Role of RNA Structure and Susceptibility to RNase E in Regulation of a Cold Shock mRNA, cspA mRNA

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Degradation of the cspA mRNA in vivo is very rapid at temperatures greater than 30°C and is moderately dependent on RNase E. Investigations in vitro show that degradosomes prepared from normal or cold-shocked cultures cleave the cspA mRNA preferentially at a single site in vitro between two stem-loops—24 residues 3′ to the termination codon and —31 residues from the 3′ end. The site of cleavage is independent of the temperature and largely independent of the phosphorylation status of the 5′ end of cspA mRNA. A 5′ stem-loop, potential occlusion of the initiation and termination codons, temperature-dependent translational efficiency, and the position of the RNase E cleavage site can explain the differential stability of the cspA mRNA.

Exposure of Escherichia coli or other mesophilic microorganisms to temperatures below 20°C induces an immediate growth arrest (“cold shock”) which is followed by a period of adaptation and subsequent resumption of growth (17, 30, 31, 35). A set of at least 26 proteins is induced immediately following the shift to low temperature (17). Among these is a family of low-molecular-weight proteins including CspA (16). CspA contains an OB-fold domain (1, 29), which is typical of many RNA binding proteins (34). Indeed, various roles in RNA metabolism have been attributed to CspA and other related cold shock proteins, including chaperoning RNAs (17, 19, 31). Although its expression can be detected at 37°C, CspA becomes one of the mostly highly expressed proteins following a shift to low temperature (4, 12). The induction of CspA at low temperature is largely independent of transcription and is dependent primarily on posttranscriptional controls, including enhanced translation (4, 14, 15, 17). In particular, its mRNA is stabilized in a strain carrying a temperature-sensitive RNase E. Investigations in vitro show that degradosomes prepared from normal or cold-shocked cultures cleave the cspA mRNA preferentially at a single site in vitro between two stem-loops—24 residues 3′ to the termination codon and —31 residues from the 3′ end. The site of cleavage is independent of the temperature and largely independent of the phosphorylation status of the 5′ end of cspA mRNA. A 5′ stem-loop, potential occlusion of the initiation and termination codons, temperature-dependent translational efficiency, and the position of the RNase E cleavage site can explain the differential stability of the cspA mRNA.

In this communication we show directly that cspA mRNA, which is normally quite labile at temperatures of 30°C or higher, is susceptible to RNase E in vivo and in vitro. We have determined the secondary structure of potential regulatory features within the cspA mRNA and have mapped the primary site of endonucleolytic cleavage to the 3′ untranslated region (UTR). Our data suggest a model for the regulation of expression of CspA that rationalizes the roles of secondary structure and RNase E.

MATERIALS AND METHODS

Strains and plasmids. All strains are derivatives of E. coli K-12. CF881 [Δlac forA trp recB1009 (ΔathA-pnc) Δrne] was obtained from Michael Cashel (NHI, Bethesda, MD). A set of isogenic strains including MG1693 (ΔthrD), SK5665 (ΔthrD recE+ [rne-50] [28]), SK5691 (ΔthrD recE+ [rne-50 G66S]), and SK7998 (ΔthrD ΔrecB) was obtained from Sidney Kushner, University of Georgia, Athens, GA. Strain KCB1008 (Δrph-131 [rneΔ585-1061] [21]) was constructed from MG1693 by P1-mediated transduction (K. E. Baker and G. A. Mackie, unpublished). To construct plasmid pCspA-1, DNA corresponding to a T7 promoter, the 5′ UTR, coding sequence, and 3′ UTR of cspA was amplified from E. coli DNA using primers CSP-A-F and CSP-A-R (Table 1). The product was purified, digested with EcoRI and BamHI, and ligated into pUC18 digested with the same enzymes. Following transformation of strain DHS5, a recombinant plasmid, pCspA-1, from a single clone was purified, and its authenticity was verified by DNA sequencing.

Enzymes and assays. “Standard” degradosomes were purified from strain CF881 grown in LB medium with vigorous aeration at 37°C as described previously (26). “Cold shock” degradosomes were purified from the same strain grown at 30°C in LB medium to an A600 of 0.4 and then mixed with an equal volume of ice-cold LB and shifted to a water bath at 15°C. Growth was continued to saturation prior to harvest. Purification was performed as described previously (32). The compositions of both preparations were checked by sodium dodecyl
TABLE 1. Sequences of oligonucleotides

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence (5'→3')</th>
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<tbody>
<tr>
<td>CSPA-F</td>
<td>CGCCGAAATTCTTAATACGACTCTAC ATAGGGTTTTGAGCTAGACGG</td>
</tr>
<tr>
<td>CSPA-R</td>
<td>GGACGATTCCTGAAAAACATTTGAAAATCCCGCC</td>
</tr>
<tr>
<td>CSPA-PE1</td>
<td>CGATATGGCGTGCTTTAC</td>
</tr>
<tr>
<td>CSPA-PE2</td>
<td>GAACACATCTTTAGAGCC</td>
</tr>
<tr>
<td>CSPA-PE3</td>
<td>TAAGCAGAGATTACAGGC</td>
</tr>
</tbody>
</table>

TABLE 2. Half-lives of cspA mRNA in different strains and conditions

<table>
<thead>
<tr>
<th>Strain</th>
<th>Allele</th>
<th>Half-life (s)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>MG1693</td>
<td>Wild type</td>
<td>ND*</td>
</tr>
<tr>
<td>SK5665</td>
<td>me-1</td>
<td>ND</td>
</tr>
<tr>
<td>KCB1008</td>
<td>me-131</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Cultures were grown in LB at 30°C and then shifted to 42°C for 20 min or to 15°C for 60 min prior to addition of rifampin. Samples were withdrawn at 45-s intervals (42°C and 30°C) or at 2.5-min intervals (15°C) and RNA extracted immediately. Half-lives were determined by primer extension using CSPA-PE1 (Table 1). The values given represent the averages of duplicate determinations.

RESULTS

Effect of rne alleles on the stability of cspA mRNA in vivo. As an initial step in determining the basis for the instability of the cspA mRNA at normal growth temperatures, we measured its half-life in vivo to determine whether it was sensitive to RNase E. Cultures of the isogenic strains MG1693 (wild type), SK5665 (rne-1), and KCB1008 (rne-131) were grown at permissive temperature (30°C) and shifted to either 42°C (non-permissive) or 15°C prior to extraction of RNA (see Materials and Methods). Half-lives of the cspA mRNA from different samples were initially determined by Northern blotting. However, to obtain sufficient sensitivity, we subsequently resorted to primer extension. A summary of the data obtained is shown in Table 2. We could not reliably detect the cspA mRNA in the total RNA extracted from cultures shifted to 42°C, as the band corresponding to full-length cspA RNA was diffuse (data not shown). Moreover, no signal could be detected after treatment with rifampin. Taken together, these observations suggest that the cspA mRNA is very labile at elevated temperature. Others have reported that the half-life of the cspA mRNA is only 10 s at 37°C (15). In contrast, cspA mRNA could be detected readily from cultures grown at 30°C. Its half-life was 47 s in the wild-type strain and increased to 80 s in both strains carrying rne alleles (Table 2). As expected, the abundance and stability of the cspA mRNA increased significantly in cultures subjected to cold shock for 60 min (i.e., shifted from 30°C to 15°C). Table 2 shows that the half-life of the cspA mRNA in MG1693 was at least 600 s. The mRNA was also equally if not more stable in the two rne alleles. More accurate estimates could not be obtained, as no significant decay occurred during the time of sampling.

Endonucleolytic cleavage of cspA mRNA in vitro. Examination of the DNA sequence of the cspA gene suggests that it should encode an mRNA of 428 nucleotides (nt) (Fig. 1a). A combination of inspection, computer-assisted folding (27), and structure mapping with T1 RNase was used to assess the potential secondary structure of the cspA mRNA, particularly at its extremities. In agreement with previous work (36), the data summarized in Fig. 1b show that this mRNA contains a moderately stable 5' stem-loop (∆G = −3.4 kcal/mol), a feature which is normally associated with enhanced mRNA stability (2, 3, 7, 11, 18, 23, 33) and the cold shock response (36). The 3' end of the mRNA contains a predicted highly stable terminator stem-loop (∆G = −14.3 kcal/mol). In addition, we predict that the AUG codon and Shine-Dalgarno sequence can be occluded by base pairing with sequences further upstream in the 5' UTR. We presume that cold shock releases this (or other) putative structural barrier to translation, consistent with cold shock sensitivity residing in the 5' UTR (12-15, 36).

In view of the demonstrated and potential secondary structure in the cspA mRNA, we tested whether it would be a substrate for degradosomes prepared from cells grown at 37°C (Fig. 2a). We also tested whether cold shock degradosomes (32) would also cleave the cspA mRNA substrate (Fig. 2b). The two degradosome preparations were normalized to contain similar quantities of RNase E. The data in Fig. 2a and b show that in both cases the cspA mRNA substrate was cleaved readily to yield a major product of ~390 residues which was relatively stable. This suggests that the major RNase E cleavage occurs ~30 to 40 residues from one end of the mRNA. In this and subsequent experiments, we noted that normal degradosomes displayed a lag in attacking the substrate. We did not pursue this observation.

To map the cleavage site, we initially subjected the products of digestion by degradosomes to further cleavage with RNase H in the presence of specific oligonucleotides. The data showed that the initial cleavage occurred ~40 residues from the 3' end of the substrate (not shown). Moreover, using three primers spaced throughout the cspA sequence (CSPA-PE [Table 1]), we were unable to detect new 5' termini in the 5' UTR.
by primer extension, a finding consistent with in vivo data (12).

Accordingly, we prepared 3′-H11032-end-labeled cspA mRNA and repeated the experiment of Fig. 2a, using 10% gels to retain the anticipated 3′ product. A time course of digestion is shown in Fig. 2c. The substrate disappeared within 10 min, and a product of ~35 residues accumulated in nearly 100% yield. Additional characterization of the product was achieved by separation on a sequencing gel, using labeled DNA oligonucleotides to provide size markers. Assuming that the mobilities of the RNA products can be compared directly to those of the DNA markers, the products migrated as a doublet of 30 and 31 nt (Fig. 2d). This would correspond to cleavage immediately 5′ to the base of the terminal stem-loop (i.e., 3′ to residue 237) but possibly further 5′ between residues 233 and 234 (Fig. 1b).

Heterogeneity in the products could reflect minor variations in the site of termination of transcription or in cleavage itself, analogous to the case for the major cleavage site in the rpsT mRNA (25). This cleavage site(s) lies ~20 residues 3′ from the termination codon between two stem-loops, somewhat reminiscent of the rpsO mRNA (6). A previous inference that the site of RNase E cleavage lies in the 5′ UTR (12) is not supported by our data.

To test whether the predicted 5′ stem-loop was functional during our assays, we measured the effect of replacing the 5′-triphosphate terminus with a 5′-monophosphorylated terminus. We reasoned that if present, the predicted 5′-proximal stem-loop should negate the stimulatory effects of monophosphorylation (23). Normal or cold shock degradosomes were assayed against ppp-cspA mRNA (Fig. 3a and b) or p-cspA mRNA (Fig. 3c and d) at 30°C. In either case, the monophosphorylated substrate was digested at least 1.5- to 2.5-fold faster than ppp-cspA mRNA (compare Fig. 3a or b with Fig. 3c or d, respectively). At 15°C, however, the rate of cleavage of p-cspA mRNA by cold shock degradosomes as well as the rate of accumulation of 390-nt product was indistinguishable from that of the ppp-substrate (Fig. 3, compare panels f [p-] and e [ppp-]). Since substrates with single-stranded 5′ termini are typically stimulated up to 30-fold by monophosphorylation (23), we conclude that the 5′ terminus of cspA mRNA is sequestered in a form that is not readily recognized by RNase E, particularly at 15°C.

**DISCUSSION**

The expression of the cspA gene appears to be regulated almost entirely by posttranscriptional mechanisms, including translational efficiency and mRNA stability, that are dependent on the temperature at which cultures are grown (17). We have focused on better understanding the basis for the instability of the cspA mRNA at normal temperatures. The avail-
able data show that the cspA mRNA is very labile in vivo at temperatures of >30°C, with half-lives of 47 s at 30°C (Table 2) and only 10 s at 37°C (15). This lability seems surprising in view of the 5′ stem-loop in the cspA mRNA, a feature that usually confers stability on mRNAs ostensibly by preventing a productive interaction with the 5′ sensor domain of RNase E (2, 3, 7, 11, 18, 23, 33). However, the 5′ stem-loop in cspA mRNA is only moderately stable (calculated ΔG = -3.4 kcal/mol). Nonetheless, the data in Fig. 3 suggest that its effectiveness as a stabilizing element improves at lower temperature, consistent with its requirement for the stabilization of cspA mRNA at low temperature (36). We also found that me mutations increase the half-life of the cspA mRNA in vivo, although relatively modestly (Table 2). Taken together, these data suggest a role for RNase E in the degradation of cspA mRNA.

Our data show clearly that cspA mRNA is nearly equally sensitive to cleavage by RNase E in vitro at either 30°C or 15°C whether the RNase E resides in normal or cold shock degradosomes. Nonetheless, cspA mRNA is no more sensitive to RNase E in vitro than other substrates we have tested (e.g., rpsT mRNA and 9S pre-tRNA). However, the initial 390-nt product of cleavage is atypically stable, although prolonged digestion results in further cleavage (Fig. 3d). The secondary structure of the cspA mRNA can rationalize these observations. RNase E cleavage essentially truncates the cspA mRNA at its 3′ end, leaving the 5′ terminus intact. The preferred RNase E cleavage site is located between two stem-loops and is separated from the termination codon by 20 residues which are incorporated into an imperfect stem-loop. This situation is reminiscent of the rpsO mRNA, where the M2 cleavage site lies 10 residues from the termination codon and is adjacent to a stable 3′ terminator stem-loop (6, 10). The cleavage site in cspA mRNA is located a significant distance from the 5′ end of cspA mRNA and occurs despite the presence of a moderately protective 5′ stem-loop. Thus, this cleavage would constitute an example of the internal entry pathway by RNase E (2, 20).
Such cleavages can occur on untranslated mRNAs (20). Moreover, the presence of a 5' stem-loop and a terminal triphosphate can explain why the 390-nt 5' fragment is relatively stable once cleaved in vitro: both features are protective, and once the preferred site is cleaved, alternative sites would display even lower affinity for RNase E. Finally, the observed cleavage at the major site in the 3' untranslated region (36) of the mRNA following cold shock by at least two means. First, the internal entry pathway is inhibited (2), and second, paused ribosomes provide protection against RNase E cleavage at the major site in the 3' UTR. In addition, CspA itself may chaperone its mRNA to provide further stabilization (17), while CsdA, a cold shock DEAD box RNA helicase, may promote translation and stabilization of cspA mRNA (5). Finally, the 5' stem-loop, whose protective effect is well known (2, 3, 11, 18, 23, 33), further stabilizes cspA mRNA.

REFERENCES


