

Chloroplast mRNA 3'-end processing by a high molecular weight protein complex is regulated by nuclear encoded RNA binding proteins

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In the absence of efficient transcription termination correct 3'-end processing is an essential step in the synthesis of stable chloroplast mRNAs in higher plants. We show here that 3'-end processing *in vitro* involves endonucleolytic cleavage downstream from the mature terminus, followed by exonucleolytic processing to a stem-loop within the 3'-untranslated region. These processing steps require a high molecular weight complex that contains both endoribonucleases and an exoribonuclease. In the presence of ancillary RNA binding proteins the complex correctly processes the 3'-end of precursor RNA. In the absence of these ancillary proteins 3'-end maturation is prevented and plastid mRNAs are degraded. Based on these results we propose a novel mechanism for the regulation of mRNA 3'-end processing and stability in chloroplasts.
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Introduction

The control of plastid mRNA stability is an important regulatory step during the development of the chloroplast from proplastids (Deng and Gruissem, 1987; Mullet and Klein, 1987; for reviews see Gruissem and Schuster, 1993; Gruissem and Tonkyn, 1993). During this transition the relative transcription rates of many plastid genes do not change significantly. However, there is a rapid accumulation of several mRNAs, due to a significant increase in their half-lives. It is likely that in the proplastid these mRNAs are synthesized and then rapidly degraded and that at the onset of chloroplast biogenesis these mRNAs become stabilized. Stabilization is thought to occur because the relevant mRNAs are correctly processed; most plastid mRNA precursors in higher plants undergo a series of complex maturation events that includes splicing of introns, cleavage of polycistronic RNAs and 5'- and 3'-end processing. As with animal and yeast mRNA 3'-end maturation, incorrect processing of plastid mRNA at the 3'-end is likely to result in rapid RNA degradation (Wahle and Keller, 1992; Gruissem and Schuster, 1993; Higgins *et al.*, 1993; Beelman and Parker, 1995; Keller, 1995).

A general characteristic of the 3'-untranslated region

(3'-UTR) of plastid mRNAs is an inverted repeat (IR) sequence that can fold into a stem-loop structure (Stern *et al.*, 1989). These stem-loops are similar to structures involved in prokaryotic transcription termination, but rather than efficiently terminating plastid transcription they act to stabilize upstream RNA sequences both *in vitro* (Stern and Gruissem, 1987; Adams and Stern, 1990) and *in vivo* (Stern *et al.*, 1991; Stern and Kindle, 1993). Formation of the *Chlamydomonas reinhardtii atpB* mRNA 3'-end involves endonucleolytic cleavage downstream of the mature terminus, followed by exonucleolytic processing to the stem-loop structure (Stern and Kindle, 1993). This suggests that the stem-loop structure acts as a barrier, preventing further degradation. However, plastid mRNA stability cannot be mediated solely by this secondary structure, because the predicted free energies of the stem-loops do not correlate with the observed stability of particular 3'-IR RNAs *in vitro* or the accumulation of the corresponding mRNA *in vivo*. This observation has led us to suggest that in addition to the stem-loop, nuclear encoded proteins imported into the plastid are involved in the formation and stability of chloroplast mRNA 3'-ends (Stern and Gruissem, 1989; Stern *et al.*, 1989; Hsu-Ching and Stern, 1991a,b).

Using the 3'-UTR of plastid mRNAs as probes in binding assays a characteristic set of proteins ranging from 24 to 100 kDa has been identified that interacts with the 3'-IR from different mRNAs (Stern *et al.*, 1989; Schuster and Gruissem, 1991; Hsu-Ching and Stern, 1991a,b; Nickelsen and Link, 1991, 1993; Chen *et al.*, 1995; reviewed by Gruissem and Schuster, 1993). However, a role for most of these proteins in mRNA 3'-end processing and stability has yet to be demonstrated. An important exception is the nuclear encoded 28 kDa RNA binding protein (28RNP). Depletion of this protein from chloroplast *in vitro* processing extracts (Schuster and Gruissem, 1991) or addition of the recombinant protein (Lisitsky *et al.*, 1995) interferes with mRNA 3'-end formation and results in RNA degradation. However, the purified protein fails to show any of the endo- or exoribonuclease activity required for 3'-end processing, suggesting that 28RNP is not itself a nuclease, but rather interacts with other proteins to direct correct 3'-end processing (Schuster and Gruissem, 1991).

In this paper we describe the purification and dissection of a novel high molecular weight enzymatic complex required for chloroplast mRNA 3'-end processing. This complex contains both a PNPase-like exoribonuclease and a site-specific endoribonuclease. We demonstrate that although this complex is necessary for the formation of a correctly processed and stable mRNA 3'-end *in vitro*, it is not sufficient. Rather, correct processing requires interaction of the complex with specific nuclear encoded RNA binding proteins; in the absence of these proteins

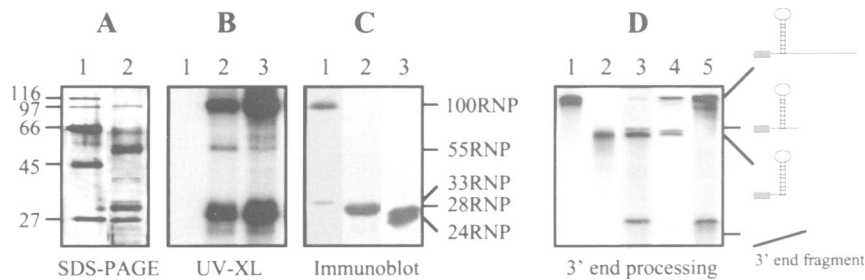


Fig. 1. RNA binding proteins co-purify with a chloroplast mRNA 3'-end processing activity. (A) SDS-PAGE and silver staining of chloroplast proteins fractionated by RNA affinity chromatography (lane 2). Lane 1, Sigma HMW protein markers. Molecular weights are indicated on the left. (B) Identification of RNA binding proteins by UV cross-linking to *petD* mRNA 3'-end precursor RNA. Lane 1, no protein; lane 2, chloroplast proteins fractionated on ssDNA-cellulose; lane 3, chloroplast proteins further fractionated by RNA affinity chromatography. (C) Immunoblot analysis of chloroplast proteins fractionated by RNA affinity chromatography. Lane 1, polyclonal antibodies against 100RNP; lane 2, polyclonal antibodies against 28RNP; lane 3, polyclonal antibodies against 24RNP. (D) Differential labeling shows that 3'-end processing is a two-step reaction. Lane 1, uniformly labeled 300 nt precursor incubated without protein; lane 2, uniformly labeled precursor incubated with protein for 60 min; lane 3, uniformly labeled precursor incubated with protein for 15 s; lane 4, 5'-end-labeled RNA incubated with protein for 15 s; lane 5, 3'-end-labeled RNA incubated with protein for 15 s. The deduced structures of the RNA precursor (top), large intermediate fragment (upper middle), mature product (lower middle) and 3'-end fragment (bottom) are illustrated on the left.

plastid mRNA is rapidly degraded. Regulation of this prokaryote-like enzymatic complex by ancillary nuclear encoded RNA binding proteins represents a unique mechanism of mRNA 3'-end formation in eukaryotic cells and may provide the means for developmental regulation of chloroplast mRNA 3'-end processing and stability.

Results

A highly purified chloroplast fraction processes plastid 3'-ends in a two-step reaction

We have previously shown that a partially fractionated chloroplast extract is capable of correctly processing plastid mRNA 3'-ends and that mRNA segments containing mature 3'-ends accumulate in the protein extract. However, in these experiments the processing extracts contained numerous proteins, many of which are unlikely to participate in mRNA 3'-end maturation (Stern and Gruissem, 1987; Stern *et al.*, 1989; Hsu-Ching and Stern, 1991a,b; Nickelsen and Link, 1991, 1993; Schuster and Gruissem, 1991; Chen *et al.*, 1995). In this study we sought to establish whether 3'-end maturation in higher plants involves both endonucleolytic and exonucleolytic processing steps and to identify those proteins responsible. To this end a fractionation scheme was developed starting with a protein extract from highly purified intact spinach chloroplasts. Total soluble proteins were fractionated by anion exchange chromatography, affinity chromatography on single-stranded DNA-cellulose and then by RNA affinity chromatography. Analysis of the RNA-bound fraction by UV cross-linking to the 3'-UTR of *petD* mRNA and by SDS-PAGE (Figure 1A, lane 2, and B, lane 3) revealed the presence of 100 kDa RNP (100RNP; Hsu-Ching and Stern, 1991a), 33 kDa RNP (33RNP; Hsu-Ching and Stern, 1991a; L.Gabay, I.Lisitsky, A.Kotler and G.Schuster, submitted for publication), 28 kDa RNP (28RNP; Schuster and Gruissem, 1991) and 24 kDa RNP (24RNP; S.Abrahamson, M.Roell, H.Tam, G.Schuster and W.Gruissem, submitted for publication), together with a small number of proteins which seem to have no direct affinity for the *petD* mRNA 3'-UTR. The presence of 28RNP and 24RNP in this fraction was confirmed by immunoblot analysis using antibodies raised against the

28RNP and 24RNP (Figure 1C, lanes 2 and 3 respectively). The 24RNP IgG fraction cross-reacts with 28RNP, presumably because these two proteins share significant regions of homology in the conserved RNA binding domain (S.Abrahamson, M.Roell, H.Tam, G.Schuster and W.Gruissem, submitted for publication). Likewise, polyclonal antibodies raised against 100RNP cross-react with 33RNP, allowing identification of both proteins in the RNA-bound fraction (Figure 1C, lane 1). This cross-reaction is not observed with affinity-purified 100RNP IgG, confirming that 33RNP is not a breakdown product of 100RNP (L.Gabay, I.Lisitsky, A.Kotler and G.Schuster, submitted for publication). As an antiserum against 55RNP is not yet available, we do not know whether the band labeled 55RNP in Figure 1B (lane 3) is the 55RNP found in the single-stranded DNA-bound fraction (lane 2) or a breakdown product of 100RNP.

The RNA-bound fraction was then tested for ability to correctly process 3'-end precursor RNAs to the stable mature RNA 3'-end (Stern and Gruissem, 1987). For these experiments we synthesized a uniformly labeled *petD* mRNA 3'-end precursor of 300 nucleotides (nt) (Figure 1D, lane 1). In a 60 min reaction the transcript was processed to completion into the mature product of 209 nt by the RNA-bound fraction (lane 2). This *in vitro* product has previously been shown to have a 3'-end coincident with the *in vivo* mature mRNA 3'-end (Stern and Gruissem, 1987, 1989; Stern *et al.*, 1989). When the incubation time was reduced to 15 s an intermediate form slightly larger than the mature product was detected (lane 3), together with a fragment of ~80–90 nt. This larger intermediate was detectable with transcripts labeled at the 5'-end (lane 4), suggesting that the difference between the processed form and the intermediate form is a short extension at the 3'-end. However, the 80–90 nt fragment is not observed, suggesting that this fragment corresponds to the 3'-end of the precursor RNA. This was confirmed by labeling the precursor RNA at the 3'-end (lane 5). Therefore, the small and unstable 80–90 nt fragment most likely results from endonucleolytic cleavage 10–20 nt downstream from the mature 3'-end. After cleavage the intermediate form is then processed to the mature form by a 3'→5' exonuclease. Together these results demon-

strate that the RNA-bound fraction is capable of correctly processing plastid mRNA 3'-ends via a two-step reaction which involves an endonuclease cleavage downstream of the 3'-IR, and 3'→5' exonucleolytic processing.

100RNP is a 3'→5' exoribonuclease essential for 3'-end maturation

The RNA affinity-purified fraction is a faithful assay system that reproducibly and correctly processes 3'-ends of *petD* mRNA in a two-step reaction. It is possible to test the involvement of individual proteins in this two-step processing reaction by depleting them from the fraction. To this end the 100RNP-specific IgG fraction (100RNP IgG) and the IgG from the pre-immune serum were attached to protein A beads and used to immunodeplete proteins from the RNA affinity-purified fraction. When the flow-through fractions from both columns were tested for correct 3'-end processing relatively little activity was detected in the 100RNP-depleted fraction (Figure 2A, lane 3). In contrast, normal 3'-end processing was observed in the flow-through from the pre-immune IgG column (lane 2), indicating that the affinity chromatography procedure does not result in non-specific depletion of proteins required for activity. This demonstrates that 100RNP is essential for correct 3'-end processing. Lack of accumulation of mature mRNA 3'-end products in the 100RNP-depleted extract was also found for the precursors of *psbA* and *rbcL* (not shown), suggesting that 100RNP may have a general function in plastid mRNA 3'-end processing and/or stability.

To identify the function of 100RNP in the 3'-end processing reaction the antibody prepared against purified 100RNP was used to screen a spinach leaf cDNA library. The longest cDNA, of ~3.2 kb, was isolated, sequenced and found to encode 781 amino acids of 100RNP (Figure 2B). Comparison of the amino acid sequence with known proteins in the GenBank database revealed a striking homology (43% identity, 63% similarity) among 100RNP and *Escherichia coli* (Régner *et al.*, 1987) and *Photobacterium luminescens* (Clarke and Dowds, 1994) polyribonucleotide phosphorylase (PNPase). The predicted amino acid sequence of 100RNP exceeds that of *E.coli* PNPase by at least 75 amino acids at the N-terminus and likely represents a unique domain of the chloroplast protein. The N-terminal sequence of mature 100RNP derived from protein sequencing (not shown) is not present in the cDNA, indicating that the partial cDNA clone does not contain the transit peptide sequence.

Escherichia coli PNPase is a 3'→5' exoribonuclease which degrades essentially any RNA to nucleoside diphosphates in a processive manner (Littauer and Soreq, 1982). 100RNP shares these properties. Figure 2C compares the effect of partially fractionated chloroplast extracts with purified 100RNP on the precursor RNA (a silver stained SDS-polyacrylamide gel of purified 100RNP is shown in the lower panel, lane P). In contrast to the chloroplast extract, 100RNP processively degrades the template RNA without production of discernible levels of processed 3'-end mRNA. The lower molecular weight product detectable in the 100RNP reactions (labeled X in the lower panel) probably results from pausing by the exoribonuclease in the stem-loop region, a characteristic shared by *E.coli* PNPase (Littauer and Soreq, 1982; Higgins *et al.*, 1993);

unlike the mature product produced by the chloroplast extract, RNA form X is rapidly degraded by 100RNP, with prolonged incubation resulting in complete degradation of both the precursor RNA and RNA form X (not shown).

The reaction products were also analyzed by thin layer chromatography as described by Carpousis *et al.* (1994). In this system RNA and oligonucleotides remain at the origin and nucleoside diphosphates are well separated (Figure 2D). The radioactive product was identified as UDP, which is the expected product of phosphorolysis of [α - 32 P]UTP-labeled RNA by a polyribonucleotide phosphorylase. Together, from the amino acid sequence homology and enzymatic activities of 100RNP, we conclude that 100RNP functions as a PNPase. However, during the *in vivo* mRNA 3'-end processing reaction in the chloroplast this activity must be modified to allow formation of stable mRNA 3'-ends.

100RNP is a component of a high molecular weight complex

To identify proteins that interact with 100RNP which might be responsible for modifying its activity *in vivo* the 100RNP IgG-bound and flow-through fractions were analyzed for RNA binding proteins by UV cross-linking to the *petD* mRNA 3'-UTR and for additional proteins by SDS-PAGE and silver staining. The 100RNP IgG-bound fraction contained 100RNP and 33RNP (Figure 3A, lane 5), together with a small number of additional proteins which are not present in the pre-immune IgG-bound fraction (Figure 3B, lane 2). Immunoblot analysis confirmed that the 24 and 28RNPs are in the 100RNP IgG flow-through fraction and not in the bound fraction (Figure 3C). Most of the 55RNP is also in the 100RNP IgG flow-through (Figure 3A, lane 6); the absence of detectable 55RNP UV cross-linking in Figure 3A (lane 5) suggests that the protein(s) migrating at 55 kDa in Figure 3B (lane 2) is not 55RNP, but some other uncharacterized protein. However, it is possible that the protein is 55RNP, but that it does not UV cross-link to the 3'-end due to the absence of one or more ancillary proteins removed during immunopurification.

To gain an insight into the size of the complex the ssDNA-cellulose-bound proteins were separated by size exclusion chromatography on a Superose 6 column. UV cross-linking to 3'-end precursor RNA revealed the 100 and 33RNPs in Superose 6 fractions 55–60 (Figure 3D), which corresponds to an apparent molecular weight of ~550 kDa. SDS-PAGE and silver staining revealed that these fractions also contained a small number of additional proteins (not shown). These fractions were found to be incapable of correctly processing the *petD* precursor RNA (not shown). 24RNP, 28RNP and 55RNP eluted in fractions corresponding to their apparent molecular weights, confirming that these proteins are not tightly bound to the high molecular weight complex. Similar results were obtained using glycerol gradient centrifugation (not shown). Since 100RNP and 33RNP both interact with RNA, we considered the possibility that their presence in fractions 55–60 was due to the interaction of these proteins with RNA, rather than a direct interaction between the proteins. However, pre-treatment of the ssDNA-cellulose fraction with micrococcal nuclease did not alter the elution profile of 100RNP and 33RNP. Although we cannot

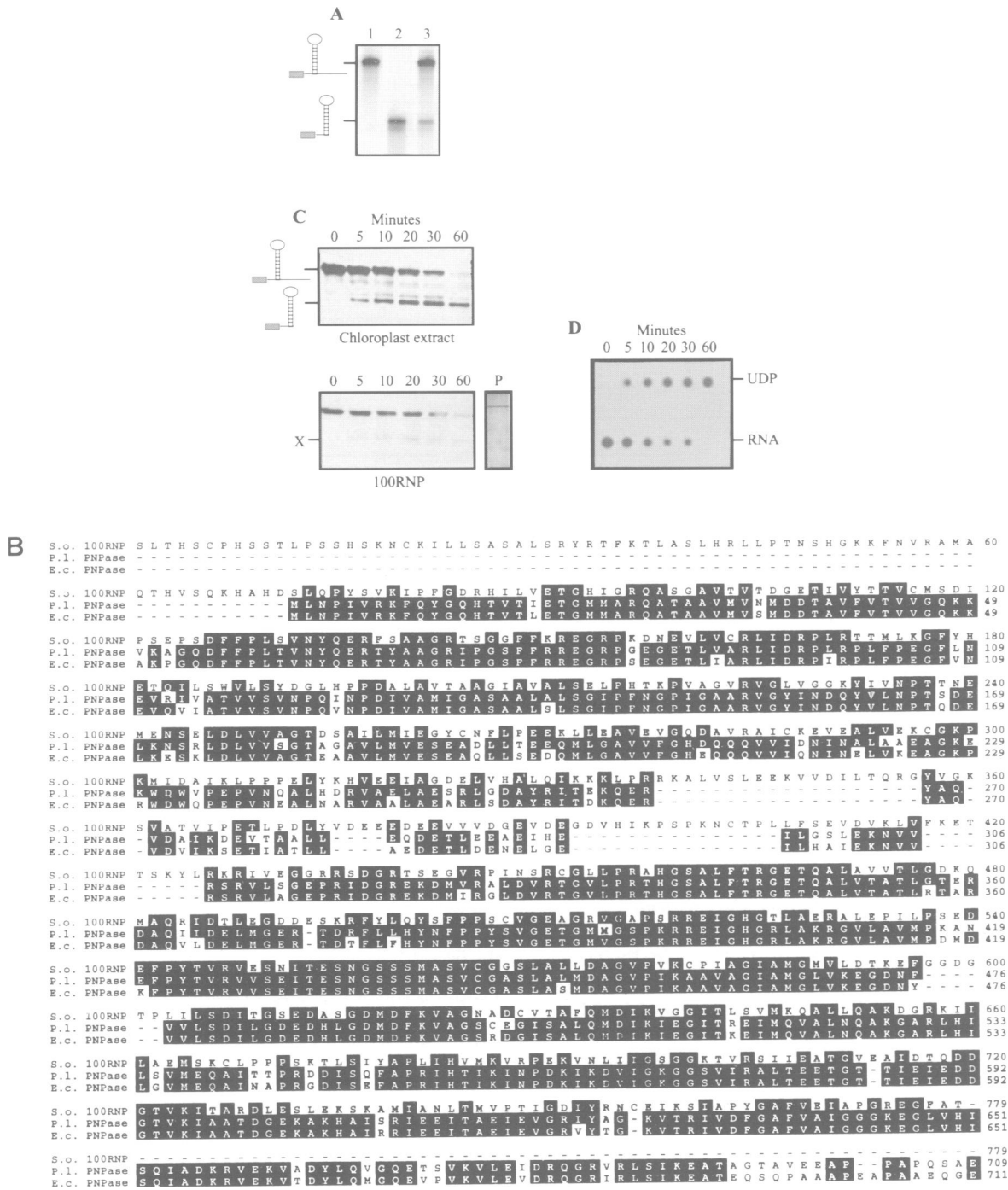


Fig. 2. 100RNP is an essential polynucleotide phosphorylase. (A) Immunodepletion of 100RNP prevents processing of *petD* mRNA 3'-end precursor mRNA. Lane 1, no protein; lane 2, RNA affinity-purified processing extract chromatographed on the pre-immune IgG column, the flow-through proteins being eluted and tested for processing activity; lane 3, RNA affinity-purified processing extract chromatographed on a 100RNP-specific IgG column, the flow-through proteins being tested for processing activity. (B) Amino acid sequence of 100RNP and its homology to PNPase. Alignment of amino acid sequences for 100RNP and PNPase from *E.coli* (Regnier *et al.*, 1987) and *Pluminescens* (Clarke and Dows, 1994) was generated using the CLUSTAL method (Higgins and Sharp, 1989) with DNASTAR software. Sequences are listed as follows: S.o., spinach, P.l., *Pluminescens*, E.c., *E.coli*. Identical amino acids are shown on a black background and similarity to the consensus sequence (≤ 2 map distance units on the PAM 250 weight table; Dayhoff, 1978) is indicated as an open box. Hyphens indicate gaps in the alignment. Amino acid position 72 in 100RNP corresponds to the initiator methionine in *E.coli* and *Pluminescens*. (C) Detection of PNPase activity. Radioactively labeled *petD* mRNA 3'-end precursor RNA was incubated with the chloroplast soluble extract (top panel) or purified 100RNP (lower panel) for the indicated times. The lower molecular weight RNA intermediate thought to be due to pausing by 100RNP is indicated by X. The deduced structures of the precursor and product are shown. Lane P in the lower panel shows a sample of purified 100RNP electrophoresed through a 10% SDS-polyacrylamide gel and silver stained. (D) Detection of PNPase activity. 3'-End precursor RNA uniformly labeled with [α - 32 P]UTP was incubated with 100RNP. Samples were withdrawn at the time points indicated and chromatographed on thin layer chromatography plates as described by Carpousis *et al.* (1994). In this system RNA and oligonucleotides remain at the origin and nucleoside diphosphates are separated. Nucleotide diphosphates were visualized by fluorescence quenching. The radioactive material migrated to exactly the same spot as the UDP marker (not shown).

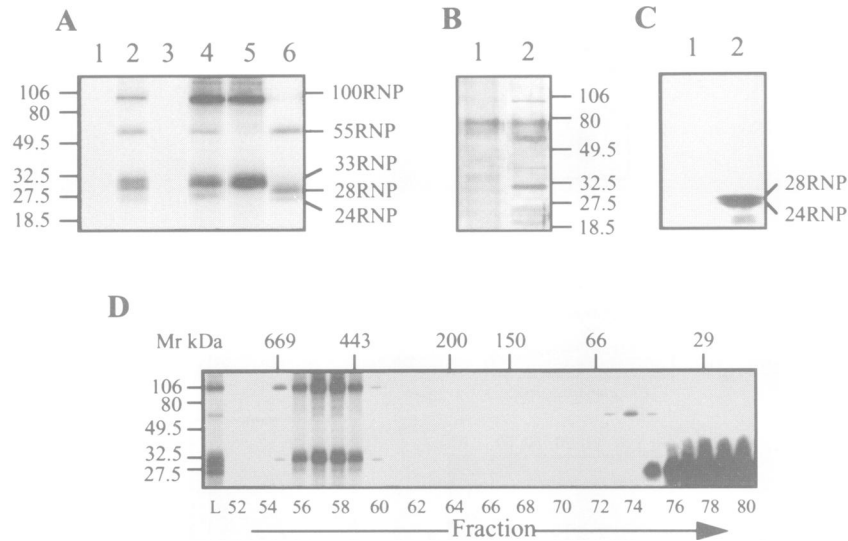


Fig. 3. 100RNP and 33RNP are essential components of a high molecular weight complex. The ssDNA–cellulose-bound 3'-end processing extract was depleted of 100RNP by immunoaffinity chromatography or separated by chromatography on a Superose 6 size exclusion column. (A) Detection of RNA binding proteins eluted from 100RNP IgG affinity columns by UV cross-linking to *petD* mRNA 3'-end precursor RNA. Lane 1, no protein; lane 2, ssDNA–cellulose-bound fraction; lane 3, pre-immune IgG column bound fraction; lane 4, pre-immune IgG column flow-through fraction; lane 5, 100RNP-specific IgG column bound fraction; lane 6, 100RNP-specific IgG column flow-through fraction. Molecular weight markers (kDa) are indicated on the left. (B) SDS–PAGE and silver staining of the pre-immune bound fraction (lane 1) and 100RNP IgG-specific bound fraction (lane 2). Molecular weight markers (kDa) are indicated on the right. (C) Immunoblot analysis of 100RNP-IgG column fractions with antibodies against 24RNP. Lane 1, 100RNP IgG column bound fraction; lane 2, 100RNP IgG flow-through fraction. Note that the 24RNP antibodies cross-react with 28RNP. (D) Size fractionation of the ssDNA–cellulose-bound 3'-end processing extract. The ssDNA-bound processing extract was fractionated by Superose 6 chromatography. Fractions were then assayed for RNA binding proteins by UV cross-linking to the precursor RNA. Fractions are indicated below the figure. L is a sample of the extract taken prior to loading onto the column. Fractions in which the protein standards eluted are indicated above the figure. Molecular weight markers (kDa) are indicated on the left.

exclude the possibility that some RNA is protected from degradation by the proteins, we believe that 100RNP and 33RNP are essential components of a higher molecular weight protein complex.

The 3'-end processing complex also contains a site-specific endoribonuclease

Recent work by Carpousis *et al.* (1994) and Py *et al.* (1994) has demonstrated that *E.coli* PNPase is closely associated with RNase E. It has been proposed that this close interaction may be biologically important during the degradation of *E.coli* mRNAs, allowing rapid turnover of unstable mRNAs (for a review see Cohen, 1995). Interestingly, Wennborg *et al.* (1995) have recently shown that human cells contain a 65 kDa endoribonuclease which can be detected by antibodies raised against RNase E. To investigate whether the chloroplast high molecular weight complex contains an RNase E-like protein we performed an immunoblot analysis of the RNA affinity-bound fraction using an antiserum which had been raised against bacterial RNase E (kindly provided by Dr G.Mackie). As shown in Figure 4A (lane 1), this antiserum detected a single band of ~67 kDa (p67). To confirm this result the experiment was successfully repeated using an antiserum against RNase E from a different laboratory (kindly provided by Dr I.B.Holland; not shown). Pre-immune antiserum from an unrelated rabbit failed to detect p67 (not shown). To demonstrate that p67 is a component of the 100RNP high molecular weight complex p67 was immunodepleted from the RNA affinity-bound fraction as described for 100RNP. The IgG fraction from the RNase E antiserum was immobilized on protein A beads. The column flow-through

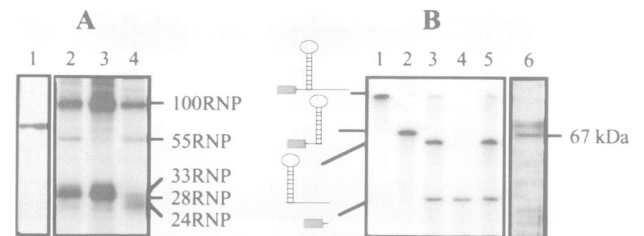


Fig. 4. The high molecular weight complex contains a 67 kDa endoribonuclease which cross-reacts with *E.coli* RNase E antibodies. (A) RNase E antibodies identify a 67 kDa component of the high molecular weight complex. Lane 1, RNA affinity-bound processing extract was fractionated SDS–PAGE and immunoblotted with polyclonal antibodies against RNase E; lanes 2–4, detection of RNA binding proteins eluted from the RNase E affinity column by UV cross-linking to *petD* mRNA 3'-end precursor RNA; lane 2, ssDNA–cellulose-bound fraction; lane 3, RNase E IgG-bound fraction; lane 4, RNase E flow-through fraction. (B) Mapping of the purified p67 endonucleolytic cleavage site in *petD* mRNA 3'-end precursor RNA. Lane 1, uniformly labeled 3'-end precursor RNA; lane 2, uniformly labeled 3'-end precursor RNA incubated with ssDNA–cellulose-bound fraction; lane 3, uniformly labeled 3'-end precursor RNA incubated with p67; lane 4, 5'-end-labeled precursor RNA incubated with p67; lane 5, unlabeled precursor RNA incubated with p67. After incubation the cleavage products were 5'-end-labeled. The products were then electrophoresed through a 8% denaturing polyacrylamide gel. The deduced structures of the precursor and 5' and 3' products are shown. Lane 6 shows a sample of the purified 67 kDa protein electrophoresed through a 10% SDS–polyacrylamide gel and silver stained. The band above p67 is due to contaminating keratin proteins (Ochs, 1983).

and bound fractions were then analyzed for RNA binding proteins. UV cross-linking to the *petD* mRNA 3'-end revealed substantial amounts of 100RNP and 33RNP in the bound fraction (Figure 4A, lane 3), confirming that

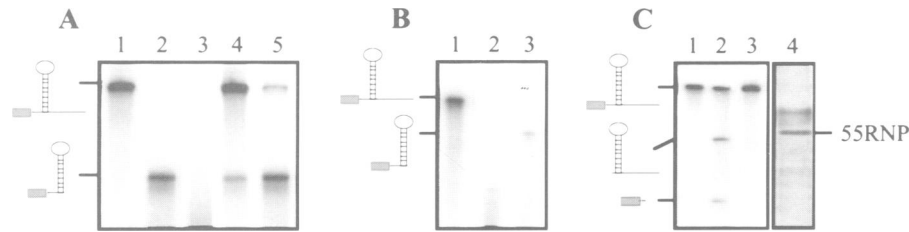


Fig. 5. Correct 3'-end processing is dependent on ancillary nuclear encoded RNA binding proteins. (A) Processing of *petD* mRNA 3'-end precursor mRNA by the high molecular weight complex. Lane 1, no protein; lane 2, ssDNA-cellulose-bound processing extract; lanes 3 and 4, ssDNA-cellulose-bound processing extract chromatographed on a 100RNP-specific IgG column. The bound fraction (lane 3, containing 100RNP, p67 and 33RNP) and the flow-through fraction (lane 4, containing 24RNP, 28RNP and all or most of 55RNP) were then tested for processing activity. Lane 5, The bound (lane 3) and flow-through fractions (lane 4) were pooled and tested for 3'-end processing. The deduced structures of the precursor and product are shown. (B) 28RNP is required for correct 3'-end processing. The precursor RNA was incubated with the 100RNP IgG-bound fraction (lane 2) or the 100RNP IgG-bound fraction in the presence of 28RNP. Lane 1 is a no protein control. The deduced structures of the precursor and 5' and 3' products are shown. (C) 55RNP inhibits endonucleolytic processing by p67. Uniformly labeled *petD* mRNA 3'-end precursor RNA was incubated with p67 (lane 2) or with 55RNP and then p67 (lane 3). Lane 1, no protein; lane 4, a sample of purified 55RNP electrophoresed through a 10% SDS-polyacrylamide gel and silver stained. The band above the 55RNP is due to contaminating keratin proteins (Ochs, 1983).

p67 is present in the high molecular weight complex together with 100RNP and 33RNP. Again, 24RNP, 28RNP and 55RNP were in the flow-through fraction (lane 4).

When purified p67 was incubated with the *petD* mRNA 3'-UTR endonucleolytic cleavage of the RNA was observed (Figure 4B, lane 3). Prolonged incubation had no further effect on the RNA, confirming the absence of contaminating exoribonucleases (not shown). When the RNA was labeled at the 5'-end prior to incubation only the lower molecular weight RNA fragment could be detected after endonucleolytic processing (lane 4). Labeling after the reaction again reveals both fragments, proving that the upper fragment is derived from internal cleavage by p67 (lane 5). From these results we conclude that the p67 is a site-specific endoribonuclease and a component of the high molecular weight complex. Surprisingly, the two resulting RNA products are most likely generated by a single endonucleolytic cleavage of the precursor RNA upstream of the stem-loop structure and not downstream of the 3'-IR, as we had expected. This suggests that p67 may not participate in initial processing of the *petD* mRNA 3'-end precursor. Instead, this protein may perform the first step in the degradation of stable mRNA.

Correct 3'-end processing is dependent on ancillary RNA binding proteins

Immunodepletion of the high molecular weight complex from partially fractionated chloroplast extracts prevents 3'-end processing, demonstrating that it is essential for 3'-end processing (Figure 2A, lane 3). When the high molecular weight complex was eluted from the 100RNP IgG column and tested for processing activity rapid degradation of the precursor RNA was observed (Figure 5A, lane 3). This clearly shows that while the complex is essential, it is not sufficient, for 3'-end maturation. It is possible that the precursor RNA is degraded by contaminating ribonucleases during the elution procedure. However, this can be considered unlikely, because the eluted pre-immune IgG-bound fraction had no detectable effect on the RNA (not shown). To prove that proteins in the 100RNP IgG column flow-through are required to direct correct 3'-end processing by the complex we supplemented the 100RNP IgG-bound fraction containing the complex (lane 3) with the flow-through fraction (lane 4) and then

tested for the ability of these pooled fractions to process precursor RNA into the mature product. As shown in Figure 5 (lane 5), a substantial amount of 3'-end product accumulated. This strongly supports our hypothesis that a protein(s) present in the flow-through fraction regulates the activity of the immunopurified complex.

To identify the protein(s) responsible for regulating processing by the complex we supplemented the 100RNP IgG-bound fraction with specific proteins from the flow-through fraction. To this end we purified 28RNP from Superose 6 fraction 78 (Figure 3D). Addition of this immunopurified protein to the complex partially restored correct *petD* mRNA 3'-end processing (Figure 5B). This confirms that 28RNP can, at least in part, direct correct processing of the 3'-end precursor RNA by the high molecular weight complex.

A substantial amount of 55RNP appears in the flow-through fraction from IgG immunoaffinity columns (Figure 3A) and in Superose 6 column fractions 73-75. This suggests that either 55RNP is loosely bound to the high molecular weight complex and readily dissociated or else that only a small proportion of the protein is bound in the complex. To test if non-bound 55RNP has any effect on processing the 100RNP IgG flow-through fraction was supplemented with 55RNP. No effect was observed (not shown). However, when 55RNP was added to purified p67 endonucleolytic cleavage of the *petD* mRNA 3'-end precursor by p67 was prevented (Figure 5C, lane 3). Therefore, as with 24RNP and/or 28RNP, 55RNP probably has a role in regulating *petD* mRNA 3'-end processing and/or stability.

Discussion

In the course of this work we have purified and characterized a high molecular weight complex involved in chloroplast mRNA 3'-end processing and degradation. We demonstrate that the ability of this complex to produce mature 3'-ends is dependent upon its interaction with additional regulatory RNPs that are loosely or transiently associated with the complex. In the absence of these RNPs correct 3'-end processing does not occur, resulting in mRNA degradation. Regulation of the plastid mRNA 3'-end processing complex by ancillary nuclear encoded RNA binding proteins represents a unique mechanism of

mRNA 3'-end formation in eukaryotic cells and may provide the means for light-dependent regulation of chloroplast mRNA processing and stability.

Plant chloroplast mRNA 3'-end processing involves a prokaryote-like enzyme complex

Our demonstration that chloroplast mRNA 3'-end maturation requires a high molecular weight complex which contains ribonucleases is similar to recent findings for *E.coli* (Carpousis *et al.*, 1994; Py *et al.*, 1994). In *E.coli* PNPase and RNase E form part of a high molecular weight complex that includes several additional proteins of unknown function. Clearly, 100RNP is a PNPase-like enzyme; both sequence homology and functional assays strongly support the idea that the protein acts as a 3'→5' exoribonuclease during plastid 3'-end maturation and degradation. Although the role of p67 in 3'-end processing and/or mRNA stability is still unclear, purified p67 is able to cleave upstream of the *petD* 3'-end mRNA stem-loop. In contrast, the high molecular weight complex containing p67 cleaves the 3'-end downstream of the stem-loop structure (not shown). These results suggest that the complex contains a second endoribonuclease responsible for downstream cleavage. An alternative possibility is that p67 is necessary for downstream cleavage, but that the protein is unable to cleave at this site in its purified form. Evidence for the former possibility comes from a recent finding that dissection of the high molecular weight complex yields a fraction which is capable of downstream cleavage but which does not contain detectable quantities of p67 (R.Hayes, unpublished results).

3'-End processing activity is regulated by ancillary RNA binding proteins

Our highly purified chloroplast extract processes the precursor RNA to a stable product. In contrast, the immunopurified high molecular weight complex degrades precursor RNA. This result implies that the complex requires additional components for 3'-end maturation. Support for this idea is provided by our finding that addition of 24RNP and 28RNP to the immunopurified high molecular weight complex restores correct 3'-end processing. 24RNP has extensive homology to 28RNP, suggesting that the two have similar roles *in vivo*, perhaps at different stages of development (S.Abrahamson, M.Roell, H.Tam, G.Schuster and W.Gruissem, submitted for publication). The 28RNP may interact with the complex via its acidic N-terminus, positioning the complex on the RNA downstream of the 3'-IR to initiate endonucleolytic cleavage and correct processing (Schuster and Gruissem, 1991). To have a role in selective mRNA degradation 28RNP would need to possess sequence specificity. It is likely that 28RNP is similar to other RNPs in having a spectrum of RNA affinities *in vitro*, but a specific RNA binding activity in the cell (Scherly *et al.*, 1990a,b; Kenan *et al.*, 1991; Dreyfuss *et al.*, 1993). The RNA affinity and specificity of an RNA binding protein can be modulated through post-transcriptional modification and/or protein-protein interactions. For example, U2-B'' RNP only binds specifically to U2 RNA in the presence of the non-RNA binding protein U2-A' (Scherly *et al.*, 1990a,b; Dreyfuss *et al.*, 1993). Alternatively, 28RNP may function by destabilizing secondary structures in the vicinity of the

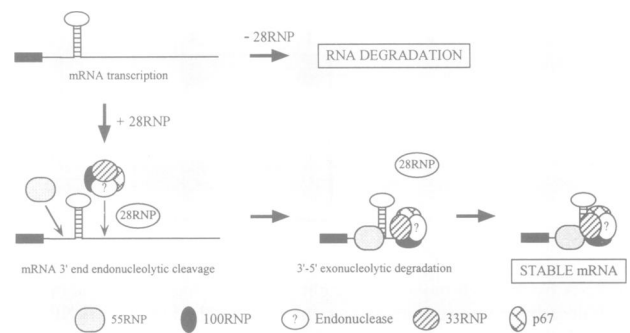


Fig. 6. Model for 3'-end processing of spinach *petD* mRNA. In this model mRNA produced by transcriptional read-through is either processed at the 3'-end by a complex containing 100RNP, p67, another endonuclease (?) and 33RNP to produce stable mRNA (in the presence of 28RNP) or is degraded by the complex (in the absence of 28RNP). The end of the *petD* mRNA open reading frame is indicated by the black box. See text for details.

downstream endonucleolytic site that inhibit cleavage (McDowell *et al.*, 1995).

Role of 55RNP and 33RNP in *petD* mRNA stability

As discussed above, we have shown that p67 is a site-specific endoribonuclease which cleaves *petD* mRNA upstream of the stem-loop structure *in vitro*. This cleavage is inhibited by 55RNP. 55RNP has been shown to bind to an AU-rich motif upstream of the *petD* mRNA stem-loop. Although the recognition site for p67 has not been studied in detail, it is possible that it cleaves at or near this sequence. The role of 55RNP may be to protect the AU-rich motif in the *petD* 3'-UTR from cleavage by p67, thereby determining the half-life of *petD* mRNA *in vivo*. For many prokaryote and eukaryote mRNAs stem-loop structures in the 3'-UTR block the processive action of exoribonucleases, thereby protecting upstream mRNA from degradation (Mott *et al.*, 1985; Newbury *et al.*, 1987; McLaren *et al.*, 1991; Nielson and Christiansen, 1992; Brown *et al.*, 1993; Binder *et al.*, 1994; for reviews see Higgins *et al.*, 1993; Beelman and Parker, 1995). The half-lives of these mRNAs are a reflection of their susceptibility to endonucleolytic cleavage (Belasco *et al.*, 1986; Chen *et al.*, 1988; Melefors and von Gabain, 1988; Chen and Belasco, 1990; Lin-Chao and Cohen, 1991; Régnier and Hajnsdorf, 1991; Bouvet and Belasco, 1992; Hajnsdorf *et al.*, 1994). It is possible that in the absence of 55RNP p67 initiates *petD* mRNA degradation by endonucleolytic cleavage upstream of the 3'-IR. Cleavage at this point would remove the 3' stem-loop, thereby allowing 100RNP within the high molecular weight complex to rapidly degrade upstream RNA. Recently a 47 kDa RNA binding protein was identified in *C.reinhardtii* which is essential for the stability of *psbD* mRNA. This protein binds to a specific segment within the 5'-UTR, thereby protecting the segment from endonucleolytic cleavage (Nickelsen *et al.*, 1994).

Purified 100RNP degrades the *petD* mRNA 3'-end precursor to completion, pausing only briefly at the stem-loop structure (Figure 3B). In contrast, exonucleolytic processing of the 3'-end *in vivo* is terminated at this secondary structure. Exoribonuclease degradation by 100RNP through this region may be prevented by 33RNP,

which has been shown to recognize the double-stranded stem in the presence of 55RNP (Hsu-Ching and Stern, 1991a). Our demonstration that 33RNP is a component of the high molecular weight complex suggests that after the initial endonucleolytic cleavage downstream of the stem-loop the complex degrades the RNA until it reaches the 3'-IR. At this point 33RNP may bind to the stem, thereby halting 3'→5' degradation. Recently a factor which impedes the exoribonucleolytic activity of PNPase at 3' stem-loop structures has been identified in bacteria (Causton *et al.*, 1994). Although this factor has not been characterized in detail, it is known to co-purify with PNPase and therefore may be equivalent to 33RNP.

Plant chloroplasts have a novel type of mRNA 3'-end processing

Our finding that the processing complex is capable of correct 3'-end formation in the presence of the RNPs allows us to propose a model for *petD* mRNA 3'-end maturation (Figure 6). In this model the majority of transcripts are produced with extended 3'-ends due to transcriptional read-through at the 3'-IR. In the effective absence of 28RNP the mRNA precursor would be degraded by the complex, which fails to recognize the structural and/or sequence elements in the RNA that direct specific 3'-end processing. In the effective presence of 28RNP precursor RNA is endonucleolytically cleaved downstream of the mature 3'-end by the complex in association with 28RNP. 100RNP in the complex then exonucleolytically processes the mRNA in a 3'→5' direction to generate the mature 3'-end. Further degradation is prevented by 33RNP and 55RNP, positioned such that 33RNP prevents continued exoribonuclease degradation by 100RNP and 55RNP prevents p67 endonucleolytic cleavage at the upstream sequence. Degradation of this RNA would involve removal of 55RNP, allowing upstream cleavage and preventing binding of the double-stranded stem by 33RNP.

Several aspects of this model will only be clarified by further study. However, it is clear from this work that correct 3'-end processing is dependent on nuclear encoded proteins which moderate the nucleolytic activity of the high molecular weight complex. Additional support for the role of 24RNP and/or 28 RNP in regulating 3'-end processing comes from two recent findings. First, immunoblot analysis demonstrates that although 100RNP is expressed in approximately equal amounts in both young and old leaves, 24RNP and 28RNP levels decrease significantly in older leaves (S.Abrahamson, M.Roell, H.Tam, G.Schuster and W.Gruissem, submitted for publication). We propose that 24RNP and 28RNP are required to moderate the activity of constitutively expressed 100RNP during the greening process, when high levels of correctly processed plastid mRNAs are essential. These proteins would not be required in older leaves during senescence and plastid mRNA degradation. Secondly, phosphorylation of 28RNP changes its affinity for RNA (Kanekatsu *et al.*, 1993; Lisitsky and Schuster, 1995). This phosphorylation step is essential for correct mRNA 3'-end formation and seems to be performed by a 28RNP-specific protein kinase (R.Hayes and W.Gruissem, submitted for publication). These findings provide further evidence that RNA binding by 28RNP is essential to direct

correct 3'-end maturation by the high molecular complex and suggests a mechanism for regulating 3'-end processing and mRNA stability during chloroplast development.

Materials and methods

Protein extract, fractionation and antibody production

Extracts were prepared from chloroplasts purified twice on linear Percoll gradients by the method of Gruijssem *et al.* (1986). Initial fractionation of the extract was carried out according to the following scheme. The protein extract (10 mg) was applied to a 5 ml Bio-Rad EconoQ column and fractionated using a Bio-Rad Automated EconoSystem. The column was developed with a gradient of 60–500 mM KCl in buffer E (20 mM HEPES, pH 7.9, 60 mM KCl, 12.5 mM MgCl₂, 0.1 mM EDTA, 1 mM dithiothreitol and 5% glycerol), as described by Schuster and Gruijssem (1991). Fractions were dialyzed against buffer E and concentrated by centrifugation in a Centricon (Amicon).

Further fractionation was carried out on a 2 ml ssDNA–cellulose column (US Biochemical). Bound proteins were eluted with a linear gradient of buffer E containing 0–500 mM KCl. RNA affinity fractionation using *petD* mRNA precursor RNA was performed as described by Schuster and Gruijssem (1991).

The molecular weights of protein complexes were estimated using Superose 6 size exclusion chromatography or sedimentation through glycerol gradients. For Superose 6 chromatography the Superose 6 column (Pharmacia) was first calibrated using high molecular weight protein markers (Sigma) in buffer E as described by the manufacturer. Protein extract (200 µl) was then applied to the Superose 6 column (Pharmacia) and fractionated at 200 µl/min in buffer E using an automated FPLC (Pharmacia). Glycerol gradient centrifugation was performed as described by Carpousis *et al.* (1994). Protein quantification was done using the Bio-Rad protein assay kit using bovine serum albumin as the standard.

To produce antibodies proteins from FPLC were separated by SDS-PAGE and electroblotted onto nitrocellulose paper. The immobilized proteins were then used as antigens as described by Schuster and Gruijssem (1991). Specific IgG fractions were prepared using affinity chromatography as described by Schuster and Gruijssem (1991).

Preparation of synthetic RNAs, in vitro processing and UV cross-linking analysis

The plasmids used for *in vitro* transcription of the mRNA 3'-ends from spinach chloroplast genes *psbA*, *petD* and *rbcL* have been described (Stern and Gruijssem, 1987; Stern *et al.*, 1989). RNAs were transcribed with T7 RNA polymerase using a Ambion Megascript kit as described by the manufacturer. Transcripts were radioactively labeled with α -³²P to specific activities of 8×10^7 c.p.m./µg for *in vitro* processing experiments or 8×10^8 c.p.m./µg for UV cross-linking analysis. The full-length transcription products were then purified on 5% denaturing polyacrylamide gels.

For end-labeling unlabeled transcripts were synthesized as described above and then labeled with [γ -³²P]ATP using T4 polynucleotide kinase, after dephosphorylation by alkaline phosphatase, to a specific activity of 8×10^7 c.p.m./µg or with [³²P]pCp using T4 RNA ligase to a specific activity of 8×10^7 c.p.m./µg (Sambrook *et al.*, 1989). The full-length transcription products were then purified on 5% denaturing polyacrylamide gels.

In vitro RNA processing experiments and UV cross-linking experiments were performed exactly as described by Schuster and Gruijssem (1991).

Construction and screening of cDNA libraries

The young spinach leaf λ Zap cDNA library (Schuster and Gruijssem, 1991) was screened with the 100RNP antibody using goat anti-rabbit alkaline phosphatase (Stratagene) as secondary antibody. The cDNA obtained by this method was sequenced using the Sequenase 2 kit (US Biochemical) and contained 445 amino acids of 100RNP. This cDNA was then used to rescreen the cDNA library for additional clones. The clone with the largest insert was rescued by superinfection with helper phage (R408) and deletions were generated using exonuclease III. DNA sequencing was performed on ALF using the AutoRead sequencing kit (Pharmacia). Nucleotide and amino acid sequences were analyzed using the GCG (Devereux *et al.*, 1984) and LASERGENE software (DNASTAR Inc., Madison, WI). Databases were searched using the FASTA (Pearson and Lipman, 1988) and BLAST (Altschul *et al.*, 1990) programs.

Detailed pairwise comparison of amino acids was done using the BESTFIT program (Devereux *et al.*, 1984).

Depletion of specific proteins from chloroplast protein extracts and protein analysis

100RNP-specific IgG from the 100RNP antiserum or IgG from the RNase E antiserum were bound to 1 ml protein A coupled to agarose beads (Pierce), washed with TBST buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl and 0.1% Tween 20) until no RNase activity could be detected in the washing solution and then covalently attached to the beads using dimethylpimelimidate (Harlow and Lane, 1988). The IgG-protein A beads (100 µl) were then suspended in buffer E and incubated for 1 h at 4°C with 50 µg chloroplast protein previously fractionated by EconoQ and ssDNA chromatography as described above.

To elute proteins specifically bound to the column the IgG-protein A beads were washed extensively with ImmunoPure Binding Buffer (Pierce). The proteins were then eluted with ImmunoPure Elution Buffer (Pierce) and dialyzed against buffer E.

For immunoblot analysis proteins were fractionated on SDS-PAGE gels and electroblotted to Immobilon P (Millipore). Goat anti-rabbit IgG horseradish peroxidase conjugate (Bio-Rad) was used as a secondary antibody and the cross-reaction was visualized using a chemiluminescence ECL kit (Amersham).

Purification of 100RNP

Soluble chloroplast proteins were fractionated on a EconoQ column as described above. Fractions containing 100RNP were applied to a 1 ml heparin column (Bio-Rad) and the bound fraction which contained 100RNP was eluted with 1 M KCl in buffer E. This fraction, which is incapable of processing *petD* mRNA 3'-end precursor RNA and which does not contain detectable amounts of the 67 kDa protein, was then fractionated on a 1 ml poly(U)-agarose column as described above. The bound fraction was then further purified on a 1 ml MonoQ column (Pharmacia) as described by Schuster and Gruissem (1991). Finally, the protein was separated from any remaining contaminating proteins by chromatography on a Superose 6 column as described above.

Purification of the 67 kDa protein

Soluble chloroplast proteins were fractionated on a EconoQ column as described above. Fractions containing 100RNP were applied to a 1 ml heparin column (Bio-Rad). The flow-through fraction, which contains the 67 kDa and which does not process *petD* mRNA 3'-end precursor RNA, was applied to a 1 ml poly(U)-agarose column as described above. The bound fraction was dialyzed against 50 mM phosphate buffer, pH 7.0, containing 2 M ammonium sulfate and then further fractionated on a 1 ml phenyl Sepharose HP column (Pharmacia). Finally, the protein was separated from any remaining contaminating proteins by chromatography on a Superose 12 column as described above.

Purification of 55RNP

55RNP was purified by chromatography on EconoQ columns as described above and then on heparin and poly(U) columns as described by Nickelsen and Link (1993).

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