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RNA Polyadenylation in Prokaryotes and Organelles; Different Tails Tell Different Tales

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The addition of poly(A) tails to RNA is a phenomenon common to almost all organisms examined as of today. In eukaryotes, a stable poly(A) tail is added to the 3′ end of most nuclear-encoded mRNAs. This process is important for mRNA stability and translation initiation. In addition, polyadenylation of nuclear-encoded transcripts in yeast was recently reported to promote RNA degradation. In prokaryotes and organelles, RNA molecules are polyadenylated as part of a polyadenylation-dependent RNA degradation mechanism. This process consists sequentially of endonucleolytic cleavage, addition of degradation-inducing poly(A)-rich sequences to these cleavage products, and exonucleolytic degradation. In spinach chloroplasts the latter two steps, polyadenylation and exonucleolytic degradation, are performed by a single phosphorolytic and processive enzyme, polynucleotide phosphorylase (PNPase), while there is no equivalent to the *E. coli* poly(A)-polymerase enzyme. This was also found to be the case in cyanobacteria, a prokaryote believed to be related to the evolutionary ancestor of the chloroplast, and also in several other bacteria. No RNA polyadenylation was detected in the halophilic archaea *Haloferax volcanii*, which lacks the exosome complex, or in yeast mitochondria, which lack PNPase. Unlike other organelles, mammalian mitochondrial transcripts are known to include stable poly(A) tails at their 3′ ends, much like the case of nuclear-encoded mRNA. However, recent data have revealed that in addition to full-length, stably polyadenylated transcripts, nonabundant, truncated, polyadenylated RNA fragments are present in human mitochondria. These results suggest that the polyadenylation-dependent RNA degradation pathway is present in human mitochondria together with the addition of stable poly(A) tails at the mature 3′ end. We describe a possible scenario illustrating the evolution of RNA polyadenylation and its related functions found in bacteria, archaea, organelles, and eukaryotes.

Keywords RNA-polyadenylation, RNA-degradation, exosome, PNPase, chloroplast, mitochondria

I. INTRODUCTION

Polyadenylation is an important post-transcriptional modification of prokaryotic, eukaryotic and organellar RNA. Considerable progress has been made over the past 15 years in the study of the various polyadenylation processes and our understanding of their biological significance. In bacteria and organelles, the relationship of polyadenylation to RNA decay has been established, and the major proteins involved identified. In the nucleus and cytoplasm, the molecular mechanism of the addition of stable poly(A) tails to the 3′ ends of most mRNAs and the importance of this process for translation initiation has been revealed. In addition, a quality control mechanism, including transient polyadenylation which targets the RNA for degradation by the exosome complex, has recently been described for the yeast nucleus (Lacava *et al*., 2005; Vanacova *et al*., 2005; Wyers *et al*., 2005). This review seeks to provide a concise comparison of our current knowledge regarding the pathways of RNA polyadenylation and decay in several systems including bacteria, archaea, chloroplasts, mitochondria and the nucleus. Indeed, a major point of this review is the assumption that a basal mechanism of polyadenylation-dependent degradation of RNA was already present in the last universal common ancestor (LUCA) of the three domains of life. During evolution, this basal mechanism was subjected to many modifications and variations which can be observed today in different organisms. In addition, different and possibly conflicting biological functions for polyadenylation were sometimes acquired, such as stabilization as opposed to degradation.

Since polynucleotide phosphorylase (PNPase) serves as a central enzyme in RNA polyadenylation, we will start with a brief description of this component. Secondly, the RNA polyadenylation pathways and their functions in different organisms and organelles are illustrated. Finally, we offer a possible scenario describing the evolution of RNA polyadenylation.

II. POLYNUCLEOTIDE PHOSPHORYLASE (PNPase)

PNPase catalyzes both processive 3′ to 5′ phosphorolysis and 5′ to 3′ polymerization of RNA (Grunberg-Manago, 1999; Littauer and Grunberg-Manago, 1999; Soreq and Littauer, 1982). In *E. coli*, PNPase is mostly active in 3′ to 5′ phosphorolysis during RNA degradation and 3′ end processing, while in spinach chloroplasts, cyanobacteria and Gram-positive bacteria, it is suggested to be the major polyadenylating enzyme (Bollenbach *et al*., 2004). PNPase was also reported to be a global regulator of virulence and persistency in *Salmonella enterica* (Clements *et al*., 2002). Part of the PNPase population in *E. coli* is associated with the endoribonuclease, RNase E, an RNA helicase, enolase, and possibly other proteins in a high-molecular complex called the degradosome (Carpousis *et al*., 1999; Coburn and Mackie, 1999; Regnier and Arraiano, 2000; Symmons *et al*., 2002). Recently, the human PNPase was identified in an overlapping pathway screen to discover genes displaying coordinated expression as a consequence of terminal differentiation and senescence of melanoma cells (Leszczyniecka *et al*., 2002; Sarkar *et al*., 2003).

The amino acid sequences of PNPases from bacteria, as well as from the nuclear genomes of plants and mammals, display a high level of homology and feature similar structures comprised of five motifs (Figure 1) (Leszczyniecka *et al*., 2004; Raijmakers *et al*., 2002; Symmons *et al*., 2000, 2002; Zuo and Deutscher, 2001; Yehudai-Resheff *et al*., 2003). X-ray crystallographic analysis revealed the three-dimensional structure of the PNPase from the bacterium *Streptomyces antibioticus*. The enzyme is arranged in a homotrimeric complex forming a doughnut shape, which surrounds a central channel that can accommodate a single-stranded RNA molecule (Figure 1). The domains of the spinach chloroplast PNPase were recently analyzed in detail (Yehudai-Resheff *et al*., 2003). It was found that the first core domain, which was predicted to be inactive in the bacterial enzymes, was active in RNA degradation but not in polymerization. The second core domain was found to be active in degrading only polyadenylated RNA. The high affinity poly(A) binding site was localized to the S1 domain.

Recently, it was suggested that the exosome, which is composed of 10 to 11 exoribonucleases and RNA-binding proteins, is

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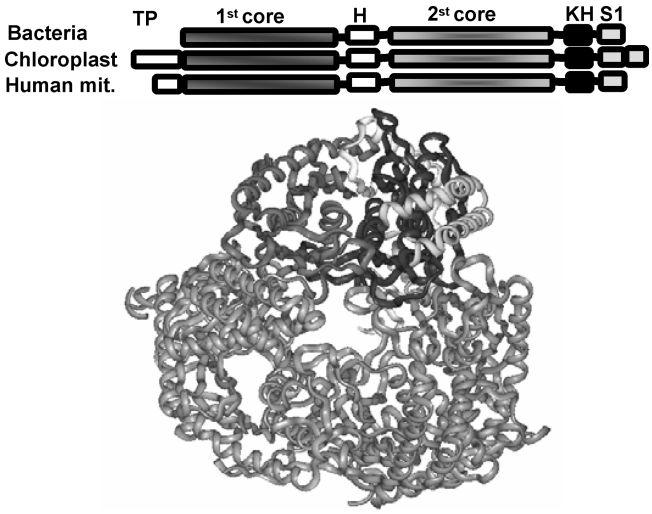

FIG. 1. The domain and homology-based structures of PNPase. The domain organization of PNPase from bacteria, spinach chloroplasts and human mitochondria is shown. TP represents chloroplast and mitochondrial transit peptides. The 1st and 2nd core represents the first and second RNase PH domains. H denotes the α-helical domain. The predicted model of the PNPase enzyme from spinach chloroplast is shown (Yehudai-Resheff *et al*., 2003). The trimeric "doughnut-like" structure is presented in an orientation allowing easy observation of the middle channel which could accommodate single-stranded RNA.

structurally similar to the trimeric PNPase complex (Aloy *et al*., 2002; Raijmakers *et al*., 2002, 2004; Yehudai-Resheff *et al*., 2003). The exosome functions in 3′ to 5′ RNA degradation in the cytoplasm and nucleus of eukaryotic cells. From these observations, it can be suggested that the PNPase and the exosome resemble a unique machine evolved in nature to degrade RNA phosphorolytically from 3′ to 5′. Indeed, an archaeal exosome-like complex displaying a structure very similar to that of PNPase was recently described (Lorentzen *et al*., 2005). The observation that this complex is responsible, in addition to RNA degradation, for the polyadenylation of RNA in the archaea *Sulfolobus solfataricus*, is also in agreement with the great similarity between the exosome and PNPase (Portnoy *et al*., 2005).

III. RNA POLYADENYLATION IN *E. COLI* AND OTHER BACTERIA

The first purification of a poly(A)-polymerase (PAP) was reported for *E. coli* many years ago (Edmond, 2002), but the topic was not immediately pursued, perhaps because a hypothesis explaining the biological role of polyadenylation in prokaryotes and organelles was lacking (Sarkar, 1997). The *E. coli* PAP belongs to the nucleotidyltransferase (Ntr) protein family and the amino acid homology to the *E. coli* Ntr is very high (Betat *et al*., 2004; Martin and Keller, 2004). Attention became refocused on polyadenylation, however, when it was discovered that in *E. coli*, mutations in *pcnB* (encoding the poly(A)-polymerase) resulted with the decrease in the copy number of ColE1 plasmids. This happened since the small RNA (RNA I) that represses plasmid DNA replication was stabilized about 10 fold because of the lack of polyadenylation (Cohen, 1995; Sarkar, 1997). Then, it was found that in *E. coli* strains lacking the exoribonuclease PNPase and RNase II activity, polyadenylated RNA accumulated to significant levels (Carpousis *et al*., 1999; Coburn and Mackie, 1999; Cohen, 1995; Sarkar, 1997). These results suggested that in contrast to the stability and translational competence imparted by polyadenylation of nuclear mRNA, polyadenylation of mRNA in *E. coli* targets it for rapid degradation.

Indeed, considerable progress has been made over the last 15 years in the understanding of the molecular mechanism of RNA polyadenylation and degradation in prokaryotes, mostly by analyzing *E. coli*. These studies revealed the proteins that are involved as well as the involvement of the polyadenylation in quality control mechanisms for transcript abundance (Table 1). Generally, RNA degradation is initiated by endonucleolytic cleavage, believed to be carried out mainly by RNase E in organisms where it is present (Figure 2). Recently, two other endoribonucleases demonstrating activity which resembles that of RNase E, termed RNase J1 and J2, were identified in the gram positive *Bacillus subtilis*, which lacks RNase E (Even *et al*., 2005). Homologous proteins were identified in various bacterial and archaeal genomes ((Even *et al*., 2005) and Table 2), and are also found in plants. Therefore, RNase J is hypothesized to be an additional endoribonuclease that is involved in the initial endonucleolytic cleavage step. In the second step, the cleavage product is polyadenylated and thereby targeted for rapid exonucleolytic degradation (Figure 2). In *E. coli*, polyadenylation is carried out mainly by Ntr-PAP, producing homopolymeric poly(A) tails, and to a certain extent, by PNPase, which produces heteropolymeric poly(A)-rich tails containing all four nucleotides (Mohanty and Kushner, 2000a). The protein Hfq, which resembles the eukaryotic Sm protein, has recently been found to be involved in the modulation of the polyadenylation activity between Ntr-PAP and PNPase (Folichon *et al*., 2005; Mohanty *et al*., 2004). The final step, exonucleolytic degradation, is performed by PNPase, RNase II and possibly also by RNase R (Figure 2) (Cheng and Deutscher, 2005). Research in *E. coli* led to the study of related mechanisms in other prokaryotes and organelles, revealing evidence for evolution and adaptation of the process.

The detailed description of RNA polyadenylation and degradation in *E. coli* is beyond the scope of this review. The reader is referred to several recent reviews describing this subject (Coburn and Mackie, 1999; Condon, 2003; Deutscher and Li, 2001; Dreyfus and Regnier, 2002; Grunberg-Manago, 1999; Kushner, 2002; 2004; Regnier and Arraiano, 2000). Compared to the extensive analysis of the RNA polyadenylation process in *E. coli*, only limited work has been performed on Gram positive bacteria. Heteropolymeric tails containing all four nucleotides were identified when transcripts of the bacterium *Streptomyces coelicolor* were analyzed, suggesting that PNPase and not Ntr-PAP is the major polyadenylating enzyme (Bralley and Jones, 2002). Accordingly, the encoded Ntr protein is active as an Ntr and not a PAP, when analyzed *in vitro* (Sohlberg *et al*., 2003). Similar

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TABLE 1
Similarities and differences between RNA polyadenylation processes among prokaryotes, Archaea, organelles and Eukaryotes

	Prokaryotes			Archaea		Organelles						Eukaryotes
	Bacteria G -	Bacteria G+	Cyano-bacteria	Haloph.	Therm.	Chloroplast Plants	Chloroplast algae	Mitochondria				Nucleus +Cytoplasm
	E.coli	*S.coe.* *B.sub.*	*Syn.*	*Volcanii*	*Sulfo.*	Spinach, *Chlam.* Arab.		Plants Arab.	yeast	Trypan.	human	yeast
Endo.	E G	E	E	E	E	E CSP41	?	?	?	?	?	- ?
Polyad-enylation	PAP I	PNP PAP?	PNP	-	Arch-Exos.	PNP PAP	PNP? PAP?	?	-	?	PAP	PAP TRAMP
Exo.	PNP II R	PNP R	PNP R	R	Arch-Exos.	PNP R	PNP R	PNP R	R	R	PNP	3' → 5' Exo 5' → 3'
Poly(A)	Hom.	Het.	Het.	-	Het.	Het. Hom.	Hom.	Hom.	-	Hom.	Hom.	Hom.
Poly(A) RNA	**Unstable**			NA	**Unstable**				NA	**Stable + Unstable**		

Within the bacteria, *E. coli* represents the Gram-negative (G−) and *Streptomyces coelicolor* (*S. coe.*) and *Bacillus subtilis* (*B. sub.*) the Gram positive (G+). Cyanobacteria are represented by *Synechocystis* (*Syn.*). Within the archaeal domain, the halophiles (Haloph.) are represented by *Haloferax volcanii (volcanii)* and the hyperthermophiles (Therm.) by *Sulfolobus solfataricus (Sulfo)*. Land plants are represented by spinach and Arabidopsis (Arab.) while algal data are from *Chlamydomonas* (*Chlam.*). For yeast, data are from *S. cerevisiae* and for trypanosomes (Trypan.), from *Trypanosoma brucei*.

Symbols and abbreviations are: (−), not present; E, proteins homologous to RNase E or RNase G of *E. coli*; G, RNase G; PAP, poly(A) polymerase; PNP, polynucleotide phosphorylase; II and R, proteins homologous to RNase II and RNase R of *E. coli*; Arc-Exos, the archaeal exosome of *Sulfolobus solfataricus*; (?), Unknown or only based on prediction from genomic sequences. Hom., homopolymeric poly(A); Het., heteropolymeric poly(A)-rich. NA-not applicable. Light gray background displays those systems where there is no RNA polyadenylation. A gray background marks systems where both stable and unstable poly(A) tails are present.

results were obtained when the encoded Ntr protein of *Bacillus subtilis* was analyzed *in vitro* (Raynal *et al*., 1998). Subsequently, heteropolymeric poly(A)-rich tails were identified in this bacterium, suggesting the involvement of PNPase (Campos-Guillen *et al*., 2005). However, analysis of PNPase-deficient *B. subtilis* revealed pronounced polyadenylation with homopolymeric poly(A) tails, suggesting that in addition to PNPase, *B. subtilis* posseses a PAP activity. Since this putative PAP is probably not an Ntr-PAP, a novel PAP type could be predicted.

IV. RNA POLYADENYLATION IN CYANOBACTERIA

Cyanobacteria are related to the evolutionary ancestor of the chloroplast (Dyall *et al*., 2004). Therefore, it was of interest to analyze RNA polyadenylation in this group of organisms. Studies of *Synechocystis* revealed that mRNA, rRNA, tRNA and the single intron located at the $tRNA^{fmet}$ undergo polyadenylation (Rott *et al*., 2003), mirroring that of the same RNA classes in *E. coli* (Li *et al*., 1998b), *Chlamydomonas* (Komine *et al*., 2000) and recently, in human mitochondria (Slomovic *et al*., 2005). Because the tails were heteropolymeric, poly(A)-rich sequences and not homopolymeric poly(A) tails, the polyadenylating enzyme was presumed to be PNPase and not an Ntr type protein (Ntr-PAP), akin to the situation in spinach chloroplasts and *Streptomyces*. Furthermore, in contrast to the situation in *E. coli* and *Bacillus subtilis*, deletion of the PNPase gene caused lethality, suggesting that PNPase is apparently the only polyadenylation enzyme in this organism.

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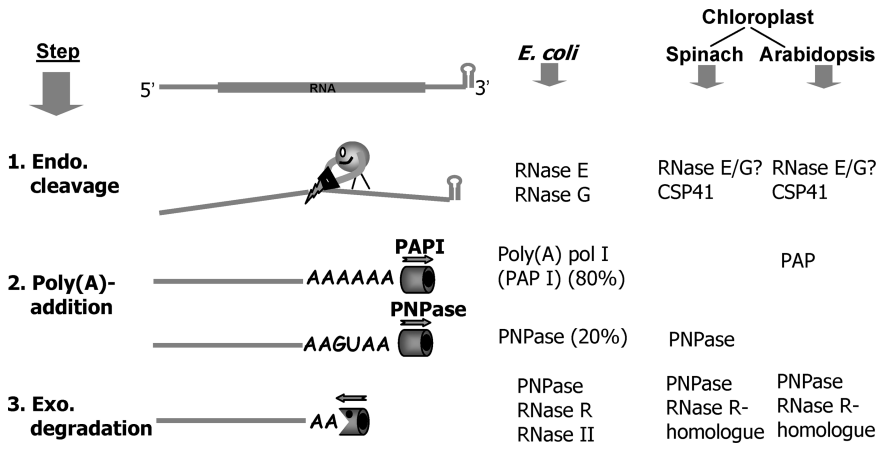

FIG. 2. A model of the polyadenylation-dependent RNA degradation pathway. A schematic representation of a bacterial/plant organellar RNA molecule is shown. The wide line represents the open reading frame and narrow lines, the 5′ and 3′ untranslated regions. The stem-loop structure located at the 3′ end is schematically presented. The initial step is believed to be endonucleolytic cleavage. The enzymes that possibly carry out this step are listed for *E. coli* and the chloroplast. A poly(A) or poly(A)-rich tail is then added to the proximal endonucleolytic cleavage product. While the poly(A) polymerase (PAP) creates homopolymeric tails, those formed by PNPase are heteropolymeric and poly(A)-rich. A division of 80% homopolymeric and 20% heteropolymeric tails has been reported for *E. coli* (Mohanty *et al*., 2004). The polyadenylated RNA molecule is rapidly degraded by an exonuclease(s).

It may be hypothesized, therefore, that *E. coli* and perhaps other proteobacteria acquired PAP relatively late in evolution, through the interconversion of a CCA-adding enzyme, as has been suggested (Yue *et al*., 1996). Indeed, RNA polyadenylation in cyanobacteria may represent a more ancient evolutionary state of the system than that which is present in *E. coli*.

V. RNA POLYADENYLATION IN ARCHAEA

Archaea, comprising the third domain of life, resemble both eukaryotes and prokaryotes in terms of RNA metabolism. Little is known about of polyadenylation and degradation mechanisms (Allers and Mevarech, 2005). Unlike prokaryotes (excluding the Mycoplasma group), no gene encoding a PNPase homologue is present in the archaeal genomes sequenced to date (Anantharaman *et al*., 2002; Koonin *et al*., 2001; Zuo and Deutscher, 2001). Hyperthermophiles of the genus *Sulfolobus* and several methanogens, however, contain a protein complex with high similarity to the eukaryotic exosome (Table 2) (Koonin *et al*., 2001). In the case of the archaeon *Sulfolobus solfataricus*, this exosome-like complex was analyzed (Evguenieva-Hackenberg *et al*., 2003), and found to resemble the bacterial and organellar PNPases in terms of structure and activity (Lorentzen *et al*., 2005). Halophilic archaea, however, do not encode homologues of the exosome components nor a PNPase (Anantharaman *et al*., 2002; Koonin *et al*., 2001; Zuo and Deutscher, 2001). There is only one Ntr gene which was shown, in several archaea, to encode a Ntr and not a PAP. Therefore, there is no obvious candidate for a polyadenylating enzyme in the sequenced genomes of the halophilic archaea. Indeed, using several methods, it was recently shown that there is no RNA polyadenylation in the halophile *Haloferax volcanii* (Portnoy *et al*., 2005). This is the first organism described that lacks all forms of RNA polyadenylation, both stable and degradation-targeting (Table 2). In the absence of PNPase and the exosome, the exoribonucleolytic activity is carried out by an RNase R homologue in a poly(A)-independent degradation pathway (Portnoy *et al*., 2005).

TABLE 2
RNA metabolism in Archaea

	Halophiles	Thermophiles and Hyperthermophiles	Methanogens
RNase E/G	+	+/−	+/−
RNase J	+/−	+/−	+/−
PNPase	−	−	−
Exosome	−	+	− and +
RNase R	+	−	−(except *M. mazei*)
Poly(A) tails	−	heterogenic	?

The presence of genes in the genomes of different archaea so far sequenced, which encode enzymes homologous to the known major enzymes involved in RNA degradation. (+/−) indicates that in some members of this group the corresponding homologue is present while not in others. The archaeal exosome of *Sulfolobus solfataricus* and the RNase R homologue of *H. volcanii* were experimentally analyzed (see text). The absence (−) or the nature of the poly(A) tails as were experimentally detected are indicated. A question mark means that there are no data yet on whether or not there is RNA polyadenylation in this group of archaea.

In contrast, heteropolymeric poly(A)-rich tails were found in the hyperthermophile *Sulfolobus solfataricus*, which were found to be generated by the archaeal exosome (Portnoy *et al*., 2005) which is structurally similar to PNPase, as mentioned above. The majority of polyadenylated molecules were not full-length transcripts; rather they were truncated, nonabundant fragments.

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Together with the fact that the gene for an RNase E homologue is present in this system, this observation suggests that the general requirements for a bacterial/organellar polyadenylation-dependent RNA degradation pathway are present in hyperthermophilic archaea. The similarities between both the structure and the polyadenylation activity of PNPase and the archaeal exosome raise several evolutionary questions. Interestingly, the yeast nuclear exosome displays very low polyadenylation activity and requires an additional poly(A)-polymerase, the Trf4p (Lacava *et al*., 2005).

VI. RNA POLYADENYLATION IN THE CHLOROPLAST

A. Heteropolymeric Poly(A)-Rich Tails are Added to Spinach Chloroplast Truncated Transcripts

Polyadenylation of RNA in spinach chloroplasts was initially examined by PCR amplification of oligo dT-primed cDNAs (Kudla *et al*., 1996; Lisitsky *et al*., 1996). These studies revealed that in contrast to the relatively short and homopolymeric poly(A) tails of *E. coli* RNA, spinach chloroplast RNA tails can reach several hundred nucleotides in length and consist of 70% adenosine, 25% guanosine, and 5% cytidine and uridine. The majority of addition sites were within transcripts rather than at the mature 3′ end. In fact, several polyadenylation sites within the spinach *psbA* RNA matched endonucleolytic cleavage sites previously mapped by primer extension using lysed chloroplast RNA (Lisitsky *et al*., 1996). Also, a polyadenylation site found in the spinach *petD* RNA was found to coincide with the major *in vitro* cleavage site of a partially purified endoribonuclease (Kudla *et al*., 1996). These results implied that most, if not all of the internal polyadenylation sites are produced by endonucleolytic cleavage of mature RNA and do not arise from polyadenylation of truncated molecules resulting from premature transcription termination (reviewed in Schuster *et al*., 1999).

Chloroplast transcripts are occasionally polyadenylated at their mature 3′ ends, but at least 50 times less frequently than at internal sites. This suggests that the mature 3′ ends of chloroplast mRNA are poor substrates for the polyadenylating enzyme. Most chloroplast mRNAs are processed at their 3′ ends, usually yielding terminal stem-loop structures. It thus seems likely that in addition to preventing initiation of exonucleolytic degradation at the 3′ end, this stem-loop structure also reduces degradation-inducing polyadenylation. In turn, this forces most mRNA degradation into the endonuclease-dependent pathway described above (Figure 2). Indeed, RNA molecules with a 3′ stem-loop structure are poorly polyadenylated *in vitro* in chloroplast protein extracts (Lisitsky *et al*., 1996, 1997b; Yehudai-Resheff *et al*., 2001).

B. RNA Polyadenylation Is Part of the RNA Degradation System in the Chloroplast

The hypothesis that polyadenylation targets endonucleolytic cleavage products for rapid exonucleolytic degradation was confirmed by experiments using the lysed chloroplast system and the polyadenylation inhibitor cordycepin (3′-dATP). When lysed chloroplasts were treated with cordycepin, full-length *psbA* transcripts were endonucleolytically cleaved but the cleavage products accumulated. Furthermore, polyadenylated cleavage products accumulated when lysed chloroplasts were incubated with yeast tRNA, a non-specific exonuclease inhibitor (Lisitsky *et al*., 1997a). These results implied that as in *E. coli*, following endonucleolytic cleavage, polyadenylation targets chloroplast mRNA to rapid exonucleolytic degradation.

The role of polyadenylation as a means of targeting RNA for degradation was also confirmed *in vivo* using the green alga *Chlamydomonas reinhardtii* (Komine *et al*., 2002). A chimeric, polyadenylated green fluorescent protein (*gfp*) gene was introduced into the *Chlamydomonas* chloroplast genome such that the 3′ end poly(A) tail would be exposed after RNase P cleavage upstream of an ectopic *trnE*. Indeed, no GFP protein nor polyadenylated *gfp* transcript could be detected in this strain, while the stability of the tagged mRNA and expression of GFP were relatively high in strains expressing a control construct either lacking a poly(A) tail or containing a heteropolymeric (A + U) tail (Komine *et al*., 2002). Similar results were obtained by modification of the endogenous *atpB* gene, which encodes a product that is required for photosynthesis. Since engineered polyadenylation conferred transcript instability, the strain became an obligate heterotroph. This could be reversed by antisense inhibition of PNPase expression, as shown by (Nishimura *et al*., 2004). This *in vivo* approach corroborated results obtained using both *in vitro* and lysed chloroplast assays and revealed the possibility of regulating RNA stability of chloroplast transgenes by genetic engineering.

During degradation competition assays in a soluble chloroplast protein extract, polyadenylated and nonpolyadenylated transcripts were degraded separately at similar rates. However, when both types of RNA were mixed, the nonpolyadenylated RNA was more stable than the polyadenylated molecules. This result implied that polyadenylated RNA was preferentially degraded by exonucleases present in the extract (Lisitsky *et al*., 1997b). The same results were obtained when competition experiments were performed using only the chloroplast PNPase, either purified from spinach or expressed as a recombinant protein in *E. coli* (Lisitsky *et al*., 1997b). The preferential degradation of polyadenylated RNA by PNPase was subsequently explained by the presence of a high affinity poly(A)-RNA binding site within its S1 domain (Yehudai-Resheff *et al*., 2003). Therefore, it is possible that as a processive enzyme, most of the PNPase population is bound to RNA in the chloroplast and its high affinity for polyadenylated RNA sequesters it from degrading nonpolyadenylated molecules.

C. PNPase May Be the Polyadenylation Enzyme in Spinach Chloroplasts

In *E. coli*, the major enzyme responsible for polyadenylation is poly(A) polymerase (Ntr-PAP), which is encoded by the

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pcnB gene (Mohanty and Kushner, 2000a). The amino acid sequence of PAP displays very high homology to the Ntr family. This similarity suggests that PAP was relatively recently derived from an Ntr by gene duplication. Further, both PAP and Ntr enzymes have the ability to specifically add a C or A to RNA without using a DNA template (Yue *et al*., 1996). In fact, the amino acid sequence identity among these family members is so high that assignment of catalytic activities as either a PAP or an Ntr must be verified biochemically. Only recently has a bioinformatic method for this analysis been developed and applied (Martin and Keller, 2004).

Initial attempts at isolating and purifying chloroplast homologs of *E. coli* PAP met with limited success (Li *et al*., 1996; Li *et al*., 1998a). In 2000, it was discovered that poly(A) tails in *pcnB* deletion strains of *E. coli* were long and heteropolymeric, very similar to those characterized in spinach chloroplasts (Mohanty and Kushner, 2000a). In the absence of Ntr-PAP, polyadenylation is performed by PNPase, which until that time was believed to be active in bacteria and chloroplasts only as an exonuclease. The observation that PNPase is able to polymerize RNA, adding heteropolymeric poly(A)-rich tails, suggested that this is the enzyme solely responsible for the polyadenylation of spinach chloroplast RNA. The finding that purification of PAP activity from spinach chloroplasts yielded only PNPase and that the activity of purified PNPase is similar to the polyadenylation activity in spinach chloroplast protein extracts, supported this hypothesis (Yehudai-Resheff *et al*., 2001).

How can one enzyme perform the opposing activities of polyadenylation and degradation? Biochemical analyses have shown that chloroplast PNPase is neither post-translationally modified nor bound to other proteins, both of which could potentially influence its activity (Baginsky *et al*., 2001). However, the directionality of this nearly freely reversible reaction which PNPase catalyzes was shown to be directly influenced by the P_i/NDP ratio (Bollenbach et al., 2004; Yehudai-Resheff *et al*., 2001, 2003). This suggests that PNPase activity may be shifted towards net exonucleolytic or polymerization activities by modulating the concentrations of its substrates.

D. RNA Polyadenylation in Arabidopsis Chloroplasts May Be Performed by a Poly(A)-Polymerase

Several recently obtained results suggest that a different situation is present in the chloroplasts of Arabidopsis where as in *E. coli*, an Ntr like PAP may be responsible for polyadenylation (Figure 2). First, the poly(A) tails identified so far in Arabidopsis chloroplasts were found to be homogenous, in contrast to those of spinach chloroplasts (unpublished results). As mentioned above, analysis of *E. coli* mutants lacking either PNPase or PAP suggested that heteropolymeric poly(A)-rich tails are produced by PNPase, whereas homopolymeric poly(A) tails are synthesized by PAP (Mohanty and Kushner, 2000b; Mohanty *et al*., 2004). A second indication of PAP presence in Arabidopsis chloroplasts was found using the bioinformatic tool mentioned above (Martin and Keller, 2004), which enabled identification of several putative PAPs in the Arabidopsis genome which are flanked by N-terminal sequences resembling chloroplast or mitochondrial transit peptides. These bioinformatic observations still await experimental verification concerning the localization of these proteins and their activity as a PAP and not an Ntr. If indeed this is the case, this implies that the conversion of Ntr to PAP occurred independently in the evolution of *E. coli* and Arabidopsis chloroplasts. Alternatively, it could be argued that this activity was present, but ceased to exist in *Synechocystis* and spinach chloroplasts.

The third observation suggesting PAP activity in Arabidopsis chloroplasts was the analysis of an Arabidopsis line in which the amount of PNPase was reduced to about 1 percent compared to wild-type plants. This was attained by overexpression of the PNPase transcript which resulted in cosuppression of its expression (Walter *et al*., 2002). These plants had no observable phenotype, but were impaired in the 3′ end processing of *psbA*, *rbcL* and 23S RNA molecules. Interestingly, a DNA gel blot of oligo(dT)-primed cDNA showed a pronounced increase in the amount of polyadenylated RNA in the cosuppressed plants.

Together, these observations show that while RNA polyadenylation is performed in spinach chloroplasts by PNPase as it is in *Synechocystis*, a PAP enzyme seems to be responsible for the parallel process which occurs in Arabidopsis chloroplasts (Figure 2). It is possible that the presence or absence of PAP activity in the chloroplasts of different higher plants resembles the diversity of chloroplast RNA editing, a process which is not conserved between different plants and therefore is believed to have evolved relatively late in evolution (Bock, 2000, 2004).

VII. RNA POLYADENYLATION IN MITOCHONDRIA

While chloroplasts are present only in plants and algae, mitochondria exist in all eukaryotes. The data obtained so far concerning the metabolism, 3′ end nature, and polyadenylation of mitochondrial transcripts, are very diverse, suggesting different evolutionary pathways (Table 1, Figure 3).

A. Higher Plant Mitochondria

RNA polyadenylation has been analyzed in the mitochondria of several plants including maize, Arabidopsis, sunflower and evening primrose (Gagliardi and Leaver, 1999; Gagliardi

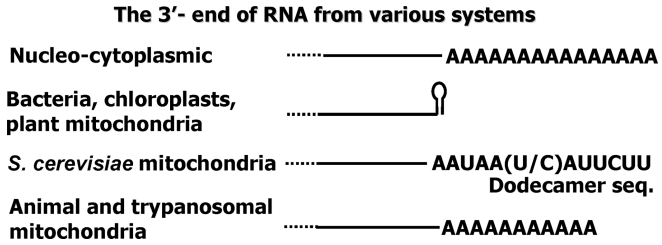

FIG. 3. The 3′ ends of RNA from various systems.

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et al., 2001; Kuhn *et al*., 2001; Lupold *et al*., 1999; Perrin *et al*., 2004). As in *E. coli*, cyanobacteria and chloroplasts, plant mitochondrial transcripts are not constitutively polyadenylated at their mature 3′ ends but contain inverted repeat sequences which can fold into relatively stable stem-loop structures. In chloroplasts, this element has been shown to be a weak substrate for polyadenylation (Figure 3). The observed polyadenylated mitochondrial transcripts contained poly(A) tails consisting almost exclusively of adenosines, suggesting that PAP, not PNPase, is the enzyme responsible. The low abundance of these polyadenylated transcripts supported a possible role in the RNA degradation process, similar to bacteria and chloroplasts (Gagliardi *et al*., 2004). The 3′ ends of yeast mitochondrial transcripts are characterized by conserved sequences, while animal mitochondrial transcripts contain stable poly(A) tails similar to nuclear-encoded RNA (Figure 3). In conclusion, mitochondria of different organisms differ in terms of RNA polyadenylation and the nature of the 3′ end of their transcripts (Gagliardi *et al*., 2004) (Table 1).

B. Yeast Mitochondria; RNA Metabolism Without Polyadenylation

Yeast mitochondria are the only organelles known to metabolize RNA without polyadenylation (Dziembowski *et al*., 2003; Gagliardi *et al*., 2004; Schafer *et al*., 2005). Interestingly, no PNPase gene is present in yeast and therefore, it is conceivable that during evolution yeast lost both the PNPase gene and with it, polyadenylation of mitochondrial RNA (Table 1), (Leszczyniecka *et al*., 2004; Yehudai-Resheff *et al*., 2003). Likewise, the halophilic archaea, *Haloverax volcanii,* has recently been described to metabolize RNA without polyadenylation (Portnoy *et al*., 2005). As described above, this archaeon lacks the genes encoding the archaeal exosome, which is very similar to the PNPase and found to be the polyadenylating enzyme in the hyperthermophile archaeon, *Sulfolobus solfataricus* (Lorentzen *et al*., 2005; Portnoy *et al*., 2005). Interestingly, the small genomes of bacteria belonging to the mycoplasma group also lack a gene encoding a PNPase and therefore the lack of RNA polyadenylation can be predicted (Zuo and Deutscher, 2001). However, this prediction has not yet been tested experimentally.

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 the mitochondria of *Saccharomyces cerevisiae* (Dziembowski *et al*., 2003). This complex might be responsible, in the absence of PNPase, for the exonucleolytic activity in this organelle. This would somewhat resemble the case of halophilic archaea, which also lack RNA polyadenylation while an RNR homologue is responsible for the exoribonucleolytic activity (Portnoy *et al*., 2005).

C. Trypanosomal Mitochondria

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 mitochondria of trypanosomes are characterized by massive editing of most protein-coding transcripts, resulting in insertion or deletion of uridines (Simpson *et al*., 2003). The addition of poly(A)-tails was detected in these organelles and interestingly, it seems that both constitutively stable and unstable poly(A)-tails are present, depending on the editing stage of the particular transcript (Kao and Read, 2005). The coexistence of stable and unstable poly(A)-tails in the same organelle was recently identified in mammalian mitochondria and yeast nuclei as well (Lacava *et al*., 2005; Slomovic *et al*., 2005; Vanacova *et al*., 2005; Wyers *et al*., 2005).

In terms of the identity of the exoribonucleases, an RNR type exoribonuclease, but not a PNPase homologue, was identified in the mitochondria of *T. brucei* (Penschow *et al*., 2004). Indeed, in the absence of PNPase, this is the only organism/organelle known to date, in which there is polyadenylation of RNA but not an exosome/PNPase (Table 1). This observation further demonstrates the uniqueness of the gene expression mechanisms of trypanosomal mitochondria. Experiments analyzing RNA degradation in protein extracts and organelle systems revealed higher degradation activity for polyadenylated RNA than nonpolyadenylated molecules, and an important role for UTP polymerization in this process (Ryan *et al*., 2003; Ryan and Read, 2005). Therefore, similar to the instance of RNA editing in trypanosomal mitochondria, the polyadenylation, UTP-polymerization and degradation of RNA in this organelle appear to have evolved and adopted characteristics unique to this system.

VIII. HUMAN MITOCHONDRIA

A. The Human Mitochondrial Genome and RNA Metabolism

The mammalian mitochondrial genome is a circular, double-stranded DNA molecule of about 16.6 kb. The DNA duplex strands can be distinguished based on G + T base composition, termed "heavy" and "light" (Anderson *et al*., 1981; Attardi and Schatz, 1988; Montoya *et al*., 1981; Ojala *et al*., 1981; Taanman, 1999). Most information is encoded on the heavy (H) strand, consisting of genes for two rRNAs, 14 tRNAs, and 12 polypeptides. The light (L) strand encodes eight tRNAs and a single polypeptide. All 13 proteins are constituents of the enzyme complexes of the oxidative phosphorylation system. Unlike the genomes of plant mitochondria, the mammalian mitochondrial genome experienced extensive condensation during evolution. There are no introns and aside from one regulatory region, the D-loop, intergenic sequences are absent or limited to a few bases. Both rRNA and tRNA molecules are unusually small. Some of the open reading frames overlap, and in several cases, transcripts are left with only partial translation termination codons which become functional only after post-transcriptional polyadenylation, described below (Figure 4) (Gagliardi *et al*., 2004; Montoya

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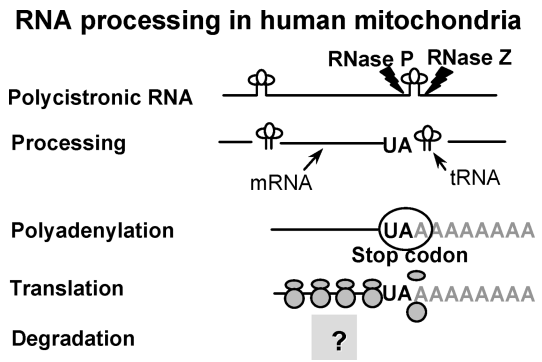

FIG. 4. RNA processing in human mitochondria.The genome is transcribed into polycistronic RNAs in which the mRNAs are punctuated by tRNAs. These tRNAs are cleaved at the 5′ and 3′ ends by the endoribonucleases RNase P and RNase Z, respectively. Several mRNAs contain incomplete translational stop codons 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. A question mark (?) indicates that the mechanism of RNA degradation in human mitochondria is still unknown.

et al., 1981). Also, there are several deviations from the standard genetic code.

Both the H and L strands are fully transcribed, resulting in polycistronic RNA molecules which are then endonucleolytically processed to produce mRNAs, rRNAs and tRNAs (Figure 4). The transcripts are then polyadenylated producing functional stop codons, or, in the case of the tRNAs, the addition of CCA and modification of internal nucleotides take place (Levinger *et al*., 2004; Montoya *et al*., 1983, 1981; Ojala *et al*., 1981). The intergenic regions of the L strand are believed to be rapidly degraded. As in the nucleus, cytoplasm, prokaryotes and chloroplasts, RNA degradation in the mitochondria is a key process for controlling the abundance of a given transcript as part of the regulation of gene expression, and is also required to eliminate aberrant transcripts (Gagliardi *et al*., 2004; King and Attardi, 1993; Taanman, 1999). However, as denoted by a question mark in Figure 4, our knowledge of the molecular mechanism of RNA degradation in human mitochondria is very limited in comparison to recently obtained data on this mechanism in the cytoplasm, nucleus, prokaryotes, chloroplasts, as well as plant and yeast mitochondria. The latter four are closely related to mammalian mitochondria, although a significant difference is the presence of stable poly(A) tails at the mature 3′ ends of mammalian mitochondrial RNAs (Figure 3).

B. RNA Polyadenylation in Human Mitochondria

The presence of a stable poly(A) tail at the 3′ end of most transcripts of human mitochondria was described more than 30 years ago (Amalric *et al*., 1978; Attardi and Schatz, 1988; Hirsch and Penman, 1973). It has been proposed that the poly(A) tail determines transcript stability, perhaps in conjunction with a putative poly(A)-binding protein (Gagliardi *et al*., 2004; Temperley *et al*., 2003). Yet, the only established function that can be attributed to the stable poly(A) tails is the formation of translational stop codons for those genes in which the encoded stop codon is incomplete (Figure 4). Although a PNPase homologue is present in human mitochondria, the homopolymeric poly(A) characteristics of the tails suggest formation by a PAP (Piwowarski *et al*., 2003). Indeed, PAP was recently identified in human mitochondria (Nagaike *et al*., 2005; Tomecki *et al*., 2004). Interestingly, in cells in which the expression of this PAP was drastically reduced by RNAi, RNA polyadenylation was still present but the length of the stable poly(A) tails was reduced from an average of 43 to eight adenosines (Tomecki *et al*., 2004). This result suggests that more than one enzyme may be responsible for the RNA polyadenylation activity in this system.

The degradation of mammalian mitochondrial RNA was anticipated to be mechanistically similar to the pathways found in other organelles and prokaryotes. If this were the case, it was hypothesized that truncated, low abundance polyadenylated fragments would be present in addition to 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 which was analyzed, including mRNA, rRNA and tRNA (Slomovic *et al*., 2005). Further, a bioinformatic tool developed to search the human EST database for cDNAs related to truncated, polyadenylated, human mitochondrial RNAs, was successful in finding hundreds of such ESTs (Slomovic *et al*., 2005) (Figure 5). The resulting ESTs represented the entire human mitochondrial transcriptome, including the L strand intergenic regions.

In all of the systems in which it has been investigated, there is 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. Thus, in terms of RNA degradation, mammalian mitochondria stay true to their prokaryotic origin (Slomovic *et al*., 2005). How does this system differentiate between stable polyadenylation and degradation-targeting poly(A) tails? What are the components responsible for this differentiation? Who is/are the polyadenylating enzyme(s) and how is PNPase/PAP involved? Which enzyme performs the initial endonucleolytic cleavage? These questions are currently being investigated in order to elucidate the RNA degradation mechanism in mammalian mitochondria.

IX. RNA POLYADENYLATION IN THE YEAST NUCLEUS

The majority of nuclear-encoded mRNA molecules in eukaryotic cells are characterized by a stable poly(A) tail at the 3′ end, which functions in translation initiation and possibly determines the transcript longevity as well (Dreyfus and Regnier, 2002; Wickens *et al*., 1997). The molecular mechanism of this stable polyadenylation pathway is beyond the scope of this

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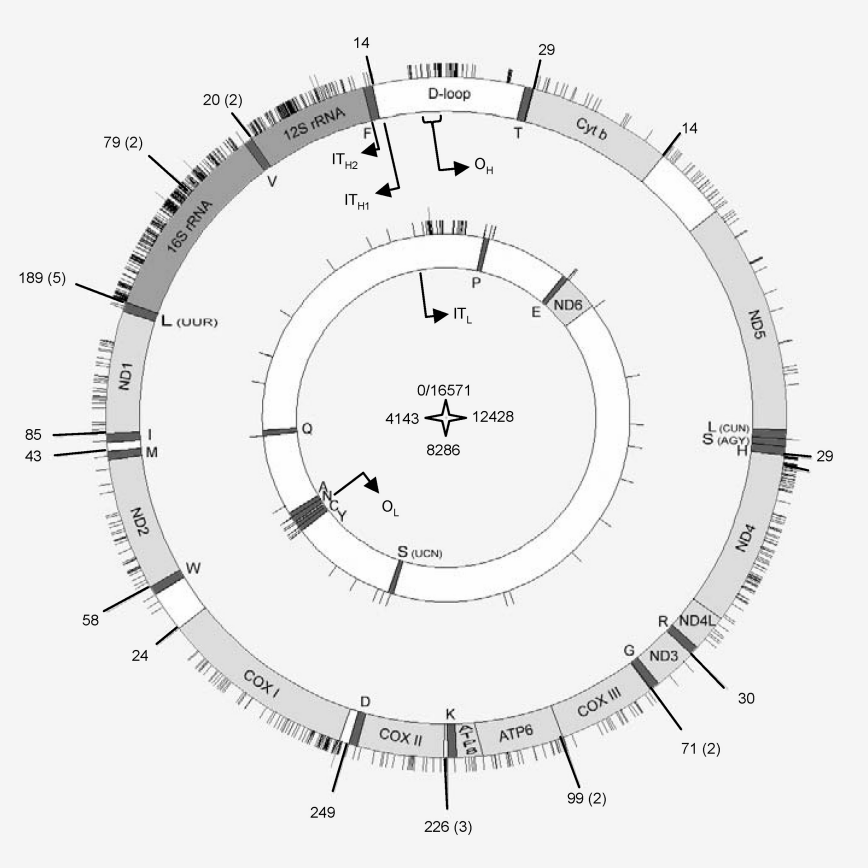

FIG. 5. RNA polyadenylation in human mitochondria. External and internal circles represent the H- and L-strands of the human mitochondrial genome, respectively. Poly(A) addition positions revealed by the bioinformatic tool, PolyAfinder (Slomovic *et al*., 2005) are indicated by radial lines. Long lines represent poly(A) sites at which >10 ESTs where found, with the precise number indicated beside the line. When several such sites are clustered, the number of sites is indicated in parentheses. The tRNA genes are indicated by single-letter symbols. The transcription initiation sites on the H- (IT_{H1}, IT_{H2}) and L- (IT_L) strands as well as the origin of replication on the H-strand (O_H) and L-strand (O_L) are indicated. For more details see Slomovic *et al*. (2005).

review and the reader is referred to several reviews describing this process (Dreyfus and Regnier, 2002; Edmonds, 2002; Mangus *et al*., 2003; Mitchell and Tollervey, 2000; Richter, 1999; Shatkin and Manley, 2000; Wickens *et al*., 1997).

Relevant to this review, however, is the recent discovery concerning the transient polyadenylation of nuclear-encoded transcripts in the yeast nucleus (Lacava *et al*., 2005; Vanacova *et al*., 2005; Wyers *et al*., 2005). It was found that tRNA molecules that are incorrectly folded, as a result of improper modification of certain nucleotides, are polyadenylated by the TRAMP complex, followed by rapid degradation by the exosome. Transcripts derived from intergenic regions were shown to undergo this polyadenylation-dependent degradation as well. Assuming that this quality control mechanism is present in the nucleus of all eukaryotes, these results suggest that the polyadenylation-dependent degradation pathway described in bacteria and organelles is present also in the nucleus. The similarities between PNPase and the exosome, described previously, and their similar involvement in the degradation of polyadenylated RNA are also evident in this system.

X. POSSIBLE EVOLUTION OF THE RNA POLYADENYLATION PHENOMENON

As described above, the addition of stable poly(A) tails to full-length RNAs transcribed from organellar genomes is unique to the mitochondria of trypanosomes and animals (Figure 3). As mentioned, in eukaryotes this feature characterizes most nuclear-encoded mRNAs. The observation that the polyadenylation-dependent RNA degradation pathway is present in bacteria, organelles, archaea and also in eukaryotic nuclei, suggests a possible evolutionary scenario concerning these two types of polyadenylation. It is proposed that the transient addition of poly(A)-tails as part of the RNA degradation process was the first form of RNA polyadenylation to appear in evolution, and probably was already present in the last universal common ancestor

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(LUCA) of the three domains of life. Perhaps thereafter animals mitochondria adopted and modified this feature for adding a stable poly(A) tails in order to establish complete stop codons left partial as a result of evolutionary pressure to condense their genomes. In agreement with this theory, the genomes of plant mitochondria are not condensed and polyadenylation seems to be restricted to the polyadenylation-dependent RNA degradation pathway, similar to chloroplasts and cyanobacteria. The stable poly(A)-tail at the 3′ end was later adopted in the eukaryotic nuclear system for the control of a number of processes, including translation initiation. Accordingly, it could be suggested that PNPase is the ancient RNA polyadenylation enzyme which functioned in bacteria and then in organelles, while the PAPs appeared later in evolution, perhaps by gene duplications of Ntrs. In parallel, the functionally PNPase-like complex, the exosome, evolved from its archaeal counterpart into the present complex of the eukaryotic cell.

XI. WHY POLY(A)?

An additional speculative scenario can be drawn in an attempt to explain why adenosine is the major constituent of the post-transcriptionally added tails, as opposed to the other nucleotides. PNPase uses nucleotides according to their relative concentrations in the surrounding solution, therefore producing heteropolymeric tails. Assuming that the original polyadenylating enzyme was indeed PNPase, the relatively high concentration of ADP, due to its function as the primary energy currency of the cell/organelle, may have been the initial reason for its dominant incorporation. Perhaps additional polyadenylating enzymes, the poly(A)-polymerases, evolved later in order to enhance the polyadenylation stage of the degradation mechanism or to accommodate modifications of the basic polyadenylation-dependent degradation pathway which occurred. These poly(A)-polymerases were already specific for the addition of ATP, therefore yielding homopolymeric poly(A) tails. In summary, the answer to the question of why adenosine was "chosen" to serve as the "energy currency" of the cell, leads to the reason why this nucleotide is the primary residue which comprises post-transcriptionally added tails.

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