Chloroplast ribonucleoproteins are associated with both mRNAs and intron-containing precursor tRNAs

Takahiro Nakamura[a], Masaru Ohta[a], Masahiro Sugiura[a], Mamoru Sugita[a,b,*]

[a] *Center for Gene Research, Nagoya University, Nagoya 464-8601, Japan*
[b] *Graduate School of Human Informatics, Nagoya University, Nagoya 464-8601, Japan*

Received 16 September 1999; received in revised form 7 October 1999

Abstract Tobacco chloroplasts possess five conserved ribonucleoproteins (cpRNPs). To elucidate the function of cpRNPs we analyzed their localization and target nucleic acid molecules in chloroplasts. Immunoprecipitation of the stromal extract and Northern analysis revealed that cpRNPs are associated in vivo with not only various species of chloroplast mRNAs but also intron-containing precursor (pre-) tRNAs. This observation strongly suggests that cpRNPs are involved in RNA processing, including mRNA stability and pre-tRNA splicing.

Key words: Chloroplast ribonucleoprotein; RNA processing; Splicing; Tobacco

1. Introduction

Most chloroplast genes of higher plants are organized in clusters and are co-transcribed as large polycistronic precursor RNAs that are subsequently processed into the shorter RNA species [1]. Precursor (pre-), intermediate and mature RNAs are relatively stable and accumulate at respective steady-state levels [2–4]. Thus, post-transcriptional RNA processing of pre-RNAs, which includes RNA cleavage/trimming, RNA splicing and RNA stabilization, is an important step in the control of chloroplast gene expression. In general, RNA processing is shown to be mediated by numerous nuclear-encoded protein factors in chloroplasts of higher plants [5,6] and the unicellular green alga *Chlamydomonas reinhardtii* [7]. We previously isolated five chloroplast ribonucleoproteins (cpRNPs) from tobacco, which are named cp28, cp29A, cp29B, cp31 and cp33 [8,9]. The cpRNPs are proteins unique to chloroplasts and consist of two consensus sequence-type RNA-binding domains (RBDs) and an acidic N-terminal domain. The tobacco cpRNPs have a strong affinity to RNA homopolymers, poly (G) and poly (U), rather than single-stranded (ss) DNA and double-stranded (ds) DNA in vitro [10,11]. Therefore, they are thought to play a role in RNA metabolism in chloroplasts. Schuster and Gruissem reported that spinach 28RNP, a counterpart of the tobacco cp28 and cp31, is required for 3′-end formation of several mRNAs [12]. This consequence was obtained using a partially purified chloroplast extract and in vitro synthesized mRNAs. Interaction of the recombinant 28RNP with the exogenous mRNA has been detected by UV-crosslinking [12–14]. However, there is no direct evidence of interaction of native cpRNPs with the steady-state RNA itself in chloroplasts.

In this report, we first demonstrated that tobacco cpRNPs are associated in vivo with various species of mRNAs and also pre-tRNAs.

2. Materials and methods

2.1. Sub-chloroplast fractionation

Intact chloroplasts were prepared from tobacco (*Nicotiana tabacum* var. Bright Yellow 4) leaves as described previously [8]. The number of isolated intact chloroplasts was counted in a hemocytometer using a light microscopy. Chloroplasts were sub-fractionated according to [15]. The intact chloroplasts (1.3 ml) were suspended in 20 ml S30 buffer (20 mM Tris-HCl, pH 8.5, 20 mM potassium acetate, 10 mM $MgCl_2$, 0.5 mg/ml heparin and 5 mM DTT). Ten ml of the suspension was layered onto 12 ml each of 20.5% and 31.5% sucrose solutions containing 50 mM Tris-HCl, pH 8.5, 25 mM KCl and 10 mM $MgCl_2$ and centrifuged at $72\,000\times g$ for 1 h at 4°C in a Hitachi SRP28SA rotor. After centrifugation, a clear brown supernatant, a yellowish green band and dark green pellet were collected as stroma, envelope and thylakoid sub-fractions, respectively. Each sub-fraction was suspended in an equal volume of S30 buffer. Alternatively, the intact chloroplasts were lysed in 2 ml of S30 buffer and centrifuged in a Beckman TLA 100.3 rotor at $30\,000\times g$ for 30 min at 2°C. The resultant supernatant (S30 fraction) was centrifuged at $120\,000\times g$ for 110 min at 2°C to separate the supernatant (S120 fraction) from the pellet (P120 fraction).

2.2. Preparation of recombinant cpRNPs and antibodies

The cDNAs encoding the mature cpRNPs [8,9] were amplified by polymerase chain reaction (PCR) using *pfu* DNA polymerase and cloned into the expression vector pMAL-c2 (New England Biolabs). Primers used for PCR are as follows: for cp28, NS281: GTTTCTGTATTATCTGAAG and SK: TCTAGAACTAGTGGATC; for cp29A, 29A2: CGGGATCCGTTACCCTTTCTGATTTTGACCAA and 29AB2: CGTCGACTCAGAATTGACGCCTGGG; for cp29B, 29B2: CGGGATCCGTTGCGCTCTCTGGTTTT and 29AB2 primer; for cp31, NS311: ATAGGATCCTGCACTGCAAGAAGAAAAC and NS312: GCAGAATTCTGACCGCTATGATTATAGAAC; for cp33, NS331: GCTTCTGTGTCTGATGG and KS: CGAGGTCGACGGTATCG. Underlines indicate restriction sites used for subcloning. PCR was performed at 94°C for 1 min, at 45°C (for cp28 and cp33) or at 50°C (for cp29A and B, cp31) for 2 min and at 72°C for 1 min for 30 cycles. The amplified fragment encoding cp28 or cp33 was digested with *Eco*RI, then ligated into a *Xmn*I-*Eco*RI-digested pMAL-c2. The amplified fragment encoding cp29A or cp29B was digested with *Bam*HI and *Sal*I, then subcloned into pMAL-2c. The amplified fragment encoding cp31 was initially cloned into pRSETB vector (Invitrogen) and then recloned into *Bam*HI-*Hin*dIII sites of pMAL-c2. The recombinant proteins fused with maltose-binding protein were produced in *Escherichia coli* XL1-blue cells and purified according to [16]. They are named re-cp28, re-cp29A, re-cp29B, re-cp31 and re-cp33. Each recombinant protein was injected several times at a week interval in a rabbit to produce the antibodies.

2.3. Western blot analysis

The proteins of sub-chloroplast fractions were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (15% polyacrylamide). The S30 fraction (100 μg protein) was treated at 37°C for 20 min with or without 120 U RNase A (Sigma) or 70 U DNase I (Promega) and separated by non-denaturing PAGE [17]. After electrophoresis, the gel was blotted onto a polyvinylidene difluoride mem-

*Corresponding author. Fax: (81) (52) 789 4779.
E-mail: sugita@info.human.nagoya-u.ac.jp

PII: S0014-5793(99)01390-3

brane (Problott, Perkin Elmer Applied Biosystems). The membrane was incubated with antibodies at a dilution of 1:5000 and immunodetection was performed using the ECL Western blotting analysis system (Amersham Pharmacia Biotech).

2.4. Immunoprecipitation

Each anti-cpRNP serum was adsorbed to 20 mg of protein A-Sepharose (PAS) beads (Amersham Pharmacia Biotech) according to [18]. The antibody-PAS beads were incubated with an aliquot of the S120 sub-fraction (1.1 mg protein) in 2 ml S30 buffer containing 0.1% Nonidet P-40 for 20 min at 4°C. The beads were washed five times with 4 ml NET-2 buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl and 0.05% Nonidet P-40), then suspended in 0.4 ml of the nucleic acid extraction buffer (0.1 M Tris-HCl, pH 9.0, 10 mM EDTA and 1% SDS) and heated for 2 min at 90°C. After centrifugation, nucleic acids in the supernatant were extracted twice with phenol/chloroform [19]. Nucleic acids co-precipitated with the cpRNPs were treated with DNase I or RNase T/RNase A and subjected to slot blot analysis. Alternatively, they were subjected to Northern blot analysis.

2.5. Northern blot analysis

RNA electrophoresis and Northern blotting onto a nylon membrane (Hybond-N+, Amersham Pharmacia Biotech) were essentially carried out as described [9]. Three kb *rrn23*-specific probe was prepared from p23S (derived from pTBa5 [21]), 1.5 kb *psbA* probe was from psbA-F (pTBa8 [21]), 1.6 kb *petD* probe from petD#1 (pTS8 [21]), 1.8 kb *rbcL* probe from rbcL-F (pTB29 [21]), 0.85 kb *rpl32* probe from pBB12 [22]. The DNA probes were ^{32}P-labelled using a random primer labelling kit (Takara Shuzo, Kyoto, Japan). For detection of pre- and mature tRNAs, synthetic oligonucleotides were used as followed. *trnF*-GAA (trnF: 5′-CCTCTGCTCTACCAACTGAGCTATCCCGGC-3′, nucleotide positions 50 239–50 268 [20]), *trnL*-UAA (W1: 5′-GTCCGTAGCGTCTACCGATTTCGCC-3′, 49 328–49 304), *trnL*-UAA intron (W2: 5′-TTTGTTAGAATAGCTTCCATTGAGT-3′, 49 503–49 479), *trnG*-UCC (G5: 5′-TTTTACCACTAAACTATACCCGC-3′, 9521–9499), *trnG*-UCC intron (Gi2: 5′-GTGTATTTGTTCTCGAATCTGTCATTGAGAGGA-3′, 9653–9621). The oligonucleotides were labelled at the 5′-termini with [γ-^{32}P]ATP and T4 polynucleotide kinase and hybridization and washing were carried out at 50°C for DNA and oligonucleotide probes [19].

2.6. mRNA 3′-end processing assay

The four antibodies against cp28, cp29A, cp31 and cp33 were adsorbed to 30 mg of PAS beads and incubated with 1 ml of the S30 fraction (9.2 mg protein) at 4°C for 1 min and were then centrifuged at low speed. The supernatant was collected and used for a mRNA 3′-end processing assay according to [23]. Plasmid pDIR includes the last 78 bp of *petD* exon II, 190 bp of its 3′-untranslated region (UTR) and the last 99 bp of *rpoA* in the opposite orientation [24]. The ^{32}P-labelled *petD* 3′-UTR that can form a stem-loop structure was produced from the *Ssp*I cut pDIR by T7 RNA polymerase using MEGA Script (Ambion). The in vitro synthesized RNA (80 000 cpm) was incubated in 40 μl each of the S30 fraction (120 μg protein) at 25°C. At the indicated time, incubation was stopped by liquid nitrogen and the RNA in each tube was extracted by phenol/chloroform. The extracted RNAs were separated by 5% polyacrylamide gel containing 7 M urea and detected by a BAS2000 Imaging analyzer (Fuji Photo Film). The radioactivity of the detected RNA was indicated as photo-stimulated luminescence (PSL) [25].

3. Results and discussion

3.1. Localization of cpRNPs in chloroplasts

To analyze the location of cpRNPs within a chloroplast, the stroma, envelope and thylakoids were prepared from tobacco chloroplasts and their polypeptides were subjected to Western blot analysis. In this study, the anti-cp28 serum cross-reacts with cp31, and vice versa, because these two proteins share significant homology in the conserved RBD and belong to group II [26]. Hence, anti-cp28 and cp31 sera were used for detection of group II cpRNPs. Likewise, the anti-cp29A serum that cross-reacts with cp29B (group I) was used for detection of group I cpRNPs (cp29A and B). The anti-cp33 serum reacts specifically with cp33 (group III). Therefore, the present immunoreactivity data support the classification of cpRNPs proposed previously based on the phylogenetic analysis [9,26]. As shown in Fig. 1A, the majority of the five cpRNPs was detected in the stroma, but not in the envelope. Although the cpRNPs were also detected in the thylakoids, they were easily dissociated from the thylakoids by washing repeatedly (data not shown). We concluded therefore that most of the cpRNPs are localized in the stroma and a small portion of them is weakly associated with thylakoids. Spinach 28RNP was also shown to localize in the stromal fraction [13]. The tobacco stromal S30 fraction was further fractionated into the pellet (P120) and soluble fraction (S120). All the five cpRNPs were distributed in the S120 (Fig. 1B). Total S30 proteins were sub-fractionated by 70% into S120 and 30% into P120.

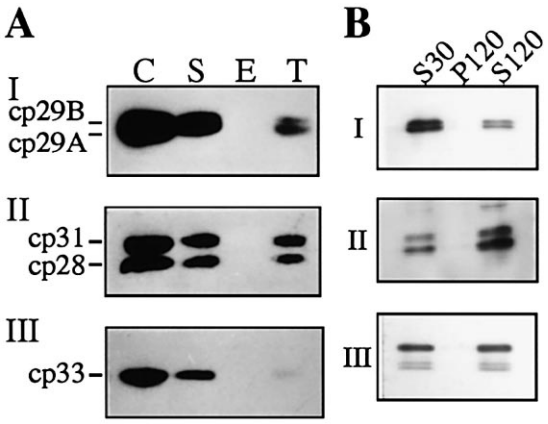

Fig. 1. Sub-chloroplast distribution of the five cpRNPs. A: Chloroplasts (C) were fractionated to stroma (S), envelope (E) and thylakoid (T). Total protein of each sample was prepared from ca. 50 000 intact chloroplasts and therefore, signal intensities indicate relative amounts of cpRNPs localized in each sub-chloroplast fraction. The anti-cp29A serum was used for detection of group I cpRNPs (I), the mixture of anti-cp28 and cp31 sera for group II (II) and the anti-cp33 serum for group III (III). B: Distribution of cpRNPs in S30, S120 and P120 fractions. The antibodies used are as (A).

3.2. cpRNPs are associated with RNA in chloroplasts

The tobacco cpRNPs were originally isolated as ssDNA-binding proteins [8,9] and shown to bind to ssDNA, dsDNA and RNA homopolymers, poly (G) and poly (U), using an in vitro nucleic acid-binding assay [10,11]. To specify whether cpRNPs bind to DNA or RNA in vivo, the tobacco S30 fraction was treated with RNase or DNase and then separated by native PAGE (Fig. 2A). When S30 was treated with DNase or without nucleases, cp28 was detected as a broad band. By contrast, cp28 in the S30 treated with RNase was detected as discrete and fast-migrated bands. This result indicates that cp28 interacts with RNA, but not with DNA in chloroplasts. Furthermore, we carried out an additional experiment to confirm this consequence. The S30 fraction was immunoprecipitated using the antibodies against cp29A, cp28/31 or cp33. The resultant pellet was treated with RNase or DNase and then subjected to slot blot analysis using ^{32}P-labelled tobacco chloroplast DNA as probe. The hybridization signals were

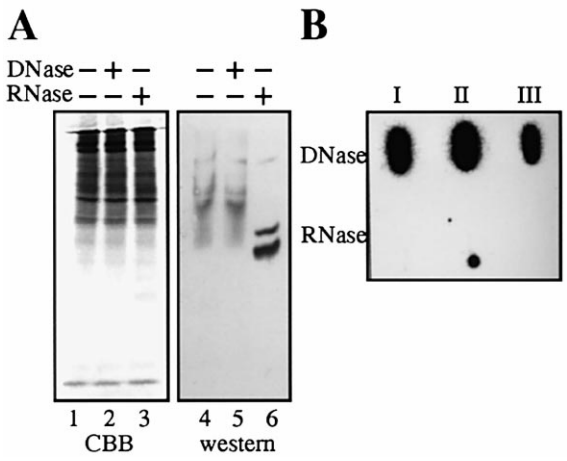

Fig. 2. The cpRNPs interact with RNA in vivo. A: The S30 fraction was treated with DNase (lanes 2 and 5), RNase (lanes 3 and 6) or not (lanes 1 and 4) and separated by native PAGE. The protein in the gel was stained by Coomassie brilliant blue (CBB) (lanes 1–3). cp28 was detected by Western blot analysis (lanes 4–6). B: The S30 was immunoprecipitated using each antibody against group I (cp29A), II (cp28/cp31) or III (cp33). The immunoprecipitated nucleic acids were incubated with DNase or RNase and subjected to slot blot analysis using ^{32}P-labelled chloroplast DNA.

detected for DNase-treated samples but did not for treatment by RNase (Fig. 2B). This result clearly confirms that the cpRNPs interact with RNAs in the stroma. Our preliminary observation showed that no cpRNPs are detected in tobacco chloroplast nucleoids, that are composed of DNA and proteins [27]. Thus, these findings clearly indicate that native cpRNPs are indeed associated with RNAs but not with DNA in the chloroplast. This is the first evidence of direct interaction of cpRNPs with chloroplast RNA in vivo.

3.3. CpRNPs interact with mRNA and intron-containing pre-tRNAs

To identify which RNA species are associated with these cpRNPs, we performed Northern analysis of the co-precipitated RNAs using chloroplast gene-specific probes. Most of 23S rRNA (95%) was recovered in P120 that contains ribosomes (Fig. 3A). In contrast, photosynthetic *psbA*, *rbcL* and *petD* mRNAs encoding the thylakoid D1 protein of the photosystem II complex, the large subunit of ribulose-1,5-bisphosphate carboxylase (RuBisCO) and subunit IV of the cytochrome b_6/f complex, respectively, were recovered in both P120 and S120. Approximately 80% of the stromal *psbA* mRNA and 60% of the *rbcL* or *petD* mRNAs are ribosome-free (Fig. 3). This observation coincides with the previous reports that *psbA* mRNA accumulates in the ribosome-free fraction in spinach [28] and barley [29]. Immunoprecipitation and Northern analysis of S120 revealed that the ribosome-free photosynthetic mRNAs were efficiently co-precipitated with the cpRNPs (lanes I, II and III, Fig. 3), but not with anti-RuBisCO antibody (lanes R, Fig. 3) and rabbit preimmune serum (data not shown). In contrast, *rpl32* mRNA

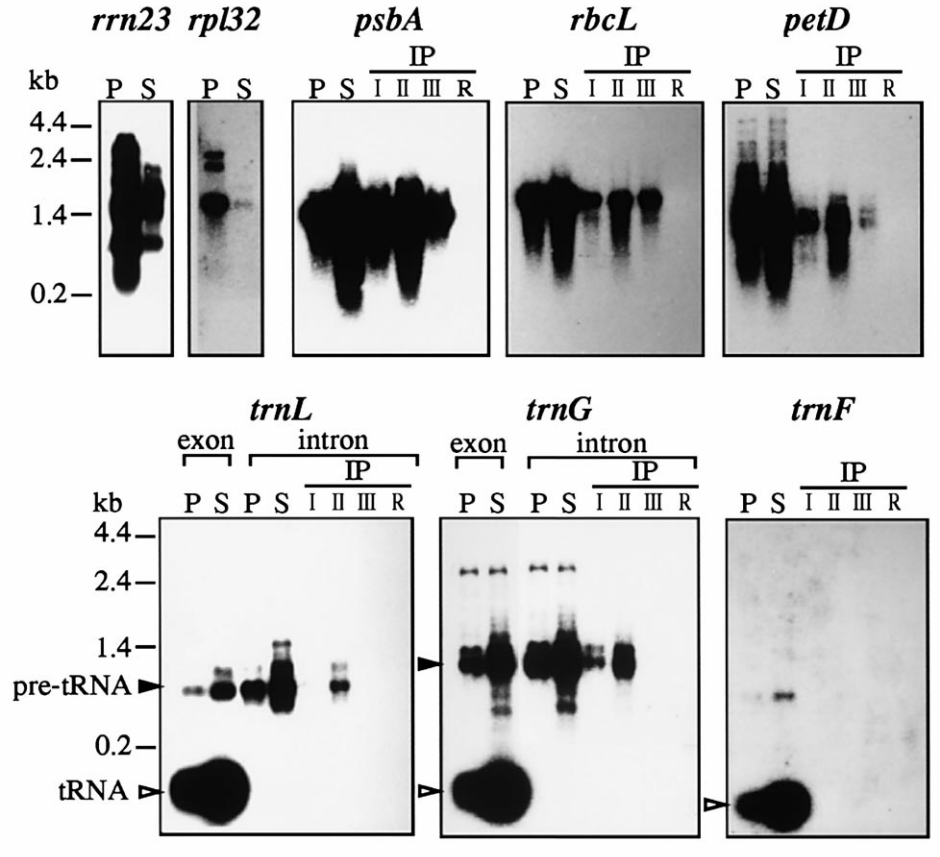

Fig. 3. Northern analysis of the RNAs co-precipitated with cpRNPs. RNAs in P120 (P), S120 (S) or immuno-pellet (IP) using each antibody against group I (I), group II (II), group III (III) cpRNPs or RuBisCO (R) were subjected to Northern blot analysis using gene-specific probes indicated above.

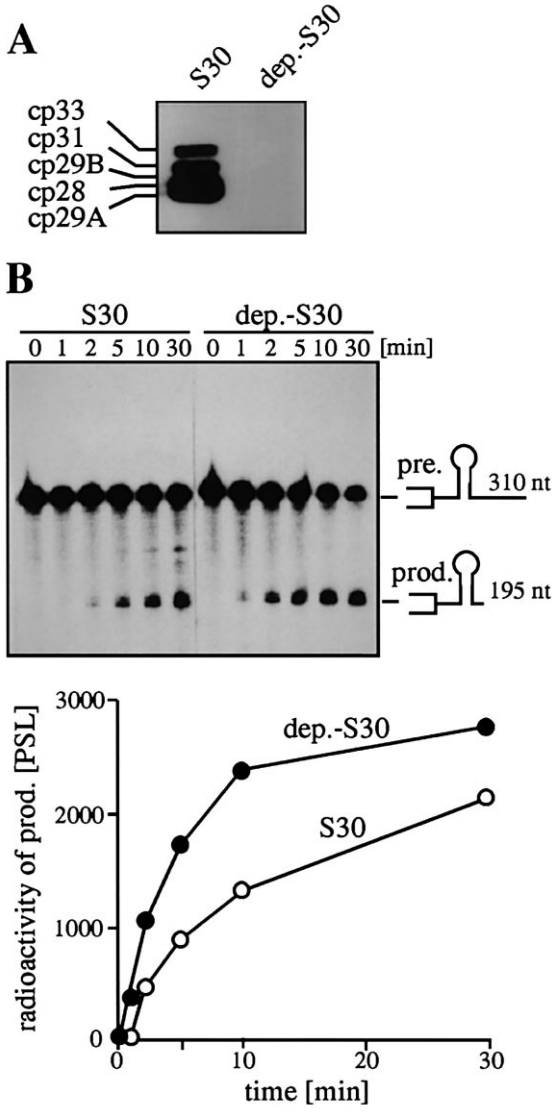

Fig. 4. Effect of cpRNPs on 3′-end formation of *petD* UTR in vitro. The S30 fraction was immunologically depleted of all the five cpRNPs and subsequently tested for *petD* mRNA 3′-end processing activity. A: Immunoblot of the S30 (S30) and cpRNPs-depleted S30 (dep.-S30) with the cpRNP antibodies. B: Processing of the 3′-UTR of *petD* mRNA was tested in the S30 or the depleted S30. Samples were removed at the indicated time (min). Pre. and prod. indicate the *petD* pre-RNA (310 nucleotides) and product (195 nucleotides) in the processing reaction. Radioactivity of the prod. was indicated as PSL [25].

encoding the ribosomal protein CL32 was detected in the P120 fraction but not in S120, indicating that most *rpl32* mRNA is associated with ribosomes. A similar result was obtained for *rpl2* (CL2) mRNA (data not shown). The transcription rate of the genes for ribosomal proteins was reported to be 2–10-fold lower than that of photosynthetic protein genes [30].

Moreover, three tRNA genes, *trnF*-GAA, *trnL*-UAA and *trnG*-UCC, were probed. The latter two genes contain a 503 bp group I intron and a 691 bp group II intron, respectively. Interestingly, unspliced pre-tRNAs were detected at substantial levels in the immunoprecipitate and mature tRNAs were not (Fig. 3), indicating that the cpRNPs, especially group II cpRNPs, are associated with unspliced pre-tRNAs. This suggests that cpRNPs are likely involved in splicing or stability of intron-containing pre-tRNAs.

In this analysis, heparin was added to a concentration of 0.5 mg/ml to avoid non-specific complex formation due to statistic interaction [31,32]. Therefore, we conclude that the cpRNPs are associated with various species of mRNAs and intron-containing pre-tRNAs in the stroma.

3.4. CpRNPs are negatively involved in RNA processing

To understand the biological significance of cpRNPs, the effect of cpRNPs on 3′-end formation of *petD* mRNA was analyzed using the cpRNPs-depleted stromal S30 fraction. Western blot analysis verified that cpRNPs were completely depleted from the S30 (Fig. 4A). The in vitro synthesized 3′-UTR of *petD* mRNA was incubated in the two different S30 fractions and 3′-end formation (mature RNA) was monitored. The cpRNPs-depleted S30 fraction was shown to retain activity of 3′-end formation, indicating that cpRNPs are dispensable for the activity. However, the 3′-end formation of *petD* was faster in the cpRNPs-depleted S30 than that in the control S30 (Fig. 4B). This result is not coincident with the previous observation by Schuster and Gruissem [12], who showed that 3′-end formation of *petD* UTR was completely inhibited by depletion of 28RNP from the spinach chloroplast extract. This discrepancy seems to be due to the difference of chloroplast extract used. They used the partially purified chloroplast extract by ammonium sulfate precipitation followed by DEAE-column chromatography. On the other hand, we used the five cpRNPs-depleted stromal S30 fraction that was rather crude. Our data suggest that all or some of the tobacco cpRNPs regulate negatively 3′-end formation of pre-mRNAs and may act as stabilizing proteins like hnRNPs in the nucleus of mammalian [33].

Acknowledgements: We thank A. Hirai, The University of Tokyo, for providing us with anti-tobacco RuBisCO antibody (T319). This work was supported in part by a Grant-in-Aid from the Ministry of Education, Science, Sports and Culture, Japan.

References

[1] Sugita, M. and Sugiura, M. (1996) Plant Mol. Biol. 32, 315–326.
[2] Westhoff, P. and Herrmann, R. (1988) Eur. J. Biochem. 171, 551–564.
[3] Meng, B.Y., Wakasugi, T. and Sugiura, M. (1991) Curr. Genet. 20, 259–264.
[4] Miyagi, T., Kapoor, S., Sugita, M. and Sugiura, M. (1998) Mol. Gen. Genet. 257, 299–307.
[5] Jenkins, B.D., Kulhanek, D.J. and Barman, A. (1997) Plant Cell 9, 283–296.
[6] Hubschmann, T., Hess, W.R. and Borner, T. (1996) Plant Mol. Biol. 30, 109–123.
[7] Rochaix, J.D. (1996) Plant Mol. Biol. 32, 327–341.
[8] Li, Y. and Sugiura, M. (1990) EMBO J. 9, 3059–3066.
[9] Ye, L., Li, Y., Fukami-Kobayashi, K., Go, M., Konishi, T., Watanabe, A. and Sugiura, M. (1991) Nucleic Acids Res. 23, 6485–6490.
[10] Li, Y. and Sugiura, M. (1991) Nucleic Acids Res. 19, 2893–2896.
[11] Ye, L. and Sugiura, M. (1992) Nucleic Acids Res. 20, 6275–6279.
[12] Schuster, G. and Gruissem, W. (1991) EMBO J. 10, 1493–1502.
[13] Lisitsky, I., Liveanu, V. and Schuster, G. (1995) Plant Physiol. 107, 933–941.
[14] Hayes, R., Kudla, J., Schuster, G., Gabay, L., Maliga, P. and Gruissem, W. (1996) EMBO J. 15, 1132–1141.
[15] Douce, R. and Joyard, J. (1982) in: Methods in Chloroplast Molecular Biology (Edelman, M., Hallick, R.B., Chua, N.H., Eds.), pp. 239–256, Elsevier Biomedical Press.

[16] Maina, C.V., Riggs, P.D., Grandea III, A.G., Slatko, B.E., Moran, L.S., Tagliamonte, J.A., McReynolds, L.A. and Guan, C.D. (1988) Gene 74, 365–373.
[17] Shigeoka, S. and Nakano, Y. (1991) Arch. Biochem. Biophys. 288, 22–28.
[18] Steitz, J.A. (1989) Methods Enzymol. 180, 468–481.
[19] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory Press.
[20] Shinozaki, K. et al. (1986) EMBO J. 5, 2943.
[21] Sugiura, M., Shinozaki, K., Zaita, N., Kusuda, M. and Kumano, M. (1986) Plant Sci. 44, 211–216.
[22] Vera, A., Matsubayashi, T. and Sugiura, M. (1992) Mol. Gen. Genet. 233, 151–156.
[23] Stern, D. and Gruissem, W. (1987) Cell 51, 1145–1157.
[24] Vera, A. and Sugiura, M. (1995) EMBO J. 13, 2211–2217.
[25] Amemiya, Y. and Miyahara, J. (1988) Nature 336, 89–90.
[26] Ohta, M., Sugita, M. and Sugiura, M. (1995) Plant Mol. Biol. 27, 529–539.
[27] Sakai, A., Saito, C., Inada, N. and Kuroiwa, T. (1998) Plant Cell Physiol. 39, 928–934.
[28] Minami, E., Shinohara, K., Kawakami, N. and Watanabe, A. (1988) Plant Cell Physiol. 29, 1303–1309.
[29] Kim, M., Gamble-Klein, P. and Mullet, J.E. (1994) Plant Mol. Biol. 25, 459–467.
[30] Rapp, J.C., Baumgartner, B.J. and Mullet, J. (1992) J. Biol. Chem. 267, 21404–21411.
[31] Pinol-Roma, S., Choi, Y.D., Matunis, M.J. and Dreyfuss, G. (1988) Genes Dev. 2, 215–227.
[32] Konarska, M.M. and Sharp, P.A. (1986) Cell 46, 845–855.
[33] Dreyfuss, G., Matunis, M.J., Pinol-Roma, S. and Burd, C.G. (1993) Annu. Rev. Biochem. 62, 289–321.