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Processing and degradation of chloroplast mRNA

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Abstract — The conversion of genetic information stored in DNA into a protein product proceeds through the obligatory intermediate of messenger RNA. The steady-state level of an mRNA is determined by its relative synthesis and degradation rates, i.e., an interplay between transcriptional regulation and control of RNA stability. When the biological status of an organism requires that a gene product's abundance varies as a function of developmental stage, environmental factors or intracellular signals, increased or decreased RNA stability can be the determining factor. RNA stability and processing have long been known as important regulatory points in chloroplast gene expression. Here we summarize current knowledge and prospects relevant to these processes, emphasizing biochemical data. The extensive literature on nuclear mutations affecting chloroplast RNA metabolism is reviewed in another article in this volume (Barkan and Goldschmidt-Clermont, this issue). © 2000 Société française de biochimie et biologie moléculaire / Éditions scientifiques et médicales Elsevier SAS

RNA stability / RNA processing / ribonucleases / RNA-binding proteins / chloroplast

1. Model organisms for chloroplast RNA biochemistry

Genetic, molecular and biochemical approaches have all been used to discern how chloroplast genes are regulated post-transcriptionally [1–3]. While molecular genetic studies can lead to inferences as to the nature of these mechanisms, their ultimate description requires in vitro approaches using either chloroplast protein extracts and/or purified regulatory factors. Genetic studies have been most prominent in *Chlamydomonas*, maize and *Arabidopsis*, whereas biochemistry has been largely carried out in spinach, tobacco and *Chlamydomonas*. This largely reflects the state of genetic/genomic resources on the one hand, and the ease of chloroplast isolation on the other hand. Eventually, a coalescence of genetic, biochemical and molecular (e.g., chloroplast transformation) techniques applied to a single organism will be most advantageous. This is because in vivo confirmation of in vitro results can only be done in organisms whose chloroplasts can be routinely transformed, currently *Chlamydomonas* [4] and tobacco [5].

The in vitro systems currently reported for chloroplasts have been primarily used to study transcription initiation, RNA maturation and translation initiation. Chloroplast transcription extracts have been made from several species including the protist *Euglena* [6], maize [7], pea [8], spinach [9] and tobacco [10, 11], and have been exploited to define promoter characteristics. RNA maturation extracts have been made from spinach [12, 13] and *Chlamydomonas* [14] chloroplasts, and while not active for intron splicing, can accurately perform certain steps of 5' and 3' end formation and RNA degradation. Most recently, a translation system was made from tobacco chloroplasts [15] and unlike those reported earlier, proved reproducible in other laboratories [16]. Together, these in vitro tools provide unparalleled opportunities to investigate chloroplast RNA metabolism.

2. The scope of mRNA processing

RNA processing is a general term to describe the modification of a newly-synthesized RNA molecule. In chloroplasts, mRNAs are subject to several forms of processing, which are not mutually exclusive. These activities form the mature 5' and 3' ends, and are also involved in RNA metabolism by recycling nucleotides of non-functional molecules. In addition, many chloroplast genes contain introns which must be spliced. The most common RNA processing reactions are carried out by ribonucleases, ubiquitous enzymes found in bacteria, organelles, and eukaryotes. There are two types of ribonucleases, exoribonucleases which processively remove nucleotides beginning at either the 5' or 3' end, and endoribonucleases, which cleave internally [17]. Endonuclease cleavage products are sometimes substrates for exonucleases. Although some introns in *Chlamydomonas*

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(i.e., 23S rRNA, *psbA*) can self-splice in vitro under non-physiological conditions, it is believed that in vivo splicing requires protein factors, including ribonucleases [3, 18]. As exemplified by intron splicing, both RNA structure and sequence are important in identifying substrates for a particular processing activity.

Another common form of RNA processing, particularly in organelles, is RNA editing. RNA editing occurs post-transcriptionally and most commonly results in C to U conversions [19]. The extent of RNA editing varies between organisms; being most extensive in trypanosome mitochondria [20], but relatively rare in the nucleus of eukaryotes [21]. In vascular plants, editing is more prevalent in mitochondria than in plastids, although it should be noted that many chloroplast mRNAs are edited [19]. Interestingly, no evidence for editing has been detected in *Chlamydomonas* chloroplasts.

3. Defining mRNA stability

RNA stability is normally expressed in units of half-life, meaning either the number of minutes or hours required for half the initial amount of the RNA being measured to disappear. Because the values vary markedly between RNAs, and depend on whether the measurement is being made in vitro or in vivo and under which conditions, studies often refer to relative RNA stability, where the half lives of two molecules are being compared. The terms 'stable' and 'unstable' RNA are often used but this measure is arbitrary: for example, the average mRNA half life is on the order of minutes in bacteria, but hours in chloroplasts. Thus, the terms should be defined in any particular application.

'Regulation' of mRNA stability generally refers to a situation in which there is a change between the relative transcription and accumulation rates of a given transcript. For example, in spinach chloroplasts it was shown that changes in the transcription of the *psbA* gene in dark-grown (etiolated) and light-grown leaves could not account for the large difference in *psbA* transcript accumulation [22]. Thus, although the actual half-life of the transcript was not measured, increased RNA stability in the light could be inferred.

4. Chloroplast ribonucleases

Ribonucleases are the enzymes that ultimately carry out RNA maturation or degradation. Their activities have been shown to be or are hypothesized to be modulated by factors such as protein modification (e.g., phosphorylation or redox state), protein-protein interactions (i.e., multiprotein complexes), and RNA secondary or tertiary structure. Here we summarize the biochemical data on chloroplast ribonucleases.

4.1. Endoribonucleases

Because chloroplast RNA processing has been frequently studied in vitro, several endonuclease activities have been loosely characterized. These activities have been proposed to carry out 3' end formation [14, 23, 24], or to initiate RNA breakdown by disrupting stabilizing stem-loop structures [25, 26]. In most cases, these activities have been studied in crude extracts, and the proteins have not been purified. One protein that has been expressed in active form from its cDNA is CSP41, a spinach enzyme which preferentially cleaves stem-loop structures [25], has specific RNA-binding properties [27], and is also related to nucleotide-sugar epimerases and hydroxysteroid dehydrogenases [28]. Another endoribonuclease is a 54 kDa protein from mustard chloroplasts, which has been proposed to carry out 3' processing and be regulated by redox poise [23, 29]. While purification of the 54 kDa protein was reported, the encoding gene has not been cloned as of this writing. Progress has also been made on a putative RNase E homologue from spinach [24], based on cross-reactivity to an antibody raised against the *E. coli* protein. RNase E has important roles in both rRNA maturation and mRNA processing in bacteria [30, 31], and homologues have been found both in *Synechocystis* [32], a relative of the organism from which the chloroplast was derived, and in the chloroplast genome of the green alga *Nephroselmis olivacea* [33]. As yet, however, there is no genetic evidence for the in vivo roles of any of these proteins in chloroplasts.

4.2. Exonucleases

The first exonuclease to be characterized in chloroplast extracts was a 3' to 5' activity responsible for trimming artificial pre-mRNAs to their mature 3' ends immediately downstream of stem-loop structures [12]. This observation led to the purification of a 100 kDa protein from spinach which when sequenced, was found to be a nucleus-encoded homologue of polynucleotide phosphorylase (PNP), a 3' to 5' exonuclease well-known from prokaryotes [24]. PNP also participates in poly(A)-stimulated RNA degradation in chloroplasts [34, 35], and has been reported to be associated with poly(A) polymerase in bacteria [36]. The two other major *E. coli* exonucleases, RNase II and oligoribonuclease, have not been reported in chloroplasts. No plant PNP mutants have been published, though it should be noted that PNP mutants in *E. coli* are viable since RNase II can substitute for it [37].

The other possible directionality for an exonuclease is 5' to 3', which unlike 3' to 5' activities, has not been found in any prokaryote. Thus it was somewhat surprising that the existence of such an activity was been implied by studies in *Chlamydomonas*, at first from examination of the nuclear mutant *mcd1*, in which the chloroplast *petD* transcript fails to accumulate [38]. When a polyguanosine$_{18}$ (pG) sequence was inserted shortly

downstream of the *petD* mRNA 5' end, *petD* mRNA accumulated even in the mutant background. Because pG had been shown to impede 5' to 3' exonuclease activity in yeast [39] and 3' to 5' exonuclease activity in *Chlamydomonas* chloroplasts [40], it seemed likely that in *mcd1* mutant cells *petD* mRNA was being degraded by a 5' to 3' exoribonuclease activity. Similar data were subsequently obtained with another *Chlamydomonas* nuclear mutant [41]. In addition, when pG was inserted at –20 relative to the 5' end of the *petD* mRNA, mRNAs with 5' ends at both –20 and + 1 were detected in wild-type cells. These and other data suggested that 5' exonuclease trimming might be involved in *petD* 5' end maturation and also in normal mRNA decay [42]. Although the finding of a chloroplast 5' to 3' exonuclease was novel, this type of activity is well-known from studies of cytosolic mRNA decay, and in yeast, it is encoded by two genes called *XRN1* and *RAT1* [43, 44]. Homologues have been found in other species including *Arabidopsis* (Genbank accession no. AAD25627), although this *Arabidopsis* protein does not appear to contain a chloroplast transit peptide.

5. 5' end maturation and 5' UTR binding proteins

Chloroplast transcription yields mRNAs lacking a 5' cap, whether they are primary transcripts or processing products derived from polycistronic precursors. However, the 5' ends of primary vs. processed transcripts can be distinguished because the primary transcripts contain a terminal di- or triphosphate group that can be capped in vitro with labeled GTP and guanylyltransferase [45], whereas 5' ends resulting from ribonucleolytic cleavage have free hydroxyl groups which cannot be capped. In spinach, for example, *psbB* mRNA has two 5' ends, one corresponding to the transcription initiation site and the other to a processing site [46]. This pattern is found for many chloroplast transcripts, although in *Chlamydomonas* it has not been possible to cap any mRNAs, consistent with all accumulating mRNAs resulting from processing. The best evidence for this has been obtained with *petD*, where endonucleolytic cleavage, and possibly 5' to 3' exonuclease trimming, have been implicated in 5' end maturation [42, 47]. In cases where two 5' ends are found for *Chlamydomonas*, only the shorter of the two RNAs appears to be competent for translation, based on mutagenesis studies with transcripts such as *psbD* [41] and *psbA* [48].

Since proteins that bind to the 5' UTR may regulate processing or RNA stability, such binding activities have been sought and characterized. One of these proteins, which binds to the spinach *psbA* 5' UTR in vitro, was identified as a homologue of the *E. coli* ribosomal protein S1, consistent with a role in translation initiation [49]. Proteins that bind to the *Chlamydomonas psbA* 5' UTR in vitro include a 47 kDa protein with homology to poly(A) binding proteins found in the cytosol [50, 51] and a disulfide isomerase [52]. It has been speculated that binding of the *psbA* protein complex couples *psbA* processing and translation [48]. The same 47 kDa protein also appears to bind to the *Chlamydomonas psbC* 5' UTR in vitro, and along with other RNA-binding proteins including a known splicing factor [53], is associated with unusual low density membranes [54]. Other studies have sought proteins with 5' UTR binding activity by using UV-cross-linking assays. By comparing binding to different substrates, correlations between binding and function can be hypothesized, for example for the spinach ATPase gene cluster [55] and for various *Chlamydomonas* RNAs [56, 57]. Ultimately, proof of function will require reverse genetic studies; for example, some intriguing data recently showed that some *Chlamydomonas* nuclear mutants lacking *psbA* 5' binding activity in vitro also failed to translate D1, the protein encoded by *psbA* [48].

6. Intercistronic processing

The enzymes involved in intercistronic mRNA processing, i.e., the creation of shorter transcripts from primary polycistronic transcripts, are thought to include both endo- and exonucleases. It might be asked whether this processing has a functional significance, since in bacteria all proteins can be translated from a primary polycistronic transcript. The tobacco in vitro translation system has been used to analyze the importance of processing within the *psaC-ndhD* intergenic region [15, 58]. In tobacco, this dicistronic mRNA is processed, and in vitro, neither *psaC* nor *ndhD* can be translated from the dicistronic mRNA. Two regions of the mRNA were identified, one in the *psaC* coding region and the other in the *ndhD* 5' UTR, that could potentially base pair and thus form a structure that inhibits translation. Support for this model was obtained by mutating the *ndhD* element (now unable to base-pair with the *psaC* element) which rendered the mRNA translatable [58]. Mutants with defects in chloroplast intercistronic processing leading to translational blocks have been identified in maize and *Arabidopsis*, and are discussed in another article in this volume [115].

Ribosomal RNAs are also transcribed as precursors that must processed in order for ribosome assembly to occur. In the maize nuclear mutants *cps1* and *hcf7*, for example, maturation of the 23S and 16S rRNAs, respectively, are affected [59]. In the ribosome-deficient *Chlamydomonas* mutant *ac20*, there is an increased accumulation of unspliced, and unprocessed 23S rRNA. The primary defect in this mutant was shown to be at the level of RNA processing, specifically failure to process the first internal transcribed spacer [60]. These data underscore the importance of nuclear control of rRNA processing, and the resultant global regulation of chloroplast translation.

7. 3' end maturation and 3' UTR binding proteins

Like their bacterial counterparts, the 3' UTRs of almost all chloroplast mRNAs contain an inverted repeat (IR) that

can form a stem-loop structure. In *E. coli*, these structures promote mRNA stability and also can function as rho-independent transcription terminators [17]. One reason why RNA processing is so prevalent in chloroplasts is that transcription termination at the IRs is very inefficient, resulting in considerable read-through transcription of sequences downstream [12, 61].

The spinach *petD* gene has been a model for studying mRNA 3' end formation and stabilization in vitro. The mature 3' end is immediately downstream of an IR and is generated by processing of *petD* pre-mRNA, either by a 3' to 5' exonuclease activity [12, 62], or by endonucleolytic cleavage followed by exonuclease trimming [24]. Gel mobility shift assays identified a complex of three proteins (CSP for chloroplast stem-loop binding protein) that bind to *petD* pre-mRNA, specifically to the stem-loop region and a downstream AU-rich element termed Box II; these proteins are CSP55, CSP41, and CSP29 [27, 63]. CSP41, discussed above, has both RNA-binding and endonuclease activities, which are likely modulated by its associations with the other CSPs. CSP55 may be related to a 54 kDa protein from mustard chloroplasts, which binds to the 3' UTR of *rps16*, *trnK* and *trnH* mRNAs and whose activity is modulated by phosphorylation and redox state [23, 29, 64]. Another study suggested that additional proteins interact with the *petD* 3' UTR, including a 33 kDa protein with consensus RNA-binding domains, PNP, and a 67 kDa putative RNAse E homologue, also discussed above [24].

3' end maturation has also been studied in detail in *Chlamydomonas*, focusing on *atpB* mRNA. In this case, a two-step process entails endonucleolytic cleavage followed by exonuclease trimming [14]. Surprisingly, extensive mutagenesis of the endonuclease cleavage site, while in some cases interfering with in vitro processing, did not affect *atpB* mRNA in vivo [65]. This suggests that multiple pathways can lead to the same result. This conclusion is consistent with other studies showing that large deletions downstream of the *atpB* 3' IR do not necessarily abrogate mRNA accumulation [66]. One interpretation is that regardless of where transcription terminates or endonucleolytic processing first occurs, 3' to 5' exonucleases can generate an identical mature 3' end. This model would also apply to 3' ends generated by internal processing of polycistronic precursors.

8. mRNA splicing and editing

8.1. Splicing

As noted above, many chloroplast genes contain introns, the majority of which are *cis*-spliced, however a few, such as *rps12* in vascular plants, are spliced in *trans* [67]. In vascular plants, the majority of introns are of group II, and splicing of group IIa introns requires ribosomes and/or translation; splicing is impaired in mutants lacking ribosomes [18, 68–70]. However, in *Chlamydomonas*, most of the introns are of group I. Although some *Chlamydomonas* group I introns can self-splice in vitro [3], splicing under physiological conditions has not been observed, suggesting that *trans* factors are required in vivo [3, 18]. Some mRNAs are edited as well as spliced, and these reactions are independent [19].

The most complex example of organellar splicing is *Chlamydomonas psaA* mRNA, which contains three exons. Maturation of *psaA* mRNA requires at least 14 nuclear loci and one chloroplast-encoded RNA molecule (*tscA*), which is proposed to form part of the intron structure linking exons I and II [71, 72]. The *psaA* pathway is rivaled in intricacy by so-called twintrons, widespread in *Euglena* chloroplasts. Twintrons require sequential splicing of an intron within an intron, for example a group II intron split by a group III intron [73]. Additionally, in *Euglena* some chloroplast introns encode maturase-like proteins, which themselves may contain additional introns [74]. Since a reliable in vitro splicing system has yet to be established, the protein factors involved have been largely identified through analyses of splicing mutants (see Barkan and Goldschmidt-Clermont [115]). However, a recent study found that antibodies directed against certain tobacco chloroplast RNA-binding proteins could immunoprecipitate not only mRNAs but also intron-containing precursor tRNAs [75]. This is a promising approach to studying RNA-binding protein function.

8.2. Editing

Editing in chloroplasts entails C to U changes that generally create start codons (*ndhD* and *rps14*), or reintroduce a conserved amino acid into the protein [19]. A role in transcriptional regulation has been suggested, since partial editing of a key residue in *rpoA*, which encodes the alpha subunit of the chloroplast-encoded RNA polymerase, was observed [76]. This implies that the degree of *rpoA* editing could influence RNA polymerase activity. Editing is largely confined to coding regions, but in one case editing in an untranslated region was found, in the primitive angiosperm *Ginkgo biloba* [77]. This type of editing might be involved in maintaining certain RNA secondary structures, akin to the role of editing in forming introns in plant mitochondria [78–80]. Analysis of editing sites has not revealed a clear consensus sequence apart from bias at positions –1 and + 2 [76]; however, this does not exclude the possibility of a canonical RNA secondary or tertiary structure which is recognized by the editing apparatus. As a reliable in vitro editing system has not been reported for chloroplasts, elucidation of *cis* sequences required for editing has been performed in vivo using chloroplast transformation [81, 82]. These data are reviewed by Bock [116] in another article in this volume.

9. Chloroplast RNA stability

9.1. 5' mRNA stability determinants

To date, mRNA stability determinants have been localized to the untranslated regions. The role of the 5' UTR has been primarily studied in *Chlamydomonas* due to the existence of several nuclear mutants with defects in mRNA stability, and the ease with which reporter gene fusions can be introduced into its chloroplast. In all cases examined, namely *psbB* [83], *psbD* [84] and *petD* [38], it was found that the 5' UTR was necessary and sufficient to respond to the nuclear genotype, suggesting that the nuclear gene encodes a protein which promotes RNA stability by binding to the 5' UTR. A reciprocal prediction is that mutagenesis of the 5' UTR should destabilize the mRNA if the binding site for the nuclear factor were disrupted. This prediction has been confirmed for *psbD*, where small deletions or base changes can destabilize the transcript in a wild-type nuclear background [41], and for *petD*, where mutations between two and nine nucleotides downstream of the mature 5' end cause RNA instability [85].

Two other aspects of 5' UTR stability determinants merit consideration, namely RNA secondary structures and RNA-binding proteins. To date, most secondary structure predictions have been based on computer folding programs and in certain cases are supported by genetic or mutagenesis data, however, these studies have concentrated on identifying translation elements (see article by Zerges [117]). A different approach was recently taken to study the 5' UTR stability determinant of *Chlamydomonas petD* mRNA mentioned above. *Chlamydomonas* cells were infiltrated in vivo with dimethyl sulfate, which methylates C and A residues not obscured by proteins or participating in base pairing. Primer extension is then carried out, and since reverse transcriptase is blocked by methylated bases, base paired or protein binding residues can be identified [86]. After analyzing the structural data along with results from site directed or deletion mutagenesis, it could be suggested that a small terminal stem-loop structure forms in *petD* mRNA and plays a role in protein binding [85]. While interpreting in vivo modification data can be challenging, unlike computer programs they do not rely solely on energetics to predict secondary structures.

Biochemical analyses of *Chlamydomonas* 5' UTR stability proteins have been limited to *psbD*, where it was shown that in the *mbd1-nac2-26* mutant, *psbD* mRNA is unstable and there is a loss of binding of a 47 kDa protein [84, 87]. How these genetically defined proteins protect the mRNA from being degraded and promote 5' end maturation, either directly, by physically binding to the mRNA, or indirectly, possibly by modulating another protein's activity, still needs to be determined. Eventually, a synthesis of structural, biochemical and genetic data will most likely reveal complex regulatory networks that link RNA processing, stability and translation.

9.2. 3' mRNA stability determinants

The role of the 3' IR in RNA stability was mentioned above, and its importance in vivo has been demonstrated by deletions in both the *atpB* and *psaB* 3' UTRs of *Chlamydomonas* [66, 88]. However, if a stable secondary structure such as the spinach *petD* 3' IR or pG was substituted for the *atpB* 3' IR, a discrete-sized mRNA accumulated and normal gene expression was observed [40, 66]. These data suggest that there is not an absolute sequence requirement. On the other hand, some 3' IRs have an orientation-dependent function [89, 90], suggesting that a secondary structure alone is not sufficient, probably because some IRs in the antisense orientation are sensitive to nucleolytic attack.

In addition to the 3' UTR binding proteins that are thought to participate in RNA processing (discussed above), several proteins that may be involved in regulating RNA stability via the 3' UTR have also been characterized. For example, 37 kDa and 38 kDa proteins were found to bind specifically to the barley *psbA* 3' UTR, upstream of the IR [91]. Since *psbA* mRNA stability increases in the light [92], it is possible these proteins play a regulatory role. Another hint that such proteins exist derives from mutants such as *Chlamydomonas crp3*, a nuclear lesion which suppresses the conditional non-photosynthetic phenotype of an *atpB* 3' IR deletion mutant [93]. Characterization of *crp3* showed that it affected 3' end formation of several other mRNAs, particularly *cemA* and *psbI* of the *atpA* gene cluster, in addition to stabilizing the *atpB* mRNA [93, 94]. Thus, CRP3 appears to participate both in RNA processing and stabilization.

9.3. Polyadenylation

A newly (re-)discovered aspect of chloroplast RNA stability is 3' end polyadenylation. Although chloroplast polyadenylation was first reported more than 20 years ago [95], PCR techniques have recently been combined with in vitro assays to paint a far more detailed picture (for reviews see [35, 96]). The results, in general, suggest that as in bacteria [31, 97, 98] and plant mitochondria [99, 100], polyadenylation in chloroplasts marks an RNA for degradation [101, 102]. This conclusion is based on in vitro assays showing instability of adenylated versus non-adenylated molecules, and is consistent with their apparently extremely low abundance, as evidenced by the fact that they have only been detected using reverse transcriptase-PCR including more than 30 PCR cycles.

In spinach, although some polyadenylation was observed at the *psbA* mRNA mature 3' end, the majority of addition sites were found in the coding region [102], or near the start of the 3' UTR [101]. Several of these sites perfectly matched endonucleolytic cleavage sites of the corresponding mRNA that were mapped during in vitro degradation assays [102]. In contrast, in *Chlamydomonas*, the majority of poly(A) addition sites mapped for *atpB*

were either at the mature 3' end or at the slightly downstream endonuclease cleavage site [103]. Taken together with data for *petD*, 5S rRNA and *trnE*, it could be concluded that in *Chlamydomonas* chloroplasts, precursor, processed or partly degraded RNAs could all serve as substrates for the polyadenylation machinery. Because only a few RNAs have been examined to date, it is not clear whether the patterns in these organisms are fundamentally different.

In terms of the role of polyadenylation in chloroplast mRNA decay, in vitro experiments in spinach, but not in *Chlamydomonas*, suggested that polyadenylated transcripts compete with nonadenylated transcripts for PNP as well as for the other 3' to 5' exoribonucleases, and that PNP subsequently reads through stable structures such as stem-loops, providing a strong 3' to 5' component of RNA decay [101, 102]. For spinach it was shown that this competition results from PNP having a relatively high affinity for poly(A), as does the bacterial enzyme [34, 104]. In experiments where chloroplasts were treated with the polyadenylation inhibitor cordycepin, it was found that exonucleolytic degradation was inhibited, resulting in the accumulation of endonucleolytic cleavage products [105]. These experiments demonstrate that polyadenylation plays a major role in targeting endonucleolytic cleavage products for degradation.

9.4. mRNA stability regulation

The earliest interest in chloroplast mRNA stability determinants arose from experiments which showed that some light regulated mRNA accumulation results from post-transcriptional rather than transcriptional processes, particularly for *psbA* [22, 106–108]. Indeed, in vivo measurements of RNA half-life showed a wide range in chloroplasts of both barley [92, 107] and *Chlamydomonas* [109], from around 30 min for *Chlamydomonas tufA* to days for *psbA* in light-grown barley. In addition, RNA stabilities varied during either plant development [110] or during light-dark cycles for *Chlamydomonas* cells, suggesting a role for RNA stability regulation during chloroplast biogenesis or in response to photosynthetic capacity, for example redox poise [111]. Because RNA stability can be (but is not inevitably) coupled to translation rates, it can be difficult to define *cis* elements important for one process versus the other. Certainly, however, the 5' UTR is a prime candidate for a developmentally regulated RNA stability determinant. On the other hand, so far chimeric genes have not ascribed such a role to the 3' UTR [41, 112, 113].

9.5. A model for chloroplast mRNA degradation

Any regulatory process acting on RNA decay cannot be interpreted without understanding the decay pathway and its rate-limiting steps. Our working model *(figure 1)* hypothesizes that the initial event is an endonucleolytic cleavage, which produces two RNA molecules, the upstream of which lacks the protective 3' IR. Since RNAs ending in a stem-loop structure were poorly polyadenylated in vitro [102], we suggest that the proximal, but not distal fragment is polyadenylated, setting the stage for degradation of the proximal fragment by PNP and/or other exonuclease(s). Degradation of the distal fragment could proceed by one of two mechanisms, which are not mutually exclusive. In the first, a 'wave' of additional endonucleolytic cleavages would follow the initial one, yielding new proximal fragments that would undergo polyadenylation and degradation. This mechanism is consistent with the strong effect of the polyadenylation inhibitor cordycepin on in vitro RNA decay in the spinach system [105], and with extremely rapid degradation of the downstream product of *atpB* 3' endonucleolytic processing in *Chlamydomonas* [14]. In an alternative mechanism, the distal part might be attacked by a 5' to 3' exonuclease activity. This mechanism is supported by the efficiency of this activity in degrading chloroplast mRNA in mutants such as *mcd1* [38], and its known activity in wild-type cells [42].

Either version of this model suggests two ways to regulate the half-life of an RNA molecule. The first is based on the assumption that once an mRNA molecule is endonucleolytically cleaved, it is rapidly targeted for degradation. In this case, the rate-limiting step would be the initial endonucleolytic cleavage, and the nature, specificity and modulation of the activity and/or expression of the endonuclease(s) would determine the half-life. Accordingly, the activities of the poly(A) polymerase and exonuclease(s) would not be rate limiting. Alternatively, a step following endonucleolytic cleavage could be rate-limiting. For example, it is still unknown if poly(A) tail length, which can amount to several hundred nucleotides, influences the degradation rate. Furthermore, the guanosine residues characteristic of the poly(A) tails of spinach chloroplast *psbA* mRNA, but which were not found in *Chlamydomonas*, may be involved in modulating degradation rates. On the other hand, they may simply reflect the specificity of the poly(A) polymerase(s) [102, 114]. In vitro experiments, in which synthetic transcribed RNA with poly(A) tails of different lengths and different proportions of guanosine residues were incubated with chloroplast protein extract, revealed remarkable differences in degradation rates (R. Rott and G. Schuster, unpublished data). The significance of these results in relation to the in vivo situation is still unclear and awaits further investigation.

10. Conclusions

Over the past few years, our understanding of chloroplast mRNA processing and degradation has progressed significantly. In many respects, these processes are similar to those of bacteria. Chloroplast gene expression, however, is regulated and coordinated by the nucleus, and the

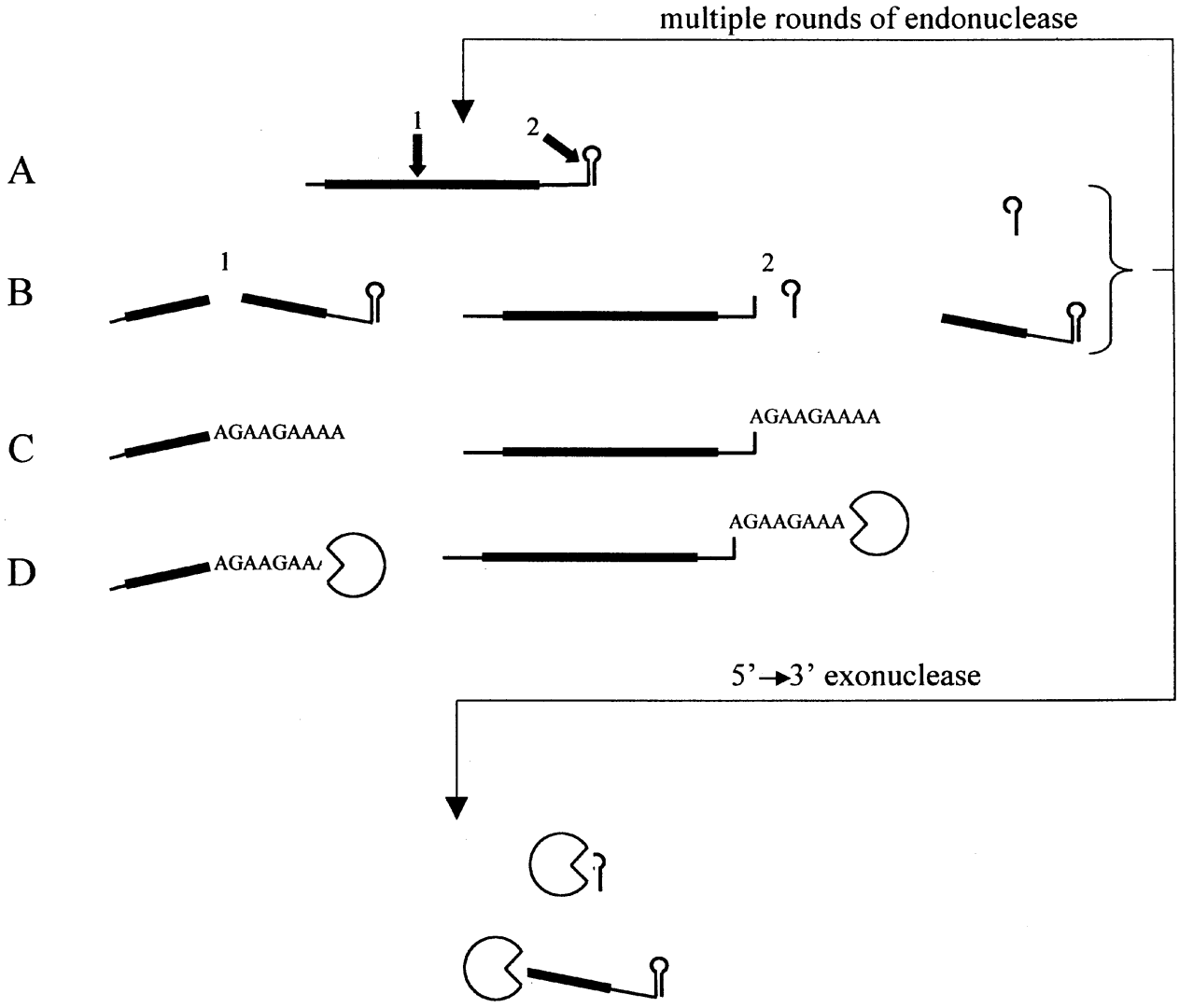

Figure 1. A working model for chloroplast mRNA degradation. The scheme is shown for a typical chloroplast mRNA with a 3' IR, such as spinach *psbA* or *Chlamydomonas atpB*. The thick portion represents the coding region. **A.** Initially, endonuclease attack occurs within the coding region (1) or the 3' IR (2), catalyzed by enzymes such as those discussed in the text. **B.** This cleavage yields proximal and distal products. The distal products are subject to further rounds of endonucleolytic cleavage (upper arrow), or may be degraded by a 5' to 3' exonuclease activity (lower arrow). **C.** The proximal products are efficiently polyadenylated with a tail up to several hundred nucleotides in length, either containing some proportion of guanosine (as in spinach) or without guanosine (as in *Chlamydomonas*). **D.** The polyadenylated RNA molecule is rapidly degraded by exonuclease(s), such as PNP.

half lives of its mRNAs more closely reflect the properties of plants than of bacteria. In addition, the 5' to 3' exonuclease activity and 5' UTR binding proteins found in chloroplasts have no known counterparts in bacteria. Therefore, the chloroplast has probably adopted an intermediate position by combining these different features, and will continue to evolve in a unique direction.

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