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RNA Polyadenylation and Decay in Mitochondria and Chloroplasts

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Mitochondria and chloroplasts were originally acquired by eukaryotic cells through endosymbiotic events and retain their own gene expression machinery. One hallmark of gene regulation in these two organelles is the predominance of posttranscriptional control, which is exerted both at the gene-specific and global levels. This review focuses on their mechanisms of RNA degradation, and therefore mainly on the polyadenylation-stimulated degradation pathway. Overall, mitochondria and chloroplasts have retained the prokaryotic RNA decay system, despite evolution in the number and character of the enzymes involved. However, several significant differences exist, of which the presence of stable poly(A) tails, and the location of PNPase in the intermembrane space in animal mitochondria, are perhaps the most remarkable. The known and

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predicted proteins taking part in polyadenylation-stimulated degradation pathways are described, both in chloroplasts and four mitochondrial types: plant, yeast, trypanosome, and animal.

Abbreviations

PAP	poly(A) polymerase
PNPase	polynucleotide phosphorylase
rNTr	ribonucleotidyl transferase
RNase E	ribonuclease E
RNase J	ribonuclease J

I. Introduction

Chloroplasts and mitochondria originated from what are considered to be the most successful symbiotic events to have occurred over the last 1.5 billion years (*1*–*3*). It is assumed that the chloroplast originated from a cyanobacterial ancestor, while mitochondria arose from a α-proteobacterium. In both symbiotic events, a prokaryotic organism entered the eukaryotic precursor and this was followed by extensive gene transfer from the organelle to the nuclear genome. Today, of the thousands of proteins present in organelles, only a very limited number remain encoded by the organellar genome. For example, only 13 proteins are encoded in the human mitochondrial genome, and about 90 in the chloroplasts of *Arabidopsis*. Still, organelles harbor a complete gene expression system, which includes DNA replication and maintenance, transcription, posttranscriptional, translational, and posttranslational activities, *albeit* much of it nucleus-encoded. The posttranscriptional components include splicing, editing, 3′, 5′, and intercistronic processing, and the addition of stable poly(A) tails in the case of animal mitochondria. Several of these events can modulate RNA half-life, which is a very significant factor in the regulation of organellar gene expression (*4, 5*).

This chapter primarily concerns the polyadenylation and degradation of organellar RNAs, as it is a central organellar process derived from prokaryotes. However, a special characteristic of animal and trypanosome mitochondria is also discussed, namely the presence of stable poly(A) tails, which have not been found in prokaryotes. The enzymes involved in RNA degradation, including exo and endoribonucleases will be described, as well as the various incarnations of the polyadenylation-stimulated degradation pathway found in organelles and prokaryotic organisms.

II. Polyadenylation of RNA

A. The Stable Poly(A) Tail of Nucleus-Encoded mRNA

The addition of a stable poly(A) tail to the 3′ end of nucleus-encoded mRNA (excluding histone mRNAs) is a well-defined and long-known phenomenon in eukaryotes (Fig. 1) (*6*). Historically, the observations that mRNA was mostly retained on an oligo(dT) column, and that cDNA could be obtained by reverse transcription of total RNA using an oligo(dT) primer, paved the way for the discovery of a stable poly(A) tail at the 3′ end. Further biochemical analysis showed that following transcription by RNA polymerase II, mRNA is cleaved and polyadenylated by a high molecular weight complex consisting of several proteins. The stable poly(A) tail functions in the transport of mRNA from the nucleus to the cytoplasm and in translation initiation. In addition, it is significantly shortened during the initial steps of RNA degradation (*7–10*). However, whether or not it is required for stability and/or determines the half-life of the transcript is still a matter of debate. It is assumed that in the nucleus and cytoplasm, the stable poly(A) tail is fully bound by the poly(A) binding protein (*11*) (Fig. 1).

B. The Polyadenylation-Stimulated Degradation Pathway

In a somewhat opposing manner to the function of the stable poly(A) tail of nucleus-encoded mRNA, the prokaryotic/organellar poly(A) tail usually functions to tag the RNA molecule for rapid exonucleolytic degradation. This phenomenon was first identified in *E. coli* (see Chapter in this volume by Hajnsdorf and Regnier), but is now well-known in all kingdoms of life including

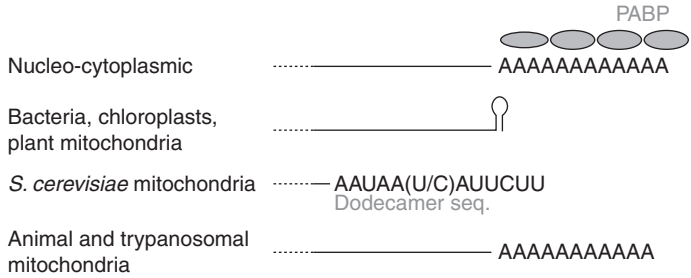

FIG. 1. The 3′ ends of mature mRNAs in various systems. The details of each are discussed in the text. "Dodecamer seq." is an encoded tag found at the end of yeast mitochondrial transcripts.

prokaryotes, archaea and organelles, and the nucleus of eukaryotic cells. Indeed, it has been found in all organisms analyzed to date, excluding the few that are described below.

In bacteria and chloroplasts, the initial event in the mRNA degradation pathway is generally thought to be an endonucleolytic cleavage (Fig. 2), preceded in some cases in *E. coli*, and probably other bacteria, by the RppH-catalyzed removal of 5′ pyrophosphate (*12, 13*). Following the initial cleavage, a wave of endonucleolytic cleavages may degrade the RNA into many fragments. These fragments are then digested by exonucleases, with or without preceding polyadenylation (Fig. 2). The different endoribonucleases that may be involved are described below. Because in some situations none of these candidate endoribonucleases have been found, for example, in plant and human mitochondria, it may be that the endonucleolytic cleavage does not take place. In these cases, RNA degradation may begin directly at the polyadenylation/exonucleolytic step (*14*).

As mentioned earlier, following endonucleolytic cleavage the RNA fragment can be polyadenylated and exonucleolytically degraded (Fig. 2). Therefore, unlike stable nuclear poly(A) tails, the polyadenylation in this context is transient. Accordingly, progressive RT-PCR amplification methods are required to detect these tails. The enzymes performing the polyadenylation step are polynucleotide phosphorylase (PNPase) and several poly(A) polymerases (PAPs) of the nucleotidyltransferase (Ntr) family. The tails can be either homopolymeric, composed exclusively of adenosines (A), or heteropolymeric, composed of the four nucleotides, with adenosine being the most abundant (poly(A)-rich tails) (Fig. 3). "Chimeric" tails where part is heteropolymeric and part homopolymeric, were also recently observed in the chloroplasts of different plants (Larum and Schuster, manuscript in preparation). Generally, homopolymeric tails are produced by PAP, while heteropolymeric tails result from PNPase activity functioning in synthetic rather than degradation mode. The pervasiveness and transience of such poly(A) tails is remarkable, and raises the question of why RNA fragments are elongated as a prelude to their degradation.

The answer is that unstable poly(A) tails are believed to serve as a platform or runway for exonucleases to bind the 3′ end of the RNA and degrade it in the 3′–5′ direction. It is very likely that the addition of the tail enables the exonuclease to digest RNA even with stem-loops and other structures that normally function as efficient barriers to exonucleases. It is also possible that this step is not built on a single polyadenylation and processive exonucleolytic degradation event, but rather on repeated cycles of polyadenylation and degradation. That is, whenever a normally processive exoribonucleases is stalled by an RNA structure, it dissociates and a new polyadenylation event occurs, adding a platform for a new molecule of the exonuclease, and perhaps modifying the secondary structure in order to weaken it.

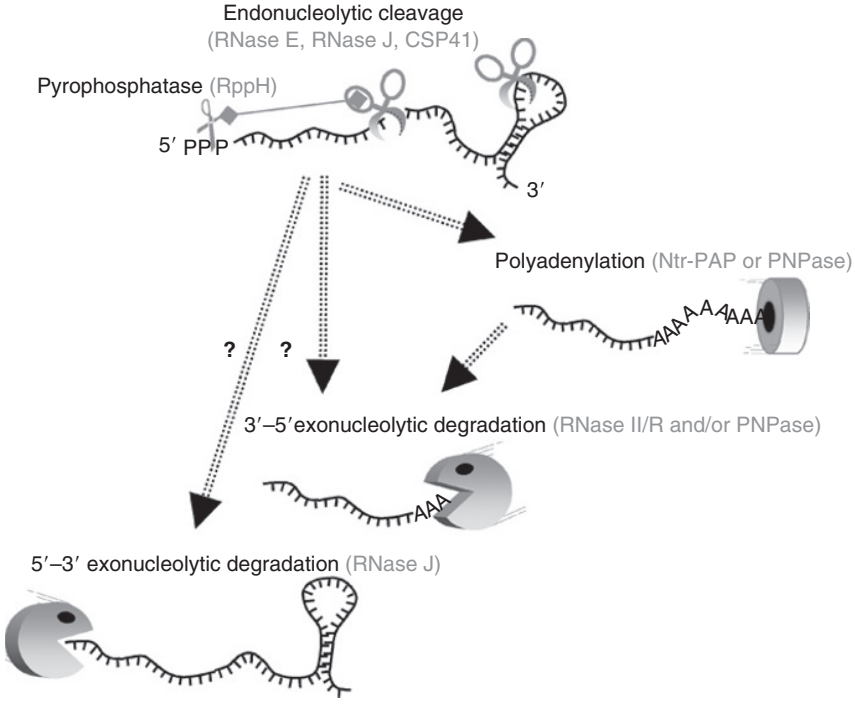

FIG. 2. The polyadenylation-stimulated RNA degradation pathway. The stages of polyadenylation-stimulated RNA turnover are: (A) endonucleolytic cleavage, (B) polyadenylation, and (C) exonucleolytic digestion. The first endonucleolytic cleavage is believed to be performed by RNase E in *E. coli* and related bacteria. In *E. coli*, it was recently shown that the removal of 5′ end pyrophosphate by RppH in some cases precedes and stimulates the RNase E cleavage. RNase J has been implicated in this function in *Bacillus subtilis*. CSP41 is an endonuclease present in the chloroplast and may be also involved in the initial cleavage. The polyadenylation is performed by Ntr-PAP, producing homopolymeric poly(A) tails or by PNPase, producing heteropolymeric poly (A)-rich tails. In hyperthermophilic and several methanogenic archaea, the heteropolymeric tails are synthesized by the archaeal exosome. The 3′–5′ exonucleolytic degradation step is carried out by PNPase and RNase II/R in bacteria and organelles. Dashed lines with a question mark indicate possible pathways and shortcuts that yet have to be shown to take place. The 5′–3′ exonucleolytic degradation is predicted to be carried out by RNase J in organisms in which it is present.

C. Is Polyadenylation A Required Step in The Degradation Pathway?

The response to this question seems to be a bit complicated. On the one hand, in organisms where the polyadenylation-stimulated degradation pathway takes place, it is very difficult to knock out the polyadenylating enzymes while still retaining viability. For example, attempts to knock out both PNPase and

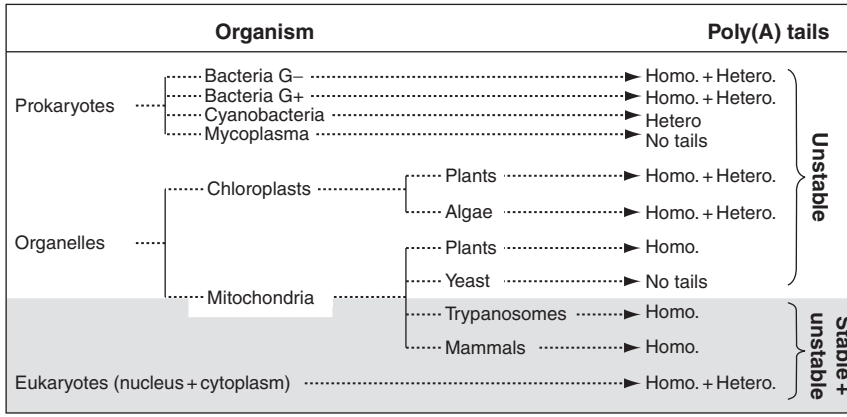

FIG. 3. Poly(A) tails in different organisms and organelles. The distribution of homopolymeric (Homo.), heteropolymeric (Hetero.), stable, and unstable tails between different organisms and organelles. "No tails" indicates that no polyadenylation takes place in the organism/organelle.

PAP in *E. coli* resulted in a significantly slower growth rate and a high rate of reversion (*15*). Therefore, temperature sensitive mutants are often used (*16*). Deletion of the only polyadenylating enzyme, PNPase, in cyanobacteria, resulted in a lethal phenotype (*17, 18*). While in *B. subtilis* there is only one gene encoding an Ntr type protein, which has been shown not be active as PAP, both hetero and homopolymeric poly(A)-tails are present (*19, 20*). A PNPase deletion mutant is viable, and the tails in this strain are homopolymeric (*20*). Therefore, if indeed the *B. subtilis* Ntr protein is not PAP, a new type of PAP that is not a member of the Ntr family, could be hypothesized. In addition, the inhibition of polyadenylation in a lysed-chloroplast system resulted in the accumulation of endonucleolytic cleavage products, a result that is similar to that obtained by the inhibition of exoribonucleases (*21*). Taken together, this limited set of experimental data suggests that in organisms and organelles in which poly(A)-stimulated degradation pathway takes place, its absence or inhibition leads to lethality or a growth defect.

On the other hand, several organisms and organelles have been described in which RNA is degraded without polyadenylation. These include yeast mitochondria, halophilic, and several methanogenic archaea, and the small-genome parasitic bacterium *Mycoplasma* (Fig. 3) (*14, 22–25*). In these systems there may be a more pronounced function of RNA helicases, which would fulfill the role of destabilizing secondary structures. It is an interesting question as to whether during evolution these organisms/organelles lost the polyadenylation process or simply never possessed it. In either case, the evolutionary pressure

leading to the present-day situation remains elusive. For example, halophilic archaea, which live at a very high salt concentration and normal temperature, that is, a condition which favors the formation of RNA secondary structure, degrade RNA without polyadenylation (*23*). However, the hyperthermophilic group, which lives at a very high temperature where the RNA is not expected to be highly folded, utilizes the polyadenylation-stimulated degradation pathway (*22, 23*).

Taken together, the polyadenylation-stimulated RNA degradation pathway is composed of several steps including a possible initial endonuclease cleavage, followed by additional cleavages, polyadenylation of the cleavage products, and exonucleolytic degradation. Polyadenylation seems to provide a platform for the processive exoribonucleases and helps them overcome highly structured RNA barriers. This pathway (with some variations) evolved and is present in all organisms and organelles but a limited few. Where the pathway is present, it appears to be very important for normal growth.

III. The Enzymes

A. Endoribonucleases

1. RNase E

RNase E is an endoribonuclease found in many bacteria, some algae and archaea, as well as in higher plants, where it is predicted to be localized in the chloroplast (Fig. 4). It plays an important role in the processing and degradation of RNA in *E. coli*. RNase E was discovered in *E. coli* as an rRNA maturation enzyme (*28*) and was later shown to be involved in the processing of numerous other RNAs, including the antisense regulator of *E. coli* plasmid replication, RNAI; the precursor of M1 RNA, which is the catalytic subunit of the RNase P; tRNAs; and small noncoding regulatory RNAs and their targets (*29–34*). In addition, RNase E alters the stability of total RNA as well as numerous specific transcripts (*35–38*). Moreover, the enzyme concentration in the cell is regulated by a feedback loop, in which RNase E controls the stability of its own mRNA (*39–41*).

The *E. coli* version of this protein is essential for cell viability and contains 1061 amino acids in two distinct domains, an amino-terminal catalytic region and a carboxy-terminal region. The latter serves as a scaffold for assembling the degradosome, a high molecular weight complex that also contains PNPase, RNA helicase B (Rhl B), and the glycolytic enzyme enolase (*42–45*). This degradosome complex, however, is not present in cyanobacteria or spinach chloroplasts (*18, 46, 47*). RNase E cleaves single-stranded RNA with a preference for A/U-rich sequences (*37, 48*). RNase G is another *E. coli* endonuclease possessing about 50% sequence similarity to the RNase E catalytic region.

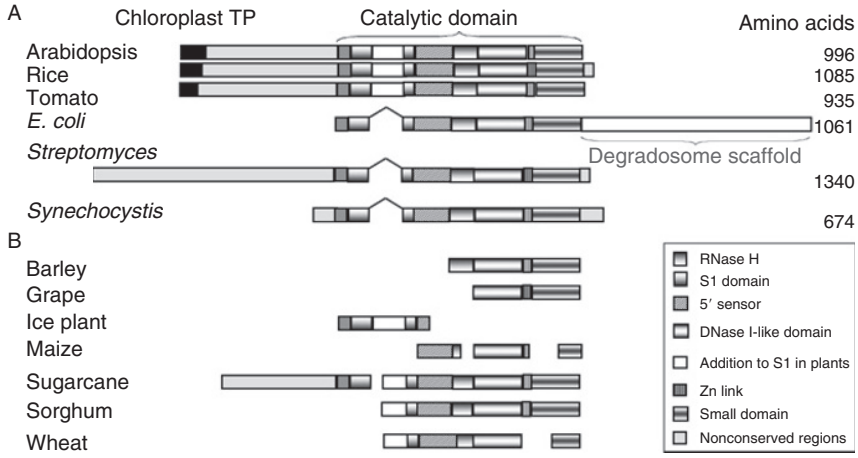

FIG. 4. Plant RNase E proteins. (A) The amino acid sequences of *Arabidopsis*, rice, and tomato RNase E homologues were aligned to those of the *E. coli, Streptomyces*, and *Synechocystis*. Regions of significant homology are shown as patterned boxes, with catalytic subdomains designated accordingly (*26*). The N-terminus of the plant proteins includes sequences shown in *Arabidopsis* and predicted in other plants to constitute a chloroplast TP (*27*). In addition, the plant proteins contain N-terminal extensions of several hundred amino acids that are not homologous between species, as well as a stretch of about 120 amino acids inside the S1 domain, which is not present in any bacterial sequence. (B) Plant ESTs (Expressed Sequence Tags). Related to RNase E, with the position of each domain in the full-length sequences indicated. When more than one EST was found, the ESTs are shown on one line aligned to the full-length sequence of *Arabidopsis*. Sequence accession numbers are as follows: *Escherichia coli*, P21513; *Arabidopsis thaliana*, NP_850987; rice (*Oryza sativa*), NP_001061542; tomato (*Lycopersicon esculentum*) (*27*); *Streptomyces*, NP_626836; *Synechocystis*, NP_439978; barley (*Hordeum vulgare*), TC141965; grape (*Vitis vinifera*), TC42553; ice plant (*Carpobrotus edulis*), TC6253; maize (*Zea mays*), TC308943, TC049636, TC284127; sugarcane (*Saccharum officinarum*), TC51842, TC68873; sorghum (*Sorghum bicolor*), TC106341; wheat (*Triticum aestivum*), TC270983, TC271418.

RNase G has overlapping, but nonidentical, cleavage specificity with RNase E (*49, 50*). Both enzymes were consequently combined into a newly named family of RNase E/G proteins. The cleavage activity of the family members depends on the number of phosphates at the 5′ end: RNA containing one phosphate is a much better substrate than RNA with three phosphates or no phosphate (*12, 51–53*). (See also Chapter in this volume by Carpousis *et al.*). The structure of the catalytic portion of *E. coli* RNase E has been solved, revealing its mode of action and the mechanism by which the number of phosphates at the 5′ end affects activity (*26, 54*).

Genes and/or ESTs encoding RNase E/G-like proteins have been found in many bacteria, cyanobacteria, red and green algae, and the nuclear genomes of higher plants, but not in eukaryotes lacking chloroplasts (Fig. 4) (*27, 55, 56*). The classification of RNase E-like polypeptides into several groups based on

the domain architecture has been proposed (*55*). In many bacteria other than *E. coli*, as well as in the nuclear genomes of several green algae and higher plants, only one member of the RNase E/G group is encoded, and it is generally termed RNase E.

The *Arabidopsis* RNase E, encoded by the At2g04270 locus, has recently been characterized (*27, 57*). Since the T-DNA null insertion mutant for this protein cannot grow without adding sucrose to the medium (*57*), *Arabidopsis* RNase E may be required for chloroplast development, similar to its requirement for viability in *E. coli*. Analysis of the N-terminal 63 amino acids revealed a canonical chloroplast transit peptide (TP) that likely directs the cytoplasmically translated protein into this organelle. Indeed, experimental analysis verified the localization of this protein to the chloroplast (*27*), and its absence can be correlated with reduced accumulation of some chloroplast transcripts (*57*). Similar proteins are also encoded by the nuclear genomes of perhaps all other plants (Fig. 4), but apparently not in *Chlamydomonas*.

The carboxyl half of the plant RNase E homologues contains the multidomain catalytic region and is similar to the amino-terminal region of *E. coli* RNase E, both in sequence and domain architecture (*26*). Interestingly, the S1 domain of the plant proteins, which is important for RNA cleavage activity, contains a uniformly located insertion of 121 nonconserved amino acids (Fig. 4). A similar insertion in the S1 domain was described before for the RNase E of α-proteobacteria (*55*). The endonucleolytic activities of the catalytic portions of the *E. coli* and *Arabidopsis* RNase E proteins were found to be very similar. Both were sensitive to the number of phosphates at the 5′ end and to substrate secondary structure. In both enzymes, replacing the two highly conserved lysine residues at positions 546 and 552 (*E. coli* residues 106 and 112), located in the S1 domain, significantly reduced catalytic activity. Therefore, the catalytic domains of the prokaryotic and chloroplast RNase E have apparently retained very similar properties despite their long evolutionary separation.

Although the sensitivity of cleavage activity to the number of phosphates located at the 5′ end of the transcripts is conserved in chloroplast RNase E, this is based on the activity of the catalytic domain without the plant-specific amino-terminal extension. While the 5′ ends of bacterial mRNAs correspond mainly to the transcription initiation site and therefore contain three phosphates, in chloroplasts mRNAs are often processed at their 5′ ends, meaning that they would have monophosphate, which would *a priori* be sensitive to RNase E cleavage. However, as best studied in *Chlamydomonas*, but perhaps also true in higher plants, chloroplast mRNAs are often protected from degradation by nucleus-encoded proteins that specifically bind the 5′ end (*56, 58–60*). It would appear, then, that the apparently poor cleavage activity of the chloroplast enzyme on triphosphorylated substrates likely protects primary transcripts, but not their processed derivatives, from undesired degradation.

2. RNase J

Many organisms lack an RNase E homologue, suggesting that another endoribonuclease is responsible for endonucleolytic processing and turnover. Recently, the purification and identification of two novel *B. subtilis* endoribonucleases, RNases J1 and J2, was described (*61*). These RNases, like the tRNA 3′ processing endonuclease RNase Z, belong to the zinc-dependent metallo β-lactamase group and *in vitro* assays suggest that they are functionally homologous to RNase E, since they have similar substrate specificity in terms of cleavage site selection in AU-rich single-stranded regions (*61, 62*). Indeed, the *B. subtilis thrS* leader mRNA, which is a substrate of RNase J, is cleaved at the same site by RNase E when it is expressed in *E. coli* (*63*).

Genes encoding RNase J homologues are widespread in eubacteria, archaea, algae, and higher plants. Although they appear to replace RNase E in many organisms such as *Chlamydomonas*, others such as *Synechocystis* and higher plants encode both types of enzyme. The *Chlamydomonas* and *Arabidopsis* nuclear genomes each contain a single *RNJ* gene (EU518648-EU518649 and At5g63420, respectively), and the N-terminus of the *Arabidopsis* gene product targets GFP to chloroplasts in transient assays (Bollenbach and Stern, unpublished data).

Surprisingly, analysis of *B. subtilis* RNase J revealed both endonuclease and 5′–3′ exonuclease activity, making it the first 5′–3′ exonuclease discovered in prokaryotes (Fig. 2) (*64*). If the analysis of green algal and higher plant RNase J proteins demonstrates chloroplast localization and 5′–3′ exoribonuclease activity, it may be possible that this is the enzyme responsible for the net 5′–3′ exonucleolytic activity that has been characterized in *Chlamydomonas* chloroplasts (*65–67*). The interplay of the endo and exonuclease activities of this protein in RNA processing and/or degradation in bacteria and possibly the chloroplast awaits further study. Moreover, the division of labor between RNase E and RNase J in cyanobacteria and higher plant chloroplasts, where both enzymes appear to be present, will be interesting to decipher.

The observation that RNase J is essential for embryo development in *Arabidopsis*—plants heterozygous for a T-DNA insertion in the *RNJ* coding sequence produce siliques containing aborted embryos (http://www.seedgenes.org)—suggests a nonredundancy with RNase E. This phenomenon may be related to a function in 16S rRNA maturation as was recently reported for *B. subtilis* RNase J (*68*).

3. CSP41

CSP41a (chloroplast stem-loop binding protein, 41 kDa) and CSP41b are widespread, highly conserved endoribonucleases, which are unique to photosynthetic organisms. The photosynthetic bacteria *Synechocystis* sp. PCC6803

and *Nostoc* sp. PCC7120 encode only a CSP41b homologue, whereas plant and algal nuclear genomes encode both CSP41a and CSP41b. Phylogenetic and motif analyses have shown that CSP41a and CSP41b are paralogs of a cyanobacterial ancestor that diverged from a bacterial epimerase/dehydratase (*69, 70*).

CSP41a was first purified from spinach chloroplasts as a *petD*-specific RNA-binding protein and a nonspecific endoribonuclease (*71, 72*). Spinach CSP41a was shown to cleave synthetic stem-loop-containing *petD*, *psbA*, and *rbcL* RNAs, and could cleave arbitrary single-stranded RNAs (*72*). This suggested that it could initiate turnover of chloroplast transcripts by endonucleolytic cleavage, the first step in the poly(A)-stimulated turnover pathway (Fig. 2). *In vitro* measurements of tobacco chloroplast mRNA degradation rates in CSP41a-deficient plants showed a 7-fold, 2-fold, and 5-fold decrease in the rates of *rbcL*, *psbA*, and *petD* transcript turnover, respectively (*73*), suggesting that CSP41a may participate broadly in chloroplast mRNA turnover. Recent analysis of a CSP41b mutant led to the suggestion that it functions to process 23S rRNA (*74*).

Most chloroplast open reading frames encode inverted repeat (IR) sequences in their 3′ untranslated regions that can fold into stable stem-loop structures. Prior research has shown that these IRs act as processing determinants and protect upstream sequences against 3′–5′ exonucleolytic degradation (*75*). As mentioned earlier, CSP41 has no sequence specificity, but displays a substrate preference for stem-loop containing RNAs from *petD*, *psbA*, and *rbcL in vitro* (*72*). This property would make CSP41 a candidate for RNA maturation leading to turnover (*73*). The analysis suggests that CSP41 has broad substrate specificity, and that stem-loop structure is a major determinant of CSP41 cleavage rates and transcript half-life in the chloroplast.

4. RNase P and RNase Z

Ribonuclease P (RNase P) is an endoribonuclease that processes the 5′ leader sequence of precursor tRNA (Fig. 5). In bacteria, RNase P is a small ribonucleoprotein complex consisting of a catalytic RNA and a protein cofactor (*76*). In human cells, a highly purified nuclear RNase P has at least ten distinct protein subunits associated with a single RNA species, the H1 RNA (*77–79*). In addition, a subset of these protein subunits is shared with RNase MRP (*80*), a mitochondrial and ribosomal RNA-processing ribonucleoprotein (*81, 82*). However, it is not known if these protein subunits are also shared with the mitochondrial form of human RNase P, a ribonucleoprotein particle shown to have an RNA moiety that is identical to H1 RNA (*83*). RNase P is an essential enzyme present in all organisms, except in some archaea that produce leaderless tRNAs (*81, 84*). There is still a debate concerning the type of RNase P in mitochondria and chloroplasts and the extent to which the organellar form contains the catalytic RNA subunit (*83, 85, 86*).

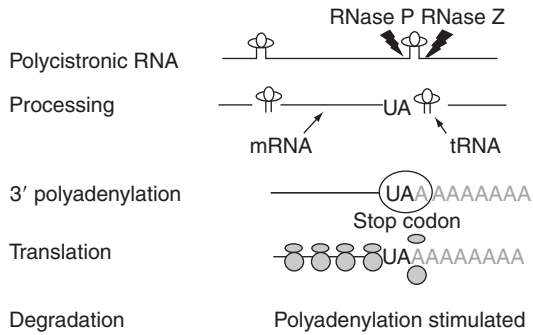

FIG. 5. RNA processing in animal mitochondria. The genome is transcribed into polycistronic RNAs in which the mRNAs are punctuated by tRNAs, which are processed as detailed in the main text. These tRNAs are cleaved at the 5′ and 3′ ends by the endoribonucleases RNase P and RNase Z, respectively. Several mRNAs contain incomplete translational stop codons composed of only U or UA instead of UAA. The addition of a stable poly(A) tail at the 3′ end creates the complete stop codon. The mRNA is then translated, and eventually degraded by the polyadenylation-stimulated degradation pathway.

RNase Z is a member of a highly conserved family of metallo-β-lactamase proteins that is found in prokaryotes, eukaryotes, and archaea (*87, 88*). This endoribonuclease is involved in the processing of tRNA precursors lacking an encoded CCA terminus at their 3′ end (*88, 89*). However, it is generally not active on tRNA precursors that contain a chromosomally encoded CCA (*90*). RNase Z cleavage of tRNA precursors generates substrates for tRNA nucleotidyl transferase-catalyzed addition of CCA to produce functional tRNA molecules (*88*).

B. Exoribonucleases

1. PNPase (Polynucleotide Phosphorylase)

PNPase (EC 2.7.7.8) was discovered during studies of biological phosphorylation in *Azotobacter vinelandii* (*91*), and was later characterized in the context of its role in *E. coli* RNA synthesis (*92*). In fact, PNPase was the first enzyme shown to catalyze the synthesis of polynucleotides from ribonucleotides; unlike RNA polymerases, PNPase catalyzes this reaction in a template-independent manner.

As a phosphorylase, PNPase catalyzes both processive 3′–5′ degradation and RNA polymerization, and participates in the degradation, processing, and polyadenylation of RNA in bacteria and organelles (*4, 25, 93–96*). PNPase has also been reported to be a global regulator of virulence and persistency in *Salmonella enterica* (*97*), and its activity in some way regulates both chloroplast isoprenoid metabolism (*98*) and the ability of *Chlamydomonas* to survive phosphate starvation (*99*). Recent work provided evidence of a possible function of PNPase in the

dedifferentiation process of human cancer cells, a possible involvement in apoptosis and a role in the protection of cellular RNA from oxidizing damage (*100, 101*). Unlike the situation in bacteria and plant organelles, where PNPase is directly involved in RNA metabolism, the human PNPase was recently shown to be localized to the mitochondrial intermembrane space. Therefore, it is not directly engaged in degrading and polymerizing mitochondrial transcripts, since these are located in the matrix (*102–104*).

Analysis of the processing and accumulation of chloroplast or mitochondrial transcripts in plants in which the corresponding PNPase was depleted, revealed dramatic changes in the 3′ end processing and accumulation of polyadenylated transcripts related to ribosomal, messenger, and transfer RNAs (*105–108*). Interestingly, these effects were not observed when the expression of the single PNPase of *Chlamydomonas reinhardtii* was down-regulated, although the accumulation of chloroplast transcripts seen in wild-type cells upon phosphate starvation, was not observed in PNPase-deficient cells (*99*).

Even though the human PNPase is located in the intermembrane space where no RNA is known to be present, the enzyme is phosphorolytically active when expressed as a recombinant protein (*109*). Moreover, siRNA-mediated knockdown of PNPase expression in human cells significantly affected the polyadenylation of mitochondrial transcripts, as well as ATP generation and other mitochondrial activities (*104*). Since mitochondrial transcripts were affected in the knockdown cells, it was suggested that PNPase phosphorolytic activity in the intermembrane space is important for proper mitochondrial functioning, perhaps by fine-tuning the phosphate and nucleotide concentrations (*102, 104, 110*). Therefore, the effects on polyadenylation and processing of mitochondrial transcripts would be indirect. This hypothesis suggests that there is a substrate for PNPase in the intermembrane space. However, since no RNA has yet been located in this compartment, one might speculate that there is a hitherto unknown substrate for this enzyme. In addition, since no gene encoding a protein related to the RNase II/R family with a mitochondrial TP has been identified in the human genome (see below), this would suggest that we do not yet have a candidate for a human mitochondrial matrix exoribonuclease.

Genes encoding PNPase homologues have been identified in almost all bacteria and eukaryotes, with the exception of *Mycoplasma*, trypanosomes, and yeast (*25*). Furthermore, there is no PNPase in archaea, although the hyperthermophiles and some methanogenic archaea contain an exosome that is structurally and enzymatically very similar to PNPase (*23, 25, 111*). The primary structures of PNPases encoded in bacteria and in the nuclear genomes of plants and mammals comprise five domains: two N-terminal core domains homologous to the *E. coli* phosphorylase RNase PH, separated by an α-helical domain, and two C-terminal RNA-binding domains (KH and S1) (*112–114*).

Structural analysis of PNPase from the bacterium *Streptomyces antibioticus* revealed a homotrimeric complex surrounding a central channel that can accommodate a single-stranded RNA molecule (*112, 113*).

Protein sequence alignments and structural observations revealed that bacterial and chloroplast PNPases are evolutionarily related to archaeal and eukaryotic exosomes. The exosome functions in 3′–5′ RNA degradation, RNA processing, and quality control of gene expression in the cytoplasm and nucleus of eukaryotic cells (*8*). It is comprised of 9–11 proteins, including six that are related to RNase PH, three related to the S1 and KH RNA-binding domains, and two others related to the hydrolytic ribonucleases, RNase II and RNase D. Overall, the core 9-subunit exosome is structurally similar to trimeric PNPase (*96, 114–118*). Therefore, PNPase, and the archaeal and eukaryotic exosomes represent functionally and evolutionarily conserved machines for 3′–5′ exonucleolytic degradation. Nevertheless, while the archaeal exosome is very similar to PNPase and is phosphorolytically active, the yeast and human exosome complexes are not (*115, 117*). Instead, the yeast and human exosomes degrade RNA only hydrolytically, and perhaps retain their circular shape as a result of evolutionary pressure for RNA binding and/or structural features (*119*).

2. RNase II/R

The RNR exoribonuclease family members, which are typified by *E. coli* RNase II and RNase R, are hydrolytically processive 3′–5′ exoribonucleases that release 5′ monophosphate nucleotides. These enzymes are widely distributed among eukaryotes, eubacteria, *Mycoplasma*, and the archaea. While most eukaryotic nuclear genomes encode at least three RNR homologues, some prokaryotic genomes encode only a single RNR-like enzyme and exceptional cases, such as *Mycoplasma*, encode a single RNR homologue as the only exoribonuclease (*24, 120*). The halophilic archaea also encode an RNR homologue, while hyperthermophiles and several methanogens contain the archaeal exosome (*22, 23*). Interestingly, no homologue of RNase II/R could be detected in those methanogens that do not contain the archeal exosome, or in the human mitochondrial matrix (*22, 96*). The *Arabidopsis* nuclear genome encodes three homologues, including RNR1, which is both plastid and mitochondria-localized, and RNR2 and RNR3, which are localized to the nucleus and cytosol, and are therefore putative exosome subunits (*107, 121–123*). Homozygous *Arabidopsis* T-DNA mutants for RNR1 can germinate only in the present of sucrose and the maturation of the 3′ ends of the 23S, 16S, and 5S rRNAs is impaired, while mRNAs appear to be unaffected (*121*). *Chlamydomonas* appears to encode two RNR members, RNB1, and RNB2. The N-terminus of the former does not target YFP significantly to an organelle, suggesting it is cytosolic and a good candidate for the exosome. RNB2, by the same criterion, is localized to the chloroplast (*99*).

In *E. coli*, the RNR family members differ in their abilities to remain processive through secondary structures (see Chapter in this volume by Arraiano and colleagues). For example, RNase II becomes distributive near stem-loops and is eventually inhibited by them, while RNase R can melt secondary structures (*124*). Therefore, although both enzymes are nonspecific exonucleases in *E. coli*, RNase II is more active on single-stranded homopolymeric transcripts such as poly(A), while RNase R has a preference for rRNAs (*124*).

An RNase II crystal structure has recently shed light on the catalytic activity and substrate specificity of RNR enzymes (*125, 126*). RNase II folds into four domains comprising two N-terminal RNA-binding moieties, a central catalytic domain, and a C-terminal S1-like RNA binding region. The N- and C-terminal domains form a clamp atop the catalytic domain, which funnels the ssRNA substrate into a narrow channel that houses the active site. Although the domain structure and sequence motifs are highly conserved among RNR family members, it is thought that differences in the clamp arrangement and thus RNA binding properties play an important role in regulating the activity on transcripts containing secondary structures.

Chloroplast/mitochondrial RNR1 is inhibited by secondary structures when assayed *in vitro* (*107, 121*). This is consistent with the fact that it participates in the processing of precursor RNAs, in particular the 3′ ends of rRNAs. Since both rRNAs and mature mRNAs often contain terminal stem-loops in both organelles (Fig. 1), any degradative action of RNR1 would require prior endonucleolytic cleavage and polyadenylation, or recruitment of an RNA helicase. The latter mechanism is employed by yeast mitochondrial Dss1, an RNase R homologue that digests secondary structures by complexing with a helicase. It should be noted that there is no PNPase in yeast mitochondria, thus Dss1 is the only exonuclease so far identified in that organelle (*127*).

RNase II, RNase R, and PNPase, which represent the major exoribonuclease activities in *E. coli*, have significantly different substrate specificities and catalytic properties *in vitro*, but share overlapping functions *in vivo*. In *Synechocystis*, there is a single RNase II/R homologue. In addition, PNPase functions as the only polyadenylation enzyme (in addition to its function in degradation). Accordingly, deletion of *Synechocystis* PNPase- or RNase II/R-encoding genes, unlike the situation in *E. coli* (*128*), leads to loss of viability (*18*). Similarly, since there is no PNPase in yeast mitochondria, deletion of the RNase II/R homologue *DSS1* leads to mitochondrial dysfunction and eventually to the loss of its genome (*127*).

Plant chloroplast PNPase and RNR1 catalyze distinguishable reactions *in vivo*, but may functionally overlap. Repression of the gene encoding chloroplast PNP, for example, leads to defects in mRNA and 23S rRNA 3′ processing, but plants are viable and grow on soil (*105*). Similar observations were made for the mitochondrial enzyme, although the growth phenotype is much stronger (*108*). In contrast, *rnr1* null mutants are defective in rRNA but not in mRNA

processing (*121, 129*). RNR1 mutants are marginally viable on soil, owing to a dependence on RNR1 for chloroplast development in cotyledons, and perhaps an effect on mitochondrial mRNA metabolism (*107*).

C. The Family of Poly(A)-Adding Enzymes

In eukaryotes, a stable poly(A) tail is added to almost all mRNAs during the transcription termination process, initiated by the cleavage of the nascent RNA chain (*130*). The addition of a stable poly(A) tail is accomplished by the so-called canonical PAP. In addition, a diverse family of related enzymes polyadenylate transcripts in various systems, mostly as part of the polyadenylation-stimulated degradation pathway. These enzymes belong to the ribonucleotidyl transferase (rNTr) family that catalyzes the nontemplated addition of homopolymeric adenosine tails to the 3′ hydroxyl group of RNAs and tags them for degradation (*131, 132*).

The rNTr superfamily includes the PAPs mentioned earlier, as well as terminal uridylyl transferases, poly(U)-polymerases and the ubiquitous CCA-adding enzymes (CCAtrs) responsible for the synthesis or repair of the 3′ terminal sequence of tRNA molecules (*131*). rNTr-PAPs are very similar in protein sequence to CCAtrs. However, one motif, which forms a predicted β-loop near the catalytic center, has been identified that seems to be PAP-specific (*133*). rNTr-PAPs are found in the β, γ, and *δ* subdivisions of the proteobacteria and several *Chlamydiales* and *Spirochaetales*, but not in Gram-positive bacteria or bacteria that diverged before the Gram-positives (*133*). In addition, no homologues can be detected in archaea. In plants, chloroplasts and mitochondria are thought to host NTr-PAP homologues encoded in the nucleus and several rNTr-PAPs were recently identified bioinformatically (*133*). For example, bioinformatic analysis of the *Chlamydomonas* genome revealed eight NTR/noncanonical PAPs, of which three are predicted to be located in the chloroplast (*134*). Similar numbers of NTr-PAPs were identified in human cells, where one was found to be mitochondrial (*132, 135–137*).

Although PNPase has been found to be primarily responsible for poly(A) addition in spinach chloroplasts (*138*), the presence of homopolymeric poly(A) tails in *Arabidopsis* and other plant chloroplasts suggests the activity of an NTr-PAP. Genes encoding putative NTr-PAPs were identified in the *Arabidopsis* nuclear genome and await experimental validation (*133*).

IV. RNA Degradation and Polyadenylation in Chloroplasts

In principle, the RNA metabolic pathways in the chloroplast were retained from their prokaryotic ancestors and therefore, the elucidation of the polyadenylation-stimulated degradation pathway in *E. coli* has paved the way

for defining this process in the chloroplast. However, chloroplast-specific variations have been observed, as discussed at the beginning of this chapter. Thus, when standard methods like oligo(dT)-primed reverse transcription PCR and others were used for the detection of nonabundant and truncated transcripts decorated with posttranscriptionally added tails, these studies surprisingly revealed heteropolymeric, poly(A)-rich tails, the first observation of such tails in any organism (*139*). Similar heteropolymeric tails, produced mainly by PNPase or the archaeal exosome, were later discovered in bacteria, archaea, and human cells (Fig. 3) (*96*).

Further studies revealed that, as in *E. coli*, both PNPase and Ntr-PAP are polyadenylating enzymes in the chloroplast, but the contribution to the tail population may differ between different plants. For example, while most of the tails in *Arabidopsis* chloroplasts are homopolymeric, suggesting a major contribution by Ntr-PAP, the majority of tails in spinach are heteropolymeric, suggesting a major contribution by PNPase (*138*) (Larum and Schuster, manuscript in preparation). It is interesting to note that PNPase is exclusively responsible for polyadenylation in cyanobacteria, which is considered the closest bacterial relative of the evolutionary ancestor of the chloroplast (*18*). Therefore, it may be suggested that Ntr-PAP evolved in the chloroplast following endosymbiosis. Since both PNPase and Ntr-PAP are active in polyadenylation in both *E. coli* and the chloroplast, it may also be suggested that the conversion of the Ntr-CCA enzyme to Ntr-PAP occurred more than once in the evolution of bacteria and organelles (*96*). Alternatively, it could be suggested that PAP was lost during cyanobacterial evolution.

The enzymes involved in the polyadenylation-stimulated degradation pathway in the chloroplast (Fig. 2) are as follows: for the initial endonucleolytic cleavage step, any of the three known endoribonucleases, RNase E, RNase J, and CSP41a/b, or perhaps the three of these together. The second step of polyadenylation is performed by PNPase (heteropolymeric tails) and perhaps Ntr-PAP (homopolymeric tails). The third step of the exonucleolytic digestion is carried out by the phosphorylase activity of PNPase and/or the hydrolytic enzyme RNase II/R. It is possible that the enzyme oligoribonuclease degrades the residual oligomers, as it does in *E. coli* (see Chapter in this volume by Danchin). This possibility is supported by the finding that homologues of the *E. coli* enzyme are encoded in both the *Arabidopsis* and *Chlamydomonas* genomes (*134*).

V. RNA Degradation and Polyadenylation in Mitochondria

A. Plant Mitochondria

The RNA degradation system in plant mitochondria resembles that of bacteria and chloroplasts in the sense that transcripts are not decorated with stable poly(A) tails. Therefore, the poly(A) tails are destabilizing. Both PNPase

and RNase II/R are present. Nonabundant polyadenylated transcripts were observed in several plants and their quantity significantly increased when the expression of the mitochondrial PNPase was impaired (*107, 140–143*). Indeed, the accumulation of truncated polyadenylated transcripts in PNPase-deficient plant mitochondria prominently reveals the normal action of the polyadenylation-stimulated degradation pathway. However, there are two major differences between plant mitochondria and chloroplasts. The first is that unlike bacteria and chloroplasts, heteropolymeric tails have not yet been detected in mitochondria, suggesting that plant mitochondrial PNPase does not work as a polymerase *in vivo* and that the tails are produced by a yet-to-be identified PAP. The second difference is that no endoribonuclease of the RNase E or RNase J type has been identified in mitochondria, although they do contain tRNA processing enzymes. Therefore, the question is whether there is any mRNA endonuclease. The observation that RNase E and RNase J are restricted to bacteria and photosynthetic organisms, where they bear predicted chloroplast TPs, argues against this, but a mitochondrial localization, or dual localization, cannot be ruled out. Alternatively, a role for the tRNA processing enzymes RNase P and/or RNase Z could be hypothesized. In summary, we speculate that the plant mitochondrial polyadenylation-stimulated degradation pathway consists simply of polyadenylation and exoribonucleolytic digestion.

B. Yeast Mitochondria: RNA Metabolism Without Polyadenylation

Yeast mitochondria are the only organelles known to metabolize RNA without polyadenylation (*14, 127, 144*). This was found to be the case both in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, suggesting that it might be a general phenomenon of fungal mitochondria. Because no *PNP* gene is present in yeast, it is conceivable that during evolution yeast simultaneously lost the *PNP* gene and mitochondrial polyadenylation (Fig. 3) (*114, 145*).

A protein complex consisting of an exoribonuclease belonging to the RNR family and an RNA helicase, defined as the mtExo or the mitochondrial degradosome, has been identified in *S. cerevisiae* mitochondria (*127*). In the absence of PNPase, this complex might be exclusively responsible for exonucleolytic activity in this organelle. This would resemble the case of halophilic archaea and the small genome parasitic bacteria *Mycoplasma*, which also lack RNA polyadenylation, but retain an RNR homologue presumably responsible for exoribonucleolytic activity (*23, 24*). The 3′ ends of yeast mitochondrial mRNAs are characterized by a conserved dodecamer sequence that is encoded in the mitochondrial genome, and is believed to be bound by a specific protein that may protect the 3′ end from exonucleolytic degradation (*14*) (Fig. 1).

C. Trypanosome Mitochondria: Both Stable and Unstable Poly(A) Tails

Trypanosomes are among the earliest branching eukaryotes and their mitochondria are of great interest both because of the unique arrangement of the mitochondrial DNA and the novel posttranscriptional events that govern gene expression. The best-known of these is the massive editing of most protein-coding transcripts, *via* the insertion or deletion of uridines (*146*). The addition of poly(A) tails was detected in these organelles and intriguingly, it seems that both constitutively stable and unstable poly(A) tails are present, depending on the editing stage of the particular transcript (*147*).

Similar to the stable 3′ end tails that characterize animal mitochondrial transcripts, two fractions of short tails composed of several adenosines (oligo (A)), and long tails (poly(A)), were identified. However, unlike human mitochondria where the tails are exclusively composed of adenosines (*104, 148*), in *Trypanosoma brucei* mitochondria the long tails contain many uridines and are therefore considered poly(A/U) extensions (*149*). The short poly(A) tails were found to be required and sufficient for maintaining the abundance of partially edited, fully edited and unedited mRNAs in mitochondria (*149*).

A PAP (KPAP1) was recently identified and characterized in *T. brucei* mitochondria (*149*). This PAP was found to be essential for parasite viability and mitochondrial function, and is engaged in a polyadenylation complex that also includes pentatricopeptide repeat (PPR) proteins (*149*). PPR proteins are characterized by multiple repeats of about 35 amino acids and are involved in the posttranscriptional regulation of gene expression, mainly in organelles (*150, 151*).

The coexistence of stable and unstable poly(A) tails in the same organelle was also observed in mammalian mitochondria and yeast and mammalian nuclei (Fig. 3) (*148, 152–154*). This implies the presence of a mechanism that can distinguish between stabilizing and destabilizing tails because in many cases, this will result in opposite functions, for example, exonucleolytic degradation as opposed to stability determination and translation. Unraveling the details of this molecular mechanism is one of the present challenges.

In terms of exoribonucleases, an RNR-type enzyme, but not PNPase, has been identified in *T. brucei* mitochondria (*155*). Experiments analyzing RNA degradation in protein extracts and organellar systems revealed higher degradation activity for polyadenylated RNA than nonpolyadenylated molecules, as well as an important role for UTP polymerization in this process (*156–158*). Therefore, by analogy to RNA editing, the polyadenylation, UTP-polymerization, and degradation of RNA in this organelle appear to have adopted unique characteristics.

D. Animal Mitochondria

The 16.6 kb circular mammalian mitochondrial genome encodes two rRNAs, 22 tRNAs, and 13 protein components of the oxidative phosphorylation complexes (*159–163*). Unlike plant mitochondrial genomes, their mammalian counterparts have been extensively condensed. They lack introns and aside from one regulatory region, the so-called D-loop, intergenic sequences are absent or limited to a few bases. Both rRNA and tRNA molecules are unusually small. In several cases, genes encode only partial translation termination codons, which become functional only after posttranscriptional polyadenylation, as described below (*14, 163*).

Both DNA strands (termed H and L) are fully transcribed, resulting in polycistronic RNA molecules, which are then endonucleolytically processed to produce mRNAs, rRNAs, and tRNAs (Fig. 5). The transcripts are then polyadenylated, producing functional stop codons. Transfer RNAs are subjected to the addition of the CCA motif and the modification of internal nucleotides (*162–165*). The intergenic regions of the L-strand primary transcript are believed to be rapidly degraded. RNA degradation is a key component of mitochondrial gene regulation, as it is also required to eliminate aberrant transcripts (*14, 25, 159, 166*). Our mechanistic knowledge of human mitochondrial RNA degradation is very limited, the most significant difference with most other organellar systems being the presence of stable poly(A) tails at the mature 3′ ends of mRNA (Figs. 1 and 5).

This stable poly(A) tail was described more than 30 years ago (*160, 167, 168*) and it has been proposed to determine transcript stability, perhaps in conjunction with a putative poly(A)-binding protein (*14, 169*). Yet, the only established function is the completion of translational stop codons where the encoded one is incomplete (*170*). Although PNPase is present in human mitochondria, the homopolymeric poly(A) nature of the tails suggest synthesis by a PAP (*171*). As mentioned earlier, PNPase was recently localized to the intermembrane space, whereas RNA metabolism occurs in the matrix (*110, 136, 137*). In cells where the expression of this PAP was drastically reduced by RNAi, polyadenylation still occurred, but tail length was reduced from an average of 43 to 8 adenosines (*104, 137*). This result suggests that the residual PAP can still produce the oligoadenylated tails, or that more than one enzyme is responsible for the polyadenylation activity in human mitochondria.

If one assumes that the degradation of mammalian mitochondrial RNA is mechanistically similar to other organellar and prokaryotic pathways, one would expect to be able to find truncated, low-abundance polyadenylated fragments in these mitochondria, in addition to the full-length RNAs with stable poly(A) tails at their 3′ ends. Indeed, analysis using oligo(dT)-primed reverse transcriptase PCR of human mitochondrial RNA, from both cancer cell lines and primary fibroblasts, revealed many such molecules derived from each gene that was analyzed,

including mRNA, rRNA, and tRNA (*148*). Furthermore, a bioinformatic tool developed to search the human EST database for cDNAs corresponding to polyadenylated truncated human mitochondrial RNAs, was successful in finding hundreds of such ESTs (*148*). The resulting ESTs represented the entire human mitochondrial transcriptome, including the L-strand intergenic regions.

In all systems for which this has been investigated, there is a strict correlation between the presence of truncated polyadenylated RNA molecules and the prokaryotic/organellar polyadenylation-stimulated RNA degradation mechanism. Therefore, this internal polyadenylation is most likely part of the RNA degradation process, meaning that in this respect, mammalian mitochondria stay true to their prokaryotic origin (*96, 148*). In this light, the discovery that PNPase is located in the intermembrane space came as a surprise, since it suggests that unlike other systems, it is not directly involved in RNA metabolism (*102, 103*). However, as described earlier, when human PNPase is expressed in bacteria, it is active as a phosphorylase, and reducing its amount in the cell by siRNA drastically affects polyadenylation and ATP production as well as other mitochondrial processes, most likely by indirect means (*102, 104, 109*). As discussed in the section on PNPase, these observations suggest that PNPase fine-tunes the nucleotide concentration in mitochondria and maintains mitochondrial homoeostasis, with mRNA metabolism being one of the processes influenced by this activity. Constitutive knockdown of PNPase in human cell lines demonstrated transcript-dependent effects on mitochondrial mRNA processing and polyadenylation (*104*). These effects, which included abnormal 5′ and 3′end processing and fluctuations in the lengths of poly(A) tails, did not seem to influence mitochondria mRNA abundance, the polypeptide synthesis rate, or protein accumulation. Since polyadenylation of the *cox1* transcript was abolished in this experiment, the results demonstrated that at least in this case, a stable poly(A) tail is not required for stabilization or translation initiation (*104*).

How do animal mitochondria differentiate between stable polyadenylation and degradation-inducing poly(A) tails? Is there a second polyadenylating enzyme? Is there an initial endonucleolytic cleavage, and if so, what is the enzyme involved? In the absence of PNPase in the matrix and the lack of a member of the RNase II/R family with a mitochondrial targeting peptide, what is the exonuclease that degrades mitochondrial transcripts, if there is one at all? These questions are currently being investigated and promise to reveal an evolutionarily unique outcome.

VI. Conclusions and Perspectives

Based on our current knowledge of RNA degradation/polyadenylation pathways in various bacteria, archaea, yeast, plants, and animals, a broader view of their evolution has been achieved. In addition, the power of

comparative genomics to understand the origin of complex RNA degradation pathways is evident. Continuing and broader investigations will reveal different combinations of enzymes, as well as the interplay between stable and unstable poly(A) tails, which in turn will help establish the role of each in a given organism.

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