Secondary Structures Common to Chloroplast mRNA 3'-Untranslated Regions Direct Cleavage by CSP41, an Endoribonuclease Belonging to the Short Chain Dehydrogenase/Reductase Superfamily*

In the chloroplasts of higher plants and green algae, gene expression is regulated primarily at the post-transcriptional level. Most chloroplast mRNAs contain an inverted repeat in their 3'-untranslated regions (UTRs) that can fold into a stable stem-loop structure. These stem-loops constitute highly specific recognition sites for nucleus-encoded RNA-binding proteins (1–3). In the absence of a stable stem-loop, or after their removal, transcripts become unstable (4), because the stem-loop and associated proteins impede the activity of processive 3'- to 5'-exonucleases (1, 5, 6). Therefore, removal of the stem-loop is likely to be a key regulatory step in chloroplast mRNA degradation (reviewed in Ref. 7). In light of this, a detailed understanding of how chloroplast ribonucleases distinguish their substrates is essential. However, although several chloroplast endoribonuclease activities (4, 8–10) and exoribonuclease activities (11) have been reported, only the exonuclease polyadenylate phosphorylase (12, 13) has undergone thorough analysis.

Previously, we reported the purification and characterization of CSP41 (chloroplast stem-loop-binding protein of 41 kDa), a chloroplast endonuclease belonging to the SDR superfamily, preferentially cleaves stem-loop-containing RNAs in vitro. This potentially directs it to the 3'-ends of mature chloroplast mRNAs, which generally possess such structures. To understand the basis for this discrimination, the RNA elements directing CSP41 cleavage of petD RNA in vitro were dissected. Substrates containing fully base-paired stem-loops were optimal substrates, whereas deletion of part of the stem-loop decreased activity by 100-fold, and deletion of the distal arm of the stem-loop abolished cleavage, even in substrates containing the primary CSP41 cleavage site. Competition assays showed that the decrease in activity resulted from decreased affinity for the RNA by CSP41. Mutations of the residues at the scissile bond and mutations and deletions at the terminal loop of the stem had a moderate effect on activity but no effect on cleavage site specificity, suggesting that CSP41 has no sequence specificity. Titration of ethidium bromide into the assay decreased activity to a basal level of ~18%, and introduction of a single base bulge into either arm of the stem-loop decreased cleavage at the primary cleavage site by up to 70%. This suggests that changing the structure of the helical stem has a mild effect on activity. Deletion analysis of CSP41 suggests that the specificity domain lies in the first 73 amino acids of the protein, a domain that also contains a putative dehydrogenase-like mononucleotide binding motif. These results are consistent with a broad role for CSP41 in the degradation of stem-loop-containing mRNAs.

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Previously, we reported the purification and characterization of CSP41 (chloroplast stem-loop-binding protein of 41 kDa), a ubiquitous endoribonuclease found in plant chloroplasts (14, 15). The amino acid sequence of CSP41 is >85% conserved in all plant species analyzed (data not shown), but it shares no homology with other proteins in the data bases. CSP41 also lacks known ribonuclease motifs but belongs to the short-chain dehydrogenase reductase (SDR) structural superfamily (16). This family consists of more than 1,600 proteins, including more than 130 in Arabidopsis thaliana (17). The best studied and closest relative of CSP41 in the SDR superfamily is Escherichia coli UDP-glucose epimerase. As such, CSP41 is predicted to contain a bidomain SDR Rossman fold. However, CSP41 lacks two sequence motifs required for epimerase activity (16, 18).

CSP41 was shown to cleave primarily within the stem-loop structures of several chloroplast RNA 3'-UTR substrates in vitro (15). Furthermore, whereas CSP41 was shown to cleave double-stranded RNA substrates, its activity was optimal with stem-loop-containing RNAs (15). Because stem-loop structures are known to be important for chloroplast mRNA stability, CSP41 was hypothesized to play a role in the initiation of RNA degradation. Three other members of the dehydrogenase family, glyceraldehyde-3-phosphate dehydrogenase and two dehydrogenases from the archaeon Sulfolobus solfataricus, have been shown to have endoribonucleolytic activity. The most prominent cleavage sites of these enzymes were in loops and bulges of the predicted secondary structure of phase T7 R1.1 RNA (19), reminiscent of the prominent cleavage sites for CSP41 within the chloroplast petD 3'-UTR. Therefore, whereas the mechanistic details regarding substrate recognition and cleavage by SDR motif-containing proteins are still somewhat vague, the evidence suggests that this motif may represent a new type of ribonuclease domain capable of binding and cleaving double-stranded RNA substrates, particularly stem-loops. Here, we have attempted to determine the basis of this substrate preference, using CSP41 as a model.

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*This work was supported by Department of Energy Biosciences Program Award DE-FG02-90ER20015. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: UTR, untranslated region; dsRNA, double-stranded RNA; EDTA, 1-ethyl-3-(3-dimethyl)aminopropylcarbodiimide; nt, nucleotide; SDR, short-chain dehydrogenase/reductase; MES, 4-morpholineethanesulfonic acid.

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EXPERIMENTAL PROCEDURES

Purification and Assay of CSP41—CSP41 was purified according to Yang and Stern (15). The enzyme was assayed at room temperature in 20 mM HEPES-KOH, pH 7.5, 10% glycerol, 20 mM of petD RNA, and 20 mM MgCl₂.

Preparation of Synthetic RNA Substrates—Templates encoding the petD 3’-UTR RNA substrates used in this study were contained between the SacI and EcoRI sites of pBlueScript KS (−). Synthetic RNAs were prepared according to Stern and Gruissem (5) after linearizing templates with HindIII. RNA substrates for CSP41 assays were synthesized with 2.5 μM [α-32P]UTP (3000 Ci/mmol) and 25 μM cold UTP. Trace-labeled RNAs were transcribed in the presence of 8 nM [α-32P]UTP and 0.5 μM cold UTP. Substrates for footprinting were synthesized in the presence of 1 μM cold rNTPs and end-labeled with [γ-32P]ATP according to Yang and Stern (15).

Lengths of RNAs were as follows: Δ50, 209 nt; Δ18, 179 nt; Δ63, 168 nt; and Δ24, 192 nt. The Dral-linearized template produced a 92-nt substrate.

Analysis of petD RNA Structure—10⁶ cpm (roughly 20 fmol) of petD RNA was incubated in the presence of 20 mM HEPES-KOH, pH 7.5, 10% glycerol and 20 mM MgCl₂ in a total volume of 10 μl. The RNA was then partially digested with either RNase A (2 ng), RNase T1 (1 unit), RNase T2 (0.24 unit), or RNase V1 (0.001 unit). The reactions were allowed to proceed for 1 min at room temperature and were then stopped with 50 μl of 5 mM aurintricarboxylic acid, 6 M urea, and 2% SDS. The reactions were extracted with phenol/chloroform and subsequently ethanol-precipitated at −20 °C in the presence of 20 μg of yeast tRNA. The precipitated RNA was collected by centrifugation and analyzed efficiently into 136- and 43-nt products, whereas the cleavage level similar to that of petDΔ63; however, cleavage occurred upstream of position 136 (marked by an asterisk). A substrate that ends at the petD translation termination codon (DraI) was not a substrate for CSP41.

The stark difference in reactivity of Δ18 versus Δ63 could be due to sequence- and/or structural differences. To map single-versus double-stranded regions of these substrates, 5'-end-labeled petDΔ18 and petDΔ63 were digested with sequence- and structure-specific endonucleases in the presence of 20 mM MgCl₂, as shown in Fig. 2. RNases A, T1, and T2 cleave in single-stranded regions only, whereas RNase V1 prefers helical RNA substrates (20). The analysis showed that upstream of the
stop codon, the cleavage patterns were similar for petDΔ18 and petDΔ63, suggesting that their secondary structures are similar (data not shown). As predicted, there was a lack of petDΔ18 cleavage with RNases A, T1, and T2 between positions 125 and 144 and between positions 151 and 170, which correspond to the petD 3′-UTR stems.

The cleavage pattern for petDΔ63 differed from that of petDΔ18 downstream of the stop codon. With petDΔ63, RNase A and RNase T1 cleaved in the proximal stem between positions 125 and 132, suggesting that petDΔ63 is single-stranded in this region. This and the lack of cleavage at the G residues at positions 133–135 are consistent with the schematic representation of petDΔ63 in Fig. 2B, although minor cleavage by RNase T1 at position 151 may suggest flexibility in the structure. Since petDΔ63 is largely single-stranded at the base of its stem under the conditions of our experiment, this suggests that the presence of a fully base-paired stem-loop is required for optimal cleavage at position 136 by CSP41 and that the smaller stem-loop still supports reduced but specific cleavage.

It was of interest to determine whether petDΔ63 and petDΔ24 were poor substrates because of inefficient binding by CSP41 or if binding was unaffected but nonproductive in terms of promoting cleavage. In vivo, nonproductive binding might trap CSP41 on nonsubstrate RNAs and in this sense might be biologically unfavorable. To address the issue, competition experiments were performed in which CSP41 was assayed in the presence of radiolabeled petDΔ18 and increasing concentrations of unlabeled competitor RNAs, as shown in Fig. 3. In Fig. 3A, the control reaction (lane −) shows the cleavage of petDΔ18 at position 136 in the absence of competitor RNA. Using an equimolar amount of unlabeled petDΔ18 RNA (Δ18–Δ1X), the accumulation of the 136-nt product decreased by 50% and decreased further as a function of increasing unlabeled petDΔ18. In the presence of a 10-fold molar excess of unlabeled petDΔ63, however, there was only a 15% decrease in accumulation of the 136-nt band compared with the control reaction (Δ63–Δ10X). Only in the presence of a 100-fold molar excess of unlabeled petDΔ63 was the cleavage of petDΔ18 significantly reduced (shown in Fig. 7C). There was no detectable decrease in cleavage of petDΔ18 in the presence of a 10-fold molar excess of petDΔ24 (Δ24–Δ10X). The results of the competition assays are summarized in Fig. 3B, which was repeated with unlabeled yeast tRNA. 50% inhibition of petDΔ18 cleavage by CSP41 occurred only in the presence of a 400-fold molar excess of...
tRNA. Taken together, these data suggest that a fully base-paired stem-loop is required for a high affinity interaction between CSP41 and petD RNA and that stem-loops in yeast tRNA do not possess the optimal structure required for binding by CSP41.

To further assess the importance of the double-stranded RNA stem-loop in recognition and cleavage of petD by CSP41, cleavage was measured in the presence of increasing concentrations of ethidium bromide, which intercalates into both dsRNA and double-stranded DNA. Intercalation of EtBr would be expected to distort the helix of A18, changing the spatial relationship of nucleotides potentially recognized by CSP41. If this treatment impeded cleavage, it might be concluded that CSP41 recognized specific sequences and/or required an undistorted helix for cleavage.

As shown in Fig. 4A, increasing EtBr concentrations caused corresponding decreases in cleavage at position 136. (Several secondary CSP41-catalyzed cleavages are also visible in this gel; however, they were not reproducible (marked with asterisks).) To control for inhibition due to direct binding and inactivation of CSP41 by ethidium, the experiment was repeated in the presence of increasing concentrations of actinomycin D. Actinomycin D, like ethidium, is a hydrophobic DNA intercalator but is not an RNA intercalator. Propidium, another hydrophobic intercalating molecule similar in structure to ethidium, has been shown to inactivate RNase A nonspecifically via interactions at an apolar site on the enzyme, whereas RNase III was not inactivated by actinomycin D under similar conditions (21, 22). When CSP41 was assayed with increasing levels of actinomycin D, cleavage at position 136 was unaffected (Fig. 4B). Furthermore, the same results were obtained whether the reaction was started with the addition of CSP41 or with RNA, suggesting that preincubation of the enzyme with EtBr did not directly inactivate CSP41.

The percentage of cleavage of petD RNA at position 136 was plotted as a function of EtBr concentration (Fig. 4C). The cleavage decreases as a function of concentration and plateaus at a level of ~18%. The concentration giving half-maximal inhibition of cleavage was ~3 μM. This is similar to the dissociation constant of 2.4 μM for EtBr binding to tRNA (23). Taken together, these data suggest that EtBr intercalates into the double-stranded stem of the petD 5′-UTR and disrupts cleavage, probably by changing the structure of the double-stranded stem.

As an independent test of the importance of three-dimensional stem structure, single base bulges were introduced independently into each strand of the inverted repeat. The petD19 RNA contains an A insertion at position 166, and petD20 contains a U insertion at position 130. The structures tested are shown in Fig. 5A, and the results of cleavage assays are shown in Fig. 5B. The cleavage position of these substrates remained the same; however, compared with the control experiment with petD18, cleavage of petD19 and petD20 was reduced by ~30% and 70%, respectively. This is consistent with the results obtained with EtBr, where cleavage at position 136 was significantly reduced but not abolished. This further emphasizes that the formation of a fully base-paired stem-loop enhances cleavage at position 136.

To gain additional insight into the relationship between RNA sequence/structure and cleavage efficiency/specificity, the cleavage rate and sequence specificity of CSP41 was measured with the mutant substrates shown in Fig. 6A. Because these mutations were introduced into petD50, the accumulation of the 136-nt product of each mutant was normalized to this substrate (Figs. 6, B and C). In petD2, the C residues at positions 160 and 161 have been mutated to G residues, which causes a complete unwinding of the minor stem loop between positions 141 and 154 (4), whereas the primary CSP41 cleavage site is retained. In petD10, the A residues at positions 136 and 137 that flank the CSP41-targeted scissile bond have been mutated to U. petDd5 and petDd6 contain internal deletions in the proximal stem downstream of the scissile bond. CSP41 cleaved petD2, petD10, petDd5, and petDd6 with the same specificity as petD50 (data not shown). As shown in Fig. 6C, the cleavage at position 136 was ~30% higher and 50% higher with petDd5 and petDd6, respectively, than with Δ50 (wild type). This suggests that mutations downstream of the scissile bond have a moderate effect on the efficiency of cleavage at position 136 and that neither the sequence nor the structure above the scissile bond, nor the nucleotides at positions 136 and 137, direct specific cleavage at position 136. Overall, the experiments to this point paint a picture of an enzyme with significant structural but little sequence specificity.

Although CSP41 is a member of the SDR structural family
based on its sequence, this does not allow the \textit{a priori} conclusion that SDR domains are involved in its function. To determine whether its SDR domains are important for specificity in binding and cleavage at position 136, we performed competition experiments in which wild type (full-length) CSP41 was assayed in competition with either inactivated full-length CSP41 or a CSP41 deletion mutant. We first performed a control experiment in which the competitor was an inactive form of CSP41. Wild type CSP41 was modified with EDAC and glycine methyl ester, which functions by modifying acidic (Asp/Glu) residues and inactivates the enzyme (data not shown). As shown in Fig. 7A, increasing amounts of unmodified CSP41 cause a corresponding increase in degradation of the full-length petD18 substrate as expected, whereas adding EDAC-modified CSP41 to the standard assay resulted in a decrease in cleavage. This suggests that catalytically inactivated CSP41 has the same binding specificity as the unmodified enzyme and that it forms a specific complex with petD18 that prevents cleavage by unmodified enzyme. We cannot completely rule out that EDAC-modified CSP41 inactivates the unmodified enzyme, for example by dimerizing, although unmodified CSP41 acts as a monomer when monitored by native gel electrophoresis or gel filtration (data not shown).

An interesting observation in this experiment is that at increasing concentrations of CSP41, there is little additional accumulation of the 136-nt fragment, which would be expected given the increase in cleavage rate of the full-length RNA. It is possible that further cleavage of the 136-nt RNA could occur at these high enzyme concentrations. When dilutions of CSP41 are made below 0.5 g/reaction, the amount of CSP41 added to the standard assay, both the degradation of full-length substrate and the accumulation of the 136-nt cleavage product for each petD mutant, with the level for petDΔ50 set to 100%. Each bar represents either a single measurement or the average of two measurements.

The experiment was repeated with increasing amounts of CSP41ΔP, a deletion mutant that contains only the N-terminal 73 amino acids (15). This portion of CSP41 contains three
highly conserved motifs, including the $\beta\alpha\beta$ mononucleotide motif responsible for binding NAD(P)H in dehydrogenases (16).

At least part of the CSP41 active site is deleted, because CSP41EAp is not catalytically active (15). Fig. 7B shows that CSP41EAp inhibits cleavage of petD18 at position 136 by wild type (full-length) CSP41 as indicated at the top of each lane. A nonenzymatic control is included for reference (C). Transcript sizes are indicated at the left. B, samples were incubated for 10 min in the presence of 0.5 μg of CSP41 and the indicated molar excess of unmodified CSP41 (−EDAC), or EDAC-modified CSP41 (+EDAC), as indicated at the top of each lane. A nonenzymatic control is included for reference (C). Transcript sizes are indicated at the left. C, samples were incubated for 10 min in the presence of 0.5 μg of CSP41 and either petD63 (at a 100-fold molar excess over radiolabeled petD18), CSP41EAp (where wild type CSP41 is present at an 8-fold molar excess over CSP41EAp), or both competitors, as indicated at the top of each lane. A nonenzymatic control (C) and a control reaction in the absence of petD63 and CSP41EAp (−) are included for reference.

### DISCUSSION

Previous results suggested that whereas CSP41 could recognize arbitrary dsRNA substrates, its cleavage activity and specificity were much lower than in the presence of petD RNA. This suggested that CSP41 recognized specialized structures and/or sequences within the petD RNA (15). Here, we have expanded on its substrate specificity and determined the RNA structural elements necessary for recognition and cleavage within the stem-loop.

A key question was whether the sequence at the scissile bond was important for cleavage, since such sequence contexts might be relatively rare in the chloroplast. In petD10, the adenine residues at positions 136 and 137, the primary cleavage sites for CSP41, were mutated to uracil (Fig. 6). This had the double effect of changing the identity of the bases at these positions and changing them from purines to pyrimidines. The cleavage of petD10 was the same as for wild type petD RNA, suggesting that CSP41 has no primary sequence specificity or preference for a particular type of residue. This is different from general endoribonucleases such as RNase A and RNase T1, which have a preference for cleavage after purines and after guanosine residues, respectively, or RNase T2, which has a slight preference for adenosine residues but will cleave after any residue (24).

Results of cleavage assays with the substrates petD24 and petD63 (Fig. 1) suggest that there is no consensus sequence upstream of the petD stem-loop that can alone direct binding and cleavage. The results of cleavage assays containing one or multiple point mutations in several positions within the stem-loop further suggest that there is no consensus sequence within the stem that is recognized by CSP41 (Fig. 6). One implication of these results is that in vivo, CSP41 might target many 3’-UTRs, since most chloroplast mRNAs terminate in stem-loops. This would further implicate CSP41 broadly in chloroplast mRNA decay rather than as a petD-specific or highly specialized enzyme.

Although we did not identify specific RNA residues that directed CSP41 cleavage, the deletion mutants showed the importance of a double-stranded stem. The optimal petD substrate was petD18, which represents the mature petD RNA in vivo. Cleavage at position 136 of petD63, which retains three base pairs below the scissile bond, was 100-fold lower, although specificity for position 136 was retained. With petD24, lacking any stem base pairs, cleavage at position 136 was abolished. This is similar to the structural requirements of the substrates of yeast Rnt1p and E. coli RNase III, both of which require a double-stranded RNA region for recognition and cleavage. Furthermore, in the presence of a 100-fold molar excess of unlabeled petD63 over radiolabeled petD18, cleavage at position 136 of petD18 was similarly affected (Fig. 7C). In the presence of both an 8-fold molar excess of wild type CSP41 over CSP41EAp and a 100-fold molar excess of unlabeled petD63 over petD18, there was no cleavage at position 136. This suggests that CSP41EAp has the same binding specificity as wild type CSP41 and that affinity is highest for a fully base-paired stem-loop.
Substrate Specificity of CSP41

therrmore, Rnt1p cleavage has been shown to require a duplex region on either side of the cleavage site, whereas the efficiency of cleavage is enhanced by a complete stem below the cleavage site (25). The results of competition experiments suggested that the low cleavage activity with petDΔ63 and with petDΔ24 reflects the inability of CSP41 to bind the mutated petD RNA rather than nonproductive binding (Fig. 3). This suggests that CSP41 contains a motif capable of discriminating between single- and double-stranded RNA substrates. To our knowledge, this is the first description of such a binding activity in an SDR protein.

The importance of the helical nature of the stem-loop was tested using either an intercalating dye, EtBr (Fig. 4), or by introducing single base bulges that could alter the helical structure of the stem (Fig. 5). Cleavage decreased in the presence of EtBr, which has been shown to cause unwinding, lengthening, and local distortion of RNA double helices (22). The fact that CSP41 cleavage was not completely abolished by saturating ethidium bromide shows that it was not inhibiting cleavage by simply intercalating at the scissile bond and preventing cleavage, a phenomenon that has been observed in similar experiments with E. coli RNase III (22).

We complemented these data by introducing single base bulges into the stem midway between the base of the stem and the primary CSP41 cleavage site, which caused modest decreases in cleavage at position 136 (Fig. 5). This suggests that whereas CSP41 requires a double-stranded stem for recognition and cleavage at a particular site, the absolute requirement for an A-form helix is relaxed with CSP41, and the RNA recognition motif on the enzyme can recognize several forms of helical RNA. This is also true for the double-stranded RNA binding motif, commonly found in dsRNA-binding proteins and double-strand-specific ribonucleases. For example, both human interferon-induced dsRNA-induced protein kinase and Xenopus RNA-binding protein A were found to bind to RNAs with secondary structure defects, provided the helix had an overall A-form geometry (26, 27). The relatively loose requirement for A-form RNA is reflected in the fact that CSP41 is able to cleave petD, psbA, and rbcL RNA stem-loops at similar rates (15). These substrates neither share primary sequence nor are predicted to share a common tertiary structure. However, unlike the dsRNA-specific (and double-stranded RNA binding motif-containing) endoribonuclease Rnt1p from yeast (28) or Staufen from Drosophila (29), CSP41 does not appear to require specific interactions with the terminal loop on the stem-loop, because mutations that either alter the three-dimensional structure of the loop or modify the sequence of the terminal loop caused at most a 50% change in the level of cleavage of petD at position 136 (Fig. 6). With Rnt1p, for example, mutation of the AGGA tetraloop terminating the 25 S rRNA 3′-ETS substrate to GUGA causes at least a 4-fold decrease in both affinity and cleavage rate (30).

CSP41 is predicted to be a member of the short chain dehydrogenase/reductase superfamily (16). Comparisons of the sequences and three-dimensional structures of many proteins in this family show that they are structurally related despite significant divergence in their amino acid sequences. We have shown here that the N-terminal 73 amino acids of CSP41 contain a domain with a high affinity and specificity for the petD double-stranded stem-loop (Fig. 7). Multiple expectation-maximum for motif elicitation (MEME) analysis using a training set of 195 SDR proteins suggested that three highly conserved structural motifs lie in this domain of CSP41. MEME is an artificial intelligence-based motif analysis tool that identifies the conserved regions that are characteristic of the data set, given a set of unaligned sequences (16). Further analysis showed that one of the conserved motifs overlapped with the ββ mononucleotide binding fold in dehydrogenases (16).

Several dehydrogenases have previously been shown to be sequence-specific RNA-binding proteins. Glyceraldehyde-3-phosphate dehydrogenase was also shown to have a high affinity for tRNA (31, 32). Recently, it was reported that yeast glycerolaldehyde-3-phosphate dehydrogenase and two dehydrogenases from the archaeon S. solfataricus, Acd-1 and Acd-5, are endoribonucleases (19). In the S. solfataricus enzymes, the active site of the enzyme was localized to the first mononucleotide binding motif, contained within the first 70 amino acids of the enzyme. However, direct comparisons between these dehydrogenases and CSP41 must be made with caution. The first mononucleotide binding motif of CSP41, which lies within the sequence of CSP41P, does not have catalytic activity, since activity requires the first 191 amino acids of the protein. Furthermore, CSP41 activity absolutely requires Mg2+, whereas glyceraldehyde-3-phosphate dehydrogenase, Acd-1, and Acd-5 do not (19). Neither the endonucleolytic activity nor the cleavage specificity of CSP41 is inhibited by mono- or dinucleotides.2

These data suggest that, whereas CSP41 contains a putative mononucleotide binding motif, the affinity of this motif for nucleotides is significantly reduced in CSP41.

REFERENCES

1. Stern, D. B., Jones, H., and Gruissem, W. (1989) J. Biol. Chem. 264, 18742–18750
2. Chen, H. C., and Stern, D. B. (1991) Mol. Cell. Biol. 11, 4380–4388
3. Memen, A. R., Meng, B., and Mullet, J. E. (1996) Plant Mol. Biol. 30, 1195–1205
4. Chen, H., and Stern, D. B. (1991) J. Biol. Chem. 266, 24205–24211
5. Stern, D. B., and Gruissem, W. (1987) Cell 51, 1145–1157
6. Stern, D. B., and Gruissem, W. (1989) Plant Mol. Biol. 13, 615–625
7. Monde, R. A., Schuster, G., and Stern, D. B. (2000) Biochimie (Paris) 82, 573–582
8. Klaff, P. (1995) Nucleic Acids Res. 23, 4885–4892
9. Kudla, J., Hayes, R., and Gruissem, W. (1996) EMBO J. 15, 7137–7146
10. Nickelsena, J., and Link, G. (1993) Plant J. 3, 537–544
11. Hayes, R., Kudla, J., Schuster, G., Gahby, L., Maliga, P., and Gruissem, W. (1996) EMBO J. 15, 1132–1141
12. Yehudah-Resheff, S., Hirsh, M., and Schuster, G. (2001) Mol. Cell. Biol. 21, 5408–5416
13. Baginsky, S., Shteiman-Kotler, A., Liveano, V., Yehudah-Resheff, S., Bellacoui, M., Settlage, R. E., Shabanowitz, J., Hunt, D. F., Schuster, G., and Gruissem, W. (2001) RNA (N Y) 7, 1464–1475
14. Yang, J., Schuster, G., and Stern, D. B. (1996) Plant Cell 8, 1409–1420
15. Yang, J., and Stern, D. B. (1997) J. Biol. Chem. 272, 12784–12880
16. Baker, M. E., Grundy, W. N., and Elkan, C. P. (1998) Biochem. Biophys. Res. Commun. 248, 250–254
17. Kalberg, Y., Oppermann, U., Jornvall, H., and Persson, B. (2002) Protein Sci. 11, 636–641
18. Bellamacina, C. R. (1998) FASEB J. 12, 1257–1269
19. Evgenieva-Hackenberg, E., Schiltz, E., and King, G. (2002) J. Biol. Chem. 277, 46145–46150
20. Lowman, B. H., and Draper, D. E. (1988) J. Biol. Chem. 263, 5396–5403
21. MacGrath, M., Casein, D., Willams, R., Johnson, D., Greene, M., and McPherson, A. (1987) Mol. Pharmacol. 32, 600–605
22. Calin-Jageman, I., Amarasinghe, A. K., and Nicholson, A. W. (2001) Nucleic Acids Res. 29, 1915–1925
23. Torgerson, P. M., Drickamer, H. G., and Weber, G. (1980) Biochemistry 19, 3957–3960
24. Uchida, T., and Egami, F. (1967) J. Biochem. (Tokyo) 61, 44–53
25. Abou-Elela, S., and Ares, M. J. (1998) EMBO J. 17, 3738–3746
26. Bevilaqua, P. C., George, C. X., Samuel, C. E., and Cech, T. R. (1998) Biochemistry 37, 6303–6316
27. Byer, J. M., and Schultz, S. C. (1998) EMBO J. 17, 7505–7513
28. Lebars, I., Lamontagne, B., Yoshizawa, S., Aboul-Elela, S., and Fourmy, D. (2001) EMBO J. 20, 7250–7260
29. Raimo, A., Gruenert, S., Adam, J., Micklem, D. R., Proctor, M. R., Freund, S., Bycroft, M., St. Johnston, D., and Varani, G. (2000) EMBO J. 19, 997–1009
30. Nagel, R., and Ares, M. Jr. (2000) RNA (N Y) 6, 1142–1156
31. Nagel, R., and Green, M. R. (1993) Science 260, 365–368
32. Nagy, E., and Rigby, W. F. C. (1995) J. Biol. Chem. 270, 2755–2763

2 T. J. Bollenbach and D. B. Stern, unpublished data.
