

# The Mechanism of Preferential Degradation of Polyadenylated RNA in the Chloroplast

THE EXORIBONUCLEASE 100RNP/POLYNUCLEOTIDE PHOSPHORYLASE DISPLAYS HIGH BINDING AFFINITY FOR POLY(A) SEQUENCE\*

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Polyadenylation of mRNA in the chloroplast has recently been shown to target the RNA molecule for rapid exonucleolytic degradation. A model has been suggested in which the degradation of chloroplast mRNA is initiated by endonucleolytic cleavage(s) followed by the addition of poly(A)-rich sequences and rapid exonucleolytic degradation. When *in vitro* transcribed RNAs were incubated with chloroplast protein extract, competition between polyadenylated and non-polyadenylated RNAs for degradation resulted in the rapid degradation of the polyadenylated molecules and stabilization of their non-polyadenylated counterparts. To elucidate the molecular mechanism governing this effect, we determined whether the chloroplast exoribonuclease 100RNP/polynucleotide phosphorylase (PNPase) preferably degrades polyadenylated RNA. When separately incubated with each molecule, isolated 100RNP/PNPase degraded polyadenylated and non-polyadenylated RNAs at the same rate. However, when both molecules were mixed together, the polyadenylated RNA was degraded, whereas the non-polyadenylated RNA was stabilized. In RNA binding experiments, 100RNP/PNPase bound the poly(A) sequence with much higher affinity than other RNA molecules, thereby defining the poly(A)-rich RNA as a preferential substrate for the enzyme. 100RNP/PNPase may therefore be involved in a mechanism in which post-transcriptional addition of poly(A)-rich sequence targets the chloroplast RNA for rapid exonucleolytic degradation.

Photosynthesis and other essential biosynthetic plant cell activities occur in the chloroplast. The chloroplast structural proteins and enzymes are encoded by both nuclear and chloroplast genomes. During its development, chloroplast gene expression is tightly regulated at many levels, including that of mRNA accumulation (reviewed in Refs. 1–4). RNA metabolism involves a series of steps that are dependent on RNA secondary structures, nucleases, and regulatory RNA-binding proteins (5, 6). Similar to bacterial mRNAs, most chloroplast mRNAs contain an inverted repeat sequence in their 3'-untranslated region that can fold into a stable stem-loop structure (7). Unlike nuclear encoded mRNA, most of the chloroplast mRNAs are non-polyadenylated at the 3'-end in their steady-state condition.

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Several RNA-binding proteins that may be involved in the processing, maturation, and degradation of chloroplast RNAs have been characterized over the past few years. For example, a family of proteins with an RNA-binding recognition sequence motif has been described, of which spinach 28RNP was most prominently characterized. Immunodepletion of this protein from the chloroplast extract or the addition of recombinant protein interfered with the *in vitro* 3'-end processing of chloroplast RNAs (8, 9). RNA binding properties of 28RNP have been studied in detail and show that phosphorylation changes the affinity of the protein for RNA (9–12). 28RNP is not itself a ribonuclease. A search for such an enzyme, involved in the 3'-end processing of chloroplast mRNAs, yielded a 100-kDa RNA-binding protein (13). The purified 100-kDa protein had biochemical properties very similar to one of the two exonucleases so far discovered in bacterial cells, the polynucleotide phosphorylase (PNPase)<sup>1</sup> (14). In addition, the deduced amino acid sequence of the chloroplast 100RNP cDNA disclosed high homology to the bacterial PNPase (13). The chloroplast RNA processing and degradation system is therefore similar to recently discovered mechanisms in *Escherichia coli* (15, 16). However, unlike bacteria, plastid mRNA metabolism and associated enzymes are controlled by the nucleus and may be regulated by both light and the redox state of the chloroplast (13).

Post-transcriptional addition of poly(A) tail to the 3'-end of mRNA has been best characterized in eukaryotic cells for nuclear encoded and viral mRNAs. The long poly(A) tail is an important determinant of mRNA stability and maturation as well as initiation of translation (17–19). Poly(A) tails have also been described for several bacterial mRNA 3'-ends and for endonucleolytic and exonucleolytic sites of the *rpsO* mRNA (20–26). In contrast to the nucleus and cytoplasm of eukaryotic cells, where the poly(A) tail seems to stabilize the nuclear encoded mRNA, the addition of poly(A) tails to bacterial mRNAs promotes their degradation.

Post-transcriptional addition of poly(A)-rich sequences to chloroplast *psbA* mRNA has recently been described (27, 28). Unlike eukaryotic nuclear encoded and bacterial RNAs, the poly(A) moiety in the chloroplast, which may be several hundred nucleotides long, was found not to be a ribohomopolymer of adenosine residues, but rather clusters of adenosines bounded mostly by guanosines and, on rare occasion, by cytidines and uridines (27). When lysed chloroplasts were incubated in the presence of yeast tRNA, thereby inhibiting exonuclease activity, distinct endonucleolytic cleavage products accumulated (29). Several of the endonuclease cleavage sites mapped by primer extension perfectly matched the poly(A)-rich

<sup>1</sup> The abbreviations used are: PNPase, polynucleotide phosphorylase; PAGE, polyacrylamide gel electrophoresis.

addition sites that were analyzed by reverse transcription-polymerase chain reaction, suggesting a degradation pathway for *psbA* mRNA in the chloroplast. In this pathway, the degradation is initiated by endonucleolytic cleavage followed by the addition of poly(A)-rich sequence to the 3'-end of the proximal cleavage product. Once the poly(A)-rich sequence is added, this RNA molecule is rapidly degraded by exonuclease(s) (27). Indeed, when synthetic polyadenylated RNA was incubated with a chloroplast soluble protein extract together with its non-polyadenylated counterpart, the polyadenylated RNA was rapidly degraded. In addition, blocking mRNA polyadenylation with cordycepin inhibited endonucleolytic degradation, and endonucleolytic cleavage products accumulated.<sup>2</sup>

In this study, we show that the chloroplast exoribonuclease 100RNP/PNPase exhibits preferential activity toward polyadenylated RNA. Purified 100RNP/PNPase degraded synthetic transcribed polyadenylated RNAs much faster than non-polyadenylated RNA. We also show this difference to be due to the high binding affinity of 100RNP/PNPase for the poly(A) sequence. These results suggest the preferential affinity of 100RNP/PNPase for poly(A)-rich sequences to be part of the mechanism in which post-transcriptional addition of poly(A)-rich sequence targets the chloroplast RNA for rapid endonucleolytic degradation.

#### MATERIALS AND METHODS

**Plant Growth, Chloroplast Isolation, and Soluble Protein Extract**—Chloroplasts were isolated on Percoll gradients from leaves of hydroponically grown spinach plants (*Spinacia oleracea* cv. Viroflay) under 10.5 h of light and 13.5 h of darkness as described previously (9). A soluble protein extract capable of 3'-end processing of chloroplast RNAs was prepared from isolated intact chloroplasts as described (30).

**Purification of 100RNP/PNPase**—Chloroplast soluble protein extract was fractionated through a size-exclusion Superdex 200 column (Pharmacia Biotech Inc.). Fractions containing protein complexes of 550–650 kDa were pooled and applied to a 1-ml heparin column (Hi-Trap, Pharmacia) developed with a linear gradient of KCl in buffer E (20 mM HEPES, pH 7.9, 60 mM KCl, 12.5 mM MgCl<sub>2</sub>, 0.1 mM EDTA, 2 mM dithiothreitol, and 17% glycerol). Proteins eluted at 0.2 M KCl were dialyzed against buffer E and applied to a Resource Q column (Pharmacia). The column was developed with a linear gradient of KCl in buffer E, and 100RNP/PNPase was eluted at 0.3 M as a single silver-stained polypeptide (see Fig. 2).

**In Vitro Transcription of RNA**—The plasmids used for *in vitro* transcription of parts of the mRNA from the spinach chloroplast genes *psbA* (encoding the D1 protein of photosystem II) and *petDΔ24* (encoding subunit IV of the cytochrome *b<sub>f</sub>* complex) have been described previously (9, 31). To generate RNA containing the *psbA* amino acid coding region (nucleotides 946–1028 according to Zurawski *et al.* (32)) with an additional 14 adenosines at the 3'-end, the reverse transcription-polymerase chain reaction fragment described by Lisitsky *et al.* (see clone 5 in Fig. 1 of Ref. 27) was used. The reverse transcription-polymerase chain reaction fragment was cloned into the pUC57-dT vector (MBI Inc.) and subcloned into pBluescript SK (Stratagene) using *Bam*HI and *Kpn*I restriction sites. To generate the RNA with 14 adenosines at the 3'-end, the plasmid was linearized with *Bam*HI and transcribed using T7 RNA polymerase. The transcribed RNA was then hybridized to an adapter oligonucleotide (27) and digested with RNase H as described previously (9). To generate RNA without the additional 14 adenosines, the above-described RNA molecule was hybridized to an oligo(dT) adapter oligonucleotide (27) and then digested with RNase H. To generate RNA with poly(A) sequence located within the molecule, the RNA transcribed from the plasmid linearized with *Bam*HI was used. Therefore, this RNA contained 18 nucleotides derived from the oligo(dT) adapter oligonucleotide (27) and 5 nucleotides derived from the pUC57 vector, 3' to the 14 adenosines. RNAs were transcribed using T7 RNA polymerase and radioactively labeled with [ $\alpha$ -<sup>32</sup>P]UTP to a specific activity of 8–10 × 10<sup>3</sup> and 8–10 × 10<sup>4</sup> cpm/fmol for RNA degradation and UV cross-linking experiments, respectively (9). The full-length transcription products were purified on 5% denaturing polyacrylamide gels. *In vitro* polyadenylation of RNA was performed by incubating the *in vitro* tran-

scribed RNA with a chloroplast protein extract (1 mg/ml) in buffer E containing 0.5 mM ATP for 30 min (27). The polyadenylated RNA was purified by denaturing PAGE.

**In Vitro RNA Degradation Assay**—*In vitro* RNA degradation experiments were carried out as described previously (27). Briefly, *in vitro* synthesized RNA (2 fmol) was incubated with the chloroplast soluble protein extract (1 mg/ml) or with isolated 100RNP/PNPase (0.4 μg/ml) for the times indicated in the figure legends. Following incubation, the RNA was isolated and analyzed by gel electrophoresis and autoradiography (9).

**UV Cross-linking Assay**—UV cross-linking of proteins to [ $\alpha$ -<sup>32</sup>P]UTP-labeled RNA was carried out as described previously (8–10). Briefly, 3 fmol of RNA (240,000 cpm) were incubated with 20 μg of chloroplast proteins in 15 μl of buffer E. To avoid the poly(A) RNA degradation by 100RNP/PNPase observed in this work, the RNA/protein mixture was immediately exposed to UV light at 4 °C without preincubation at room temperature. Indeed, when UV cross-linking was performed with preincubation at room temperature, no differences were observed in the binding of 100RNP/PNPase to the different RNA molecules (data not shown) (33). Following 1.8 J of UV irradiation in a UV cross-linking apparatus (Hoefer Scientific Instruments), the RNA was digested with 5 μg of RNase A at 37 °C for 1 h, and the proteins were fractionated by SDS-PAGE. The label transferred from the RNA to the proteins was detected by autoradiography and quantified with a Fuji imaging analyzer.

**Antibodies to 100RNP/PNPase**—The 100RNP/PNPase cDNA (13) was subcloned into a pMal-cR1 vector (Bio-Lab Inc.) using the *Bam*HI/*Hind*III sites. The recombinant fusion protein was expressed and purified on amylose resin according to the manufacturer's protocol, with an additional purification step using a heparin column. Polyclonal antibodies were generated in rabbit as described previously (8). Unlike the mature protein, the maltose-binding 100RNP/PNPase-fused recombinant protein exhibited neither RNA binding nor exonuclease activity (data not shown).

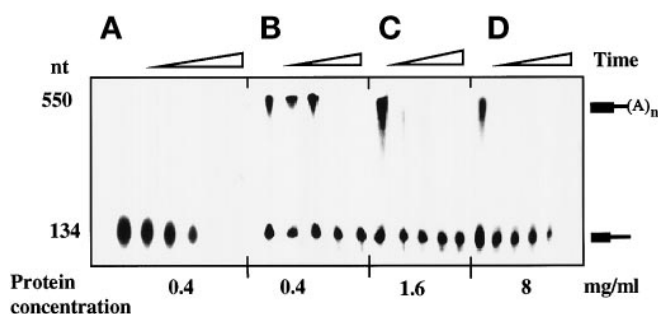
SDS-PAGE fractionation and detection of 100RNP/PNPase on immunoblots probed with specific antibodies were performed as described previously (8). Protein concentration was determined using the Bio-Rad protein assay kit.

**Binding of 100RNP/PNPase to Immobilized Ribohomopolymers**—Chloroplast protein extract (1 mg) was applied to immobilized ribohomopolymer columns (0.1 ml; Sigma). Following extensive washing of the columns with buffer E, the bound proteins were successively eluted with 0.5, 1, and 2 M KCl in buffer E. Proteins were fractionated by SDS-PAGE, transferred to a nitrocellulose membrane, and probed with specific antibodies to 100RNP/PNPase.

#### RESULTS

**Polyadenylated RNA Is Specifically Degraded in Chloroplast Protein Extract**—Chloroplast soluble protein extract has been used to analyze transcription, 3'-end processing, and polyadenylation of *in vitro* transcribed RNA corresponding to chloroplast genes (7, 9, 13, 27, 28, 31, 34, 35). *In vitro* transcribed RNA without a stem-loop structure at the 3'-end is rapidly degraded in this extract (31). In a previous work, we showed that when polyadenylated and non-polyadenylated RNAs were incubated in this extract, the polyadenylated RNA was rapidly degraded, whereas its non-polyadenylated counterpart was stabilized (27). This may have been because the polyadenylated RNA had a higher affinity for the degradation enzyme(s), thereby excluding it from the non-polyadenylated RNA. To verify this hypothesis, the same experiment was performed with increasing amounts of chloroplast extract (and consequently, increasing amounts of RNA ribonucleases). When an RNA molecule corresponding to part of the *petD* mRNA was incubated in the chloroplast extract at a concentration of 0.4 mg/ml, it was rapidly degraded (Fig. 1A). The same RNA was then mixed with its polyadenylated counterpart in such a way that an identical number of molecules were incubated in each reaction mixture. As compared with the system including only non-polyadenylated RNA (Fig. 1A), the polyadenylated RNA was degraded, whereas the non-polyadenylated RNA was stabilized (Fig. 1B). Increasing protein concentrations resulted in accelerated degradation of the polyadenylated RNA, but no

<sup>2</sup> I. Lisitsky, P. Klaff, and G. Schuster, submitted for publication.

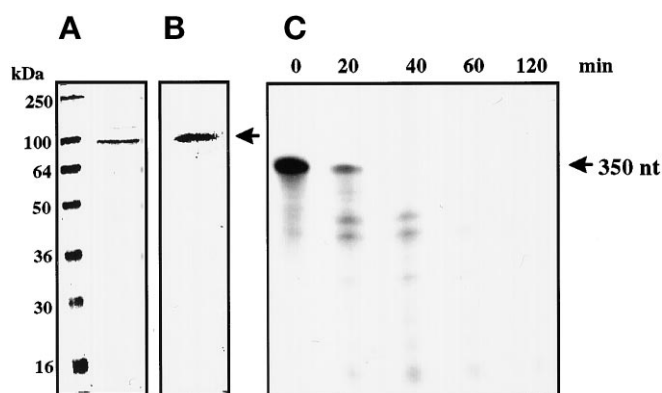


**FIG. 1. Polyadenylated RNA is preferably degraded in a chloroplast protein extract.** *In vitro* transcribed RNA corresponding to part of the *petD* mRNA (*petDΔ24*) was polyadenylated *in vitro* as described under "Materials and Methods." Non-polyadenylated and polyadenylated RNAs were mixed and incubated for 0, 5, 10, 20, and 40 min in a chloroplast protein extract at the protein concentrations indicated. The same number of RNA molecules, either polyadenylated or not, was incubated in each reaction. Following incubation, the RNA was isolated, fractionated by PAGE, and exposed to x-ray film. A schematic representation of the RNA molecules is shown on the right. The **bold line** represents the amino acid coding region, and the **thin line** represents the 3'-untranslated region sequence. *nt*, nucleotides.

degradation of the non-polyadenylated RNA. Finally, at a concentration of 8 mg/ml, 20 times greater than that originally used, the polyadenylated RNA disappeared within 5 min, and the non-polyadenylated RNA was also degraded, albeit more slowly than when incubated alone at 0.4 mg/ml (Fig. 1, A and D). Variance in the stability of the RNAs was not due to the difference in their lengths since the addition of other nucleotides has no such effect (27, 36). Since the two RNAs differed only in the presence or absence of a poly(A) tail, this result suggested that the poly(A)-tail has a higher affinity than non-polyadenylated RNA for the ribonucleases in the chloroplast protein extract. Because we had previously shown the poly(A) tail to be a target for exonucleolytic degradation of RNA (27),<sup>2</sup> we questioned whether a purified exoribonuclease would exhibit such a preference.

**100RNP/PNPase Exonuclease**—A nuclear encoded chloroplast 100-kDa RNA-binding protein exhibiting exoribonuclease activity and amino acid sequence homologous to the bacterial polynucleotide phosphorylase (100RNP/PNPase) has recently been described (13). To analyze the activity of 100RNP/PNPase in degrading polyadenylated RNA and since we were unsuccessful in expressing a recombinant active 100RNP/PNPase in *E. coli*, the enzyme was purified to homogeneity from chloroplast protein extract (Fig. 2A). Purified 100RNP/PNPase consisted of one polypeptide (which sometimes appeared as a doublet) that reacted with specific antibodies generated against the recombinant maltose-binding protein fused to 100RNP/PNPase (Fig. 2B). When the purified protein was incubated with *in vitro* transcribed RNA, the RNA was rapidly degraded (Fig. 2C) (13).

In addition to exoribonuclease activity, the *E. coli* PNPase disclosed *in vitro* RNA polymerase activity when incubated in the presence of nucleosides (14). The polymerase activity is activated when there are sufficient diphosphates and a very low concentration of inorganic phosphate (14). Chloroplast 100RNP/PNPase also showed RNA polymerase activity when incubated with RNA in the presence of ADP (data not shown). This result suggested an interesting possibility: 100RNP/PNPase might also be involved in post-transcriptional addition of poly(A)-rich sequences to RNA in the chloroplast. However, we had previously shown that depletion of 100RNP/PNPase from the chloroplast protein extract did not interfere with *in vitro* polyadenylation activity (27). In addition, a poly(A) polymerase



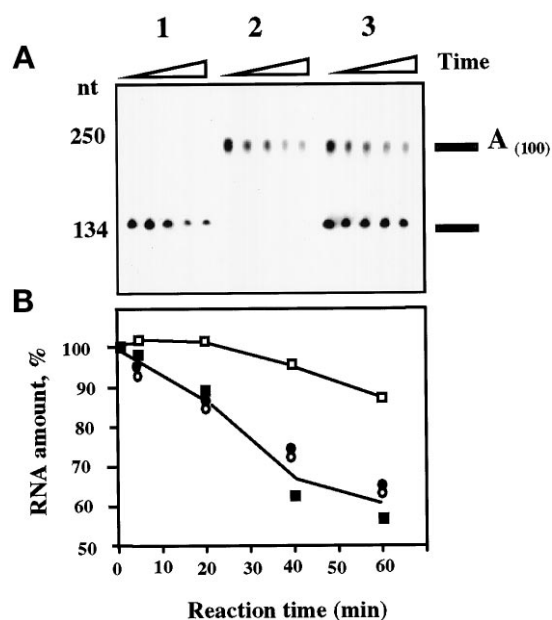
**FIG. 2. Purification of the 100RNP/PNPase exoribonuclease.** 100RNP/PNPase was purified from spinach leaves as described under "Materials and Methods." A, shown is a silver-stained gel of the purified protein. The migration of molecular mass markers is shown on the left. B, shown is a Western blot using specific antibodies to 100RNP/PNPase. C, *in vitro* transcribed RNA corresponding to the 3'-end of *psbA* was incubated with purified 100RNP/PNPase for the times indicated. Following incubation, the RNA was isolated and analyzed by denaturing PAGE and autoradiography. *nt*, nucleotides.

had been purified from spinach chloroplast extract (37).<sup>3</sup> If the chloroplast 100RNP/PNPase activity is modulated similar to the *E. coli* PNPase, then the concentrations of inorganic phosphate and nucleotide diphosphate in the chloroplast would promote the degradation rather than the polymerization activity.<sup>2</sup> Combined, these results show that additional experiments are required to clarify the *in vivo* mode of action of 100RNP/PNPase.

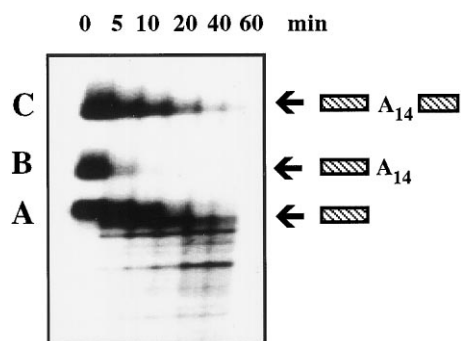
**Polyadenylated RNA Is Specifically Degraded by Purified 100RNP/PNPase**—There are two possible ways of accounting for the observed competition of degradation enzymes for polyadenylated RNA in the presence of chloroplast protein extract (Fig. 1). The preferential activity toward polyadenylated RNA could be a phenomenon intrinsic to the exonuclease polypeptide(s) itself. Alternatively, additional auxiliary proteins that confer this preferential activity may be required. To determine which of these scenarios was valid, competition experiments were performed using the isolated enzyme. *In vitro* transcribed RNA corresponding to part of the *petD* mRNA and the same RNA with the addition of 100 adenosines were incubated with purified 100RNP/PNPase, separately or in combination. As shown in Fig. 3, the RNAs were degraded at similar rates when incubated alone. However, when they were combined, the non-polyadenylated RNA was stabilized (Fig. 3B). Moreover, under our experimental conditions, the polyadenylated RNA was degraded at the same rate when incubated separately or with non-polyadenylated RNA (Fig. 3). Increasing the 100RNP/PNPase concentration led to accelerated degradation of the RNA molecules, similar to the situation shown in Fig. 1 for the protein extract (data not shown) (compare with Fig. 4, where the protein/RNA ratio was 2.5 times higher than that in Fig. 3). These results demonstrated that the purified exonuclease 100RNP/PNPase preferentially degrades polyadenylated RNA and suggested that competition for degradation of polyadenylated RNA is due to the enzyme's intrinsic properties, rather than to auxiliary proteins.

**RNA Is Not Rapidly Degraded When the Poly(A) Stretch Is Not at the 3'-End of the RNA Molecule**—100RNP/PNPase, like the bacterial PNPase, is an exonuclease that processively degrades the RNA from the 3'-end (13, 14). Since the experiments described above suggested that chloroplast 100RNP/PNPase

<sup>3</sup> S. Yehudai, A. Anbussi, I. Lisitsky, and G. Schuster, manuscript in preparation.

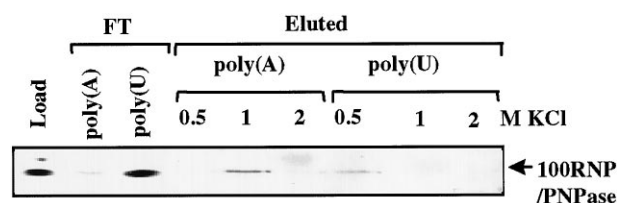


**FIG. 3. Degradation of polyadenylated and non-polyadenylated RNAs by isolated 100RNP/PNPase.** *A*, RNA corresponding to part of *petD* (*petD* $\Delta$ 24) and the same RNA with the addition of 100 adenosines (1.5 fmol of each) were incubated with purified 100RNP/PNPase (0.4  $\mu$ g/ml), and their degradation rates were followed (panels 1 and 2, respectively). In panel 3, 0.75 fmol of each RNA were mixed and incubated with purified 100RNP/PNPase. At 0, 5, 20, 40, and 60 min, the reaction was terminated, and RNA was isolated and analyzed by denaturing PAGE and autoradiography. A schematic representation of the RNA molecules is shown on the right. The narrow rectangles represent *petD* $\Delta$ 24. *B*, the RNA degradation kinetics shown in *A* was quantitated using a Fuji imaging analyzer. White and black squares indicate non-polyadenylated *petD* $\Delta$ 24 RNA incubated together with polyadenylated RNA and alone, respectively. White and black circles indicate polyadenylated *petD* $\Delta$ 24 RNA incubated together with non-polyadenylated RNA and alone, respectively. *nt*, nucleotides.



**FIG. 4. Internal stretch of poly(A) does not trigger rapid degradation by 100RNP/PNPase.** RNAs (0.2 fmol) corresponding to part of the *psbA* mRNA (as described under "Materials and Methods") without poly(A) (designated *A*), with the addition of 14 adenosines (designated *B*), and with the addition of 14 adenosines and 23 other nucleotides (designated *C*) were mixed and incubated with purified 100RNP/PNPase (0.4  $\mu$ g/ml). At the time points indicated, the reaction was terminated, and RNAs were isolated and analyzed by denaturing PAGE and autoradiography. A schematic representation of the RNA molecules is shown on the right.

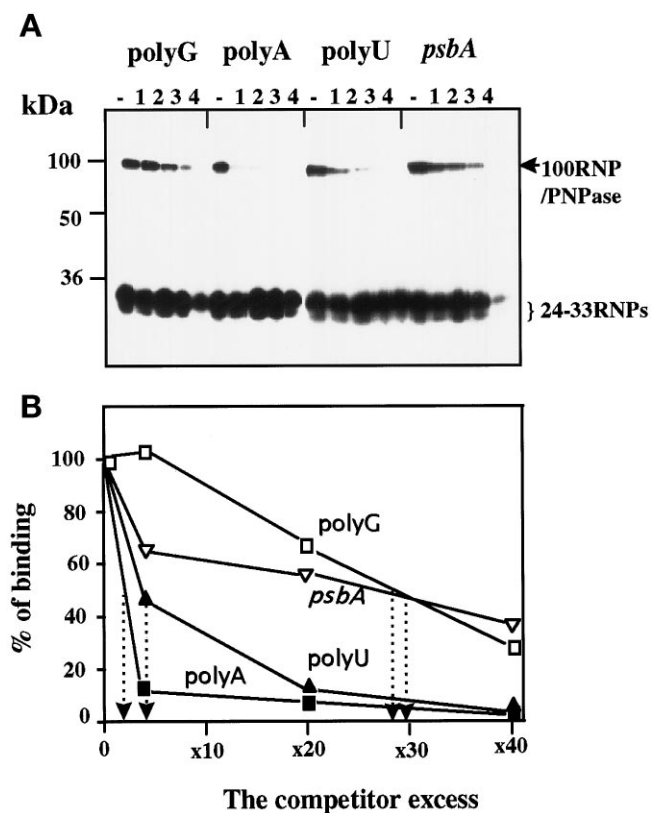
has a higher affinity for polyadenylated RNA, we questioned whether or not the poly(A) stretch needs to be located at the 3'-end of the RNA molecule. To this end, RNA molecules representing part of the *psbA* amino acid coding region with or without the addition of 14 adenosines as well as with the addition of 14 adenosines and 23 bases, including all four nucleotides, at the 3'-end were synthesized (see "Materials and Methods") (marked *A*, *B*, and *C* in Fig. 4, respectively). These molecules were mixed and incubated with purified 100RNP/



**FIG. 5. 100RNP/PNPase binds poly(A) with higher affinity than poly(U).** Chloroplast protein extract was fractionated on a poly(A)- or poly(U)-agarose column as described under "Materials and Methods." The bound proteins were eluted with increasing KCl concentrations of 0.5, 1, and 2 M. Proteins in the loading extract (*Load*) and in the flow-through (*FT*) and salt-eluted (*Eluted*) fractions were fractionated by SDS-PAGE and blotted onto a nitrocellulose membrane, which was decorated with specific antibodies to 100RNP/PNPase.

PNPase. The results presented in Fig. 4 show that the non-polyadenylated RNA and the RNA with the internal poly(A) stretch were degraded at a similar rate, whereas the RNA with the poly(A) at the 3'-end was much more rapidly degraded. Therefore, to compete for 100RNP/PNPase degradation activity, the poly(A) tail needs to be located at the 3'-end of the RNA molecule.

**Binding Affinity of 100RNP/PNPase for Poly(A)**—Two possible mechanisms could explain the preferential degradation of polyadenylated RNA by 100RNP/PNPase. In the first, the enzyme discriminates between substrates because, once bound to the RNA molecule, it degrades polyadenylated RNA much faster than non-polyadenylated RNA. The second mechanism implies similar degradation rates, but higher binding affinity of 100RNP/PNPase for polyadenylated RNA. Therefore, a higher affinity for poly(A) sequence would result in preferential activity toward the polyadenylated molecules when the enzyme concentration is limited. The first potential mechanism can be excluded by the observation that polyadenylated and non-polyadenylated RNAs were degraded at a similar rate when incubated separately with isolated 100RNP/PNPase (Fig. 3). Nevertheless, we intended to determine whether or not this protein displays a higher affinity for poly(A). To measure the binding affinities of 100RNP/PNPase for different RNA molecules, it was fractionated through immobilized ribohomopolymer columns. Chloroplast protein extract was applied to a poly(A)- or poly(U)-agarose column, and the bound proteins were eluted with increasing salt concentrations. The amount of 100RNP/PNPase in both the unbound and bound fractions was detected by probing an immunoblot with specific antibodies. Fig. 5 shows that most of the 100RNP/PNPase was depleted from the extract by the poly(A) column, whereas a much smaller amount was depleted by the poly(U) column. In addition, 100RNP/PNPase was eluted from the poly(A) column with 1 M KCl, whereas a concentration of only 0.5 M eluted the protein from the poly(U) column. No binding activity of 100RNP/PNPase on the poly(G) or poly(C) column was observed under these conditions (data not shown). To define the binding activities of 100RNP/PNPase for different RNA molecules in an additional system, we carried out UV cross-linking competition experiments (9, 10). Chloroplast protein extract was incubated with [<sup>32</sup>P]UTP-labeled RNA corresponding to the *psbA* 3'-end. 100RNP/PNPase is strongly labeled in this UV cross-linking binding assay (13). Increasing amounts of a competitor were used to define a quantitative *I*<sub>50</sub> number, defined as the concentration of competitor required to reduce the UV cross-linking signal to 50% (9, 10). Due to the preferential activity of 100RNP/PNPase toward polyadenylated RNA, these experiments were performed as rapidly as possible and at a low temperature. Nevertheless, the results obtained with poly(A) should be taken as a minimum value because the concentration of poly(A) in the mixture was lower than that added to the



**FIG. 6. 100RNP/PNPase preferentially binds to poly(A) RNA.** *A*, [<sup>32</sup>P]UTP-labeled RNA corresponding to the *psbA* 3'-end was mixed with increasing amounts of the competitors poly(G), poly(A), poly(U), and *psbA* 3'-end (same RNA as the radioactive one) and 20  $\mu$ g of chloroplast protein extract. The competitor/*psbA* 3'-end ratios (mol/mol) were as follows: none (lane -), 4-fold excess (lane 1), 20-fold excess (lane 2), 40-fold excess (lane 3), and 200-fold excess (lane 4). The mixture was immediately UV-cross-linked on ice and digested with RNase A, and the proteins were analyzed by SDS-PAGE and autoradiography. The radioactive signals of 100RNP/PNPase and the group of 24–33-kDa ribonucleoproteins (RNPs) are indicated on the right. *B*, shown is a graphical representation of the competition experiment presented in *A*. The amount of *psbA* RNA 3'-end cross-linked to 100RNP/PNPase was quantified by a Fuji imaging analyzer and plotted as a function of the competitor excess. White squares, poly(G); black squares, poly(A); black triangles, poly(U); inverted white triangles, *psbA* 3'-end.  $I_{50}$ , defined as the concentration of competitor that inhibits binding of 100RNP/PNPase to the *psbA* RNA 3'-end by 50% under our experimental conditions, was 2-, 5-, 28-, and 30-fold for poly(A), poly(U), *psbA*, and poly(G), respectively. However, the value for poly(A) was even smaller since >90% inhibition was obtained at the first point and due to rapid degradation of poly(A) in the chloroplast extract (see "Results").

system due to degradation. The results presented in Fig. 6 concurred with the column binding studies, showing the higher binding affinity of 100RNP/PNPase for poly(A). The affinity for poly(U) was about three to four times lower, and that for poly(G) and for RNA corresponding to the 3'-end of *psbA* was 15–20 times lower compared with the values obtained with poly(A). Taking the poly(A) binding affinity as a minimum value and because maximal competition had already been obtained with a 4-fold excess of poly(A) (Fig. 6), the actual differences in binding affinities are assumed to be even greater. Taken together, these results demonstrated that 100RNP/PNPase binds poly(A) with much higher affinity than any other RNA sequence. Therefore, it competes for the degradation of polyadenylated RNA molecules by binding to poly(A) and by being excluded from the non-polyadenylated RNA molecules.

#### DISCUSSION

A possible pathway for the degradation of mRNA in the chloroplast has recently been suggested (27, 28).<sup>2</sup> The first

event is endonucleolytic cleavage(s), which produces RNA with no stem-loop structure at the 3'-end. Following these cleavages, the proximal fragments are polyadenylated at their 3'-end by the addition of poly(A)-rich sequences. The polyadenylated RNAs are then rapidly digested by the exonuclease(s), possibly due to the higher enzyme affinity for the poly(A)-rich sequence. Indeed, blocking the polyadenylation of RNA in an RNA degradation assay based on lysed chloroplasts had the same effect as the addition of yeast tRNA, an exonuclease inhibitor (29): RNA degradation was inhibited, and endonucleolytic cleavage products accumulated.<sup>2</sup> Moreover, polyadenylated RNA was specifically degraded in a chloroplast protein extract and competed with non-polyadenylated RNA for the degradation machinery (Fig. 1) (27). In this work, we provide a more profound understanding of the last step of this degradation mechanism. We show that an exonuclease isolated from spinach chloroplasts, 100RNP/PNPase, uses RNA with a poly(A) tail as the preferred substrate, suggesting that specific degradation of polyadenylated RNA is a phenomenon intrinsic to the exonuclease and that other proteins are not required. Thus, polyadenylation of RNA degradation products at their 3'-end increases their binding affinity for the exonuclease 100RNP/PNPase, rendering these RNAs more susceptible to the enzyme's activity.

Analysis of the proteins involved in the processing of the 3'-end of the RNA in the chloroplast revealed a high molecular mass complex harboring 100RNP/PNPase and an endonuclease that cross-reacted with antibodies prepared against the *E. coli* endonuclease RNase E (13). RNA maturation and degradation in the chloroplast may therefore resemble the prokaryotic process, preserving its ancestral origin. Indeed, recent studies have confirmed the important role played by polyadenylation in the decay of *E. coli* transcripts. These poly(A) tails are added to at least some transcripts at multiple sites, rendering them extremely susceptible to degradation by an as yet undefined mechanism (20–26). A significant proportion of this polyadenylation occurs at endonucleolytic cleavages sites within the coding and 3'-ends of the transcripts (26). Taken together, these results suggest that the mechanism of polyadenylation-dependent exonucleolytic RNA degradation may be similar in bacteria and chloroplasts.

To date, two exonucleases have been identified in bacteria, both working in the 3' to 5' direction (5, 38). The isolation of a chloroplast exonuclease homologous to the bacterial PNPase and the similarity of their RNA degradation systems suggest that the situation in the chloroplast is similar to that in the bacterial cell. In this work, we show that purified 100RNP/PNPase preferably degrades polyadenylated RNA, similar to the situation in the chloroplast extract. However, the identification and characterization of other chloroplast exonucleases are required to determine whether the preferred poly(A) RNA degradation phenomenon is also shared by the other exonucleases. Indeed, higher *in vitro* degradation activity of bacterial RNase II for polyadenylated RNA was recently detected (39). In that direction, a potential RNase II homologue from spinach chloroplast is under purification.<sup>4</sup>

Isolated 100RNP/PNPase was active as a poly(A) polymerase (data not shown). However, depletion of 100RNP/PNPase from the chloroplast extract by heparin or single-stranded DNA columns did not interfere with *in vitro* polyadenylation activity (27). Moreover, a poly(A) polymerase has been isolated from chloroplast extract (37).<sup>3</sup> A similar situation of *in vitro* polymerase activity of the PNPase and the existence of poly(A) polymerases was reported for bacteria (14). RNA polyadenylation

<sup>4</sup> A. Kotler and G. Schuster, unpublished data.

was even enhanced in bacteria cells where the PNPase was inactivated (26). Combined, these results suggest that *in vivo*, 100RNP/PNPase might be active only as an exonuclease, probably by inhibition of its polyadenylation activity by an as yet unknown mechanism. Another possibility is that the degradation as opposed to the polymerization activity is modulated by the concentration of phosphate ions. The polymerization activity of the bacterial PNPase and chloroplast 100RNP/PNPase is highly inhibited by a relatively low concentration of inorganic phosphate (13–15). Taking into account the concentrations of inorganic phosphate and nucleoside diphosphates in the chloroplast, the degradative reaction is highly favored. Therefore, detailed biochemical characterization of the 100RNP/PNPase action in the high molecular mass complex and the factors that modulate its activity will probably help to resolve this issue.

100RNP/PNPase, like the bacterial PNPase, is a processive exoribonuclease that binds to the 3'-end and digests the RNA nucleotide by nucleotide, without disconnecting from the molecule (13). Competition experiments showed high binding affinity of 100RNP/PNPase for poly(A) (Figs. 5 and 6). The experiment presented in Fig. 4 shows that in order for the non-polyadenylated RNA to compete for 100RNP/PNPase, the poly(A) stretch must be located at the 3'-end. Taken together, these results suggest that the high affinity of 100RNP/PNPase for the poly(A) stretch directs the enzyme to the poly(A) part of the RNA, and only if this part is at the 3'-end of the molecule can the enzyme start digesting the RNA. Since 100RNP/PNPase works processively along the RNA, it remains bound to it and is thereby excluded from the non-polyadenylated molecules.

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