing of the CCA anticodon in Trp-tRNA to produce the UCA anticodon that serves to recognize both codons (Alfonzo et al. 1999; Gaston et al. 2007).

The structure corresponding LSU rRNA helix 95 (H95), also known as α-sarcin/ricin stem–loop, is well conserved in the 12S rRNA structure. This rRNA region is known to interact with elongation factors (Moazed et al. 1988), apparently to trigger GTPase activities of the EF-Tu:GTP:aa-tRNA ternary complex, or in the EF-G:GTP complex. However, the spatial position of H95 in Lmr is significantly different from eubacterial ribosome, being shifted by ~30 Å toward the peptidyl-transferase center (Sharma et al. 2009). Perhaps reflecting this, the amino acid sequence of the trypanosomatid mitochondrial EF-Tu (identifiable in the trypanosomatid genomic databases) contains an insertion that might play an accommodating role in its

interactions with Lmr. The homologs of elongation factors EF-Ts and EF-G are also quite distinct in trypanosomatids.

As other mitochondrial ribosomes, Lmr is expected to interact with the inner mitochondrial membrane. The base of the body in the SSU and the nascent polypeptide exit tunnel (PES, polypeptide exit site) of the LSU contain Lmr-specific proteins, reflecting peculiar aspects of these interactions (Fig. 10.3). Yet, the universal features are also observed in trypanosomatids; the homologs of yeast insertase Oxa1, a member of the YidC/Alb3/Oxa1 family of integral membrane proteins interacting with ribosomes close to PES and mediating cotranslational insertion of newly synthesized polypeptides (Stuart 2002), are conserved in trypanosomatids, and so are ribosomal proteins L22, L23, L24, and L40 that participate in forming the PES opening (Nissen et al. 2000; Ott and Herrmann 2010). In addition, the homologs of two membrane-bound proteins Mdm38/LETM1 and Cox11 that mediate ribosome attachment (Herrmann and Neupert 2003; Ott and Herrmann 2010) are also present in trypanosomatid databases. However, the yeast ribosome receptor Mba1 that binds to the LSU next to PES (Ott et al. 2006) is not. An additional interesting feature in Lmr is the extra opening of the nascent peptide tunnel, termed PAS (polypeptide-accessible site). As also observed in mammalian mitoribosomes (Sharma et al. 2003), PAS is located ~25 Å away from the conventional opening (PES) on the solvent side, nearly opposite to the interface with the SSU. The exact function of PAS is unclear, but it is conceivable that this additional opening plays a role in cotranslational insertion of nascent polypeptides into the membrane.

10.4 Higher-Order Interactions of Mitochondrial Ribosomes and the mRNA Recognition Problem

The kinetoplastid mitochondrial ribosome faces a challenging task of recognizing the correct initiation codon in a fully edited and nonedited mRNA while discriminating against relatively abundant pre-edited and partially edited transcripts. In trypanosomes, some initiation codons are created by editing (e.g., pan-edited ND8 in *L. tarentolae*) and others are encoded (e.g., pan-edited G4 mRNA in *L. tarentolae*) (Fig. 10.4) (Gao et al. 2001). Moreover, the correct initiation codon can be preceded by an upstream codon to be avoided, as in the ND8 example above. Finally, the typically short 5′ untranslated regions do not possess discernable universal sequences such as the Shine-Dalgarno element. They are 5′ monophosphorylated and uncapped. Hence, the cap-dependent ribosome recruitment mechanism (Campbell et al. 2003) does not operate in trypanosome mitochondria. The mammalian mitochondria mode of initiation, in which the 5′-untranslated regions are short and the most 5′-proximal AUG (or AUA) serves for initiation (Spremulli et al. 2004) is not directly applicable either. The codon recognition problem may look more similar to that in mRNA of yeast which contain

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Pan-edited ND8 mRNA (520 nt, encodes 145 aa product)
pre-ed: 5′-UAAACAUAUAUAAUGUAUUAGAUUAAAAGUAA G   G   A GA     G UUUU...
edited: 5′-UAAACAUAUAUAAUGUAUUAGAUUAAAAG*AAuGuuuGuuuAuGAuuuuuGuUUUU...
                                         M  F  V  Y  D  F  C  F   ...

Pan-edited G4 mRNA (537 nt, encodes 169 aa product)
pre-ed: 5′-CAAAUACAUUAAAACACAUAUAUGUAUAAUUGUAAAAG G  G  G     GG        ...
edited: 5′-CAAAUACAUUAAAACACAUAUAUGUAUAAUUGUAAAAGuGuuGuuGuuuuuGGuuuuuuu...
                               M  Y  N  C  K  S  V  V  V  F  G  F  F  ...
```

Fig. 10.4 Partial sequences of fully edited ND8 and G4 mRNA and the inferred amino acid sequences in *L. tarentolae* LEM125. Encoded nucleotides are shown with *upper-case letters*, inserted uridylates with *lower-case* u's and a deleted uridylate with an *asterisk*. The initiation codons are *underlined*. The AUGs *highlighted* in *yellow* point to AUG codons that are to be avoided

long 5′ UTR in which the initiation codon may be preceded by out-of-frame AUGs (Dunstan et al. 1997; Fox 1996; Green-Willms et al. 1998). The logistic requirement to coordinate the initiation at the proper codon with completion of the U-insertion/deletion editing downstream must also be accommodated. That poses a problem of signaling between these two events since editing does not necessarily affect the sequences at the very 5′ end. This is exemplified by the G4 mRNA when the same pre-existing AUG codon needs to be avoided in the pre-edited mRNA, but recognized in the fully edited mRNA (Fig. 10.4).

The initiation codon recognition in yeast mitochondria is aided by highly specific translation activators that interact with the mRNA's 5′ UTR (Green-Willms et al. 2001; Sanchirico et al. 1998; Siep et al. 2000). The COX1 mRNA is recognized by TPR/PPR protein Pet309, the COX2 mRNA by Pet111, and the COB mRNA by a set of several activators. The translation activators represent peripheral or integral membrane proteins that are assembled into a large (~1 MDa) complex tethered to the inner mitochondrial membrane, thereby also bringing the mRNA close to the membrane (Krause et al. 2004; Naithani et al. 2003). However, the yeast mRNA activators are not evolutionally conserved. Therefore, the mRNA recognition factors, if there are any in trypanosome mitochondria, must be trypanosome-specific.

Besides the 5′ end, the *cis*-signals identifying translatable (fully edited and nonedited) mRNA may be located at the 3′ end. Two types of 3′-end tails had been found in trypanosomatid mitochondrial transcripts: the shorter 20–30 nt and, the longer, 200–300 nt tails (Bhat et al. 1991, 1992). The tails' lengths correlate with the editing status of an mRNA: the short tails are found in pre-edited and partially edited mRNA, whereas the long tails are observed in the fully edited type, as well as in non-edited transcripts (Bhat et al. 1992; Etheridge et al. 2008; Militello and Read 1999; Read et al. 1994a). The longer tail represents an extension of the short tail by addition of an A/U heteropolymer upon completion of editing. It was suggested that the long tail represents a "hallmark of translationally competent mRNA" (Etheridge et al. 2008). This hypothesis was verified in a more recent work (Aphasizheva et al. 2011). The long-tail extensions were shown to be produced by the concerted actions of KPAP1 poly(A) polymerase and RET1 terminal uridylyl transferase directed by two PPR proteins termed KPAF1 and 2. Ablation of these

proteins in procyclic trypanosomes resulted in a cessation of the long-tail formation and an inhibition of the de novo protein synthesis. In bloodstream trypanosomes that lack cytochromes and oxidative phosphorylation, the transcripts for subunits cytochrome *c* oxidase and cytochrome bc_1 are devoid of long tails, and de novo synthesis of the corresponding subunits could not be detected. The latter observation has also been reported recently by Richterová and coworkers (2011). However, translation is still expected to occur in bloodstream trypanosomes, at least of the A6 mRNA, which is productively edited and A/U tailed in this stage (Cristodero et al. 2010; Hashimi et al. 2010; Schnaufer et al. 2005).

The results described above have established the long poly(A/U) tails as the general *cis*-elements that identify fully edited mRNAs as templates suitable for translation. The question is still open as to what mediates the interaction between the long-tailed mRNA and the mitoribosomes. Are mRNA-specific factors involved as well? PPR proteins have emerged lately as a class of specific sequence-recognition factors that mediate various aspects of mRNA processing, editing, and translation in plants (Schmitz-Linneweber and Small 2008). Although not so abundant in other organisms, they have also been shown to play similar roles (Delannoy et al. 2007; Lightowlers and Chrzanowska-Lightowlers 2008). In this respect, it is remarkable that trypanosomes contain more than 30 PPR proteins, noticeably more than any other investigated nonplant organism (Mingler et al. 2006; Pusnik et al. 2007). It is conceivable that this abundance is related to a higher complexity of mRNA recognition problem in trypanosomatid mitochondria that is imposed by high rate of RNA editing as compared to that in yeast or mammalian systems. Several PPR proteins were identified in *L. tarentolae* as part of the 45S SSU* complexes (Maslov et al. 2007). It should be noted however that these particles may actually represent a part of a larger complex that is not stable enough to withstand a rigorous isolation procedure. Thus, RNAi-based depletion of several additional PPR proteins in *T. brucei* resulted in a reduction of the 9S or 12S rRNA levels suggesting that these proteins also associate with the ribosomes (Pusnik et al. 2007). This view was further strengthened by the observation that ablation of some of these proteins in turn leads to the degradation of others, as expected, if they are components of the same complex. A relatively gentle affinity isolation procedure using ribosomal proteins S17 and L3 as TAP-fusion "baits" resulted in identification of a large number (nearly 140) of protein components associated with ribosomal particles in *T. brucei*, including 16 PPR proteins (Zíková et al. 2008). This list has recently been extended to 21 ribosomal PPR proteins out of 36 proteins found by database mining and the total list of proteins associated with the mitoribosomes to nearly 300 (Aphasizheva et al. 2011). While the function of the ribosome-associated PPR proteins is unknown, it is conceivable that some of the PPR proteins represent mRNA-specific activators. The long list of mitoribosome-associated proteins suggests that a multitude of tertiary and quaternary protein–protein contacts are made by these particles. However, most of these ~300 proteins do not form integral components of the mitoribosome itself, but may directly or indirectly interact with the mitoribosome as components of the

translation activator complexes, polyadenylation/polyuridylation complexes, or RNA-editing complexes.

The notion that the translating ribosome is substantially larger than the 50S "core" Lmr is further supported by the finding of >70S ribosomal complex in mitochondrial lysates of *T. brucei* (Aphasizheva et al. 2011). This complex represents only a fraction of the total rRNA-containing particles, which are broadly distributed across the gradients with the bulk of them found in the 55–65S range. The >70S complexes stand apart because they cosediment with translatable fully edited mRNA identifiable by the presence of the long 3′-end poly(A/U) tails. Moreover, the same RNP fraction also contains the functional tRNA identifiable as those with repaired CCA-ends (Alfonzo and Soll 2009). The direct association of the tRNA and the large ribosomal RNP was demonstrated by affinity pull-down. However, the physical interaction of the long-tailed mRNA with these complexes was more difficult to demonstrate, as these interactions did not withstand lengthy isolation procedures, and even a relatively gentle TAP method or immunoprecipitation from the gradient fractions resulted in a loss of the associated mRNA (Aphasizheva et al. 2011). However, by applying a rapid cryogenic homogenization followed by affinity pull-downs of the TAP-tagged ribosomes, it was possible to demonstrate a preferential association of the long-tailed mRNA with the small subunits, and that of pre-edited mRNA, as well as components of the edited machinery, with the large subunit (Aphasizheva et al. 2011). Although most of the details of these interactions still need to be uncovered, the emerging view is that of a highly integrated system, in which the process of mRNA maturation is tightly coupled with translation.

Acknowledgments The work in the authors' laboratories has been supported by National Institutes of Health grants AI088292 (to DAM) and GM61576 (to RKA). We thank R. Aphasizhev and L. Simpson for the critical comments on the manuscript.

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